

# Identification and characterization of $\beta$ -adrenergic receptors in isolated primary equine tracheal epithelial cells

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#### 1 Abstract

2 Responses and functions of airway epithelial cells are stimulated by  $\beta_2$ -agonists via the  $\beta_2$ -3  $(\beta_2$ -ARs)-G<sub>s</sub>-protein-cAMP-system, adrenergic receptors thus. affecting airway inflammation such as in asthma and equine recurrent airway obstruction (RAO). Though 4 5 horses can be used as large animal model for human asthma, evaluation of the expression and functions of the  $\beta$ -AR system in primary equine airway epithelial cells has not been yet 6 carried out. Thus, for the first time, we determined the  $\beta$ -AR density and subtype 7 distribution by  $[^{125}\Pi$ -iodocyanopindolol (ICYP) binding, examined  $\beta$ -AR function by 8 9 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 10 11 horses and cultured subsequently. The specific ICYP binding was saturable and of high 12 affinity: in freshly isolated cells the receptor density ( $B_{max}$ ) and ICYP affinity ( $K_D$ ) for  $\beta$ -ARs were  $12,727 \pm 883$  binding sites/cell and  $31.78 \pm 6.57$  pM, respectively, and in 13 cultured ETEC 3,730  $\pm$  212 binding sites/cell and 15.26  $\pm$  3.37 pM, respectively. The  $\beta$ -AR 14 subtype assessed by  $\beta_1$ -selective (CGP 20712A) and  $\beta_2$ -selective (ICI 118.551) adrenergic 15 16 receptor antagonists demonstrated that the  $\beta_2$ -AR subtype predominated (> 95%) in both cell populations (p < 0.001). The  $\beta$ -AR agonists increased cAMP formation with a rank 17 order of potency: isoproterenol > epinephrine > norepinephrine. ICI 118.551 (100 nM) 18 19 significantly blocked (p < 0.05) isoproterenol-induced cAMP accumulation but not CGP 20 20712A (300 nM). Western blot analyses and immunocytochemical staining further 21 indicated the expression of the  $\beta_2$ -AR subtype in both cell preparations. Our data indicate that in acutely dissociated and primary cultured ETEC the  $\beta_2$ -AR-AC system is expressed, 22 23 but varies considerably between the two preparations.

24

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

#### 1 1. Introduction

2 Airway epithelial cells (AEC) are specialized cells that serve as physicochemical barrier 3 and contribute to the integrated control of the respiratory function and to non-specific 4 defence mechanism and homeostasis in airways. AEC have many metabolic and 5 biochemical functions including mucus secretion [1], ion and fluid transport [2] and 6 modulation of smooth muscle function [3], [4] and [5]. Moreover, several investigations 7 have shown that the airway epithelium plays a critical role in airway inflammatory diseases 8 like asthma, chronic obstructive pulmonary disease (COPD) and equine recurrent airway 9 obstruction (RAO) as target of injury and as source of inflammatory mediators [6], [7], [8] 10 and [9]. Such activities and responses of the airway epithelium are known to be regulated 11 by the  $\beta$ -adrenergic receptor ( $\beta$ -ARs)-G-protein-adenylate cyclase system; thus, epithelial 12 damage in airway disorders and altered  $\beta$ -AR function lead to airway hyper-responsiveness 13 [10] and [11]. Epithelial  $\beta$ -ARs affect the airway smooth muscle tone and epithelial cell 14 functions. Since some respiratory diseases like asthma or the equine RAO seem to be 15 associated with the dysfunction of the  $\beta$ -AR-G-protein-adenylate cyclase system [12], [13], [14] and [15], bronchodilator drugs ( $\beta$ -AR agonists) are, therefore, beneficial by acting on 16 17  $\beta$ -AR located mainly on smooth muscle cells and on airway epithelial cells [16]. The 18 expression and function of the  $\beta$ -AR system of the airway epithelial cells from asthmatic 19 human or animals might differ from that of normal individuals, thus, information with regard to this receptor system should provide the physiological, pathological and 20 21 pharmacological importance of airway epithelial cells.

The expression of functional  $\beta$ -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  $\beta$ -AR

1 system is expressed and functional in airway epithelial cells lining the tracheobronchial 2 branch is not studied in isolated cells. The present study, hence, investigated the density, 3 subtype as well as the expression of  $\beta$ -ARs and cAMP accumulation in freshly isolated and 4 primary cultures of equine tracheal epithelial cells (ETEC) from adult horses.

#### 1 **2. Materials and Method**

#### 2 **2.1. Chemicals**

(-)-[<sup>125</sup>I]-iodocyanopindolol (specific activity: 2200 Ci/mmol) and AlphaScreen cAMP 3 assay kit were purchased from PerkinElmer (Boston, MA, USA). ICI118.551 (erythro-4 5 (+/-)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol),  $(\pm)$ -CGP12177  $((\pm)$ -4-(3-CGP20712A (1-[2-((3-6 tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on], 7 carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethylimidazolyl)-8 phenoxy]-2-propanol), (-)-isoproterenol bitartrate, (-)-epinephrine and (-)-norepinephrine 9 and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (Sigma-Aldrich 10 Chemie GmbH, Steinheim, Germany). All other chemicals are of analytical grade and are 11 commercially available.

12

#### 13 **2.2. Epithelial cell isolation and culture**

14 Primary equine tracheal epithelial cells (ETEC) were dissociated as recently described [23]. 15 Briefly, tracheae were removed from slaughter adult horses of both sexes (n=19) with no 16 signs of cardiopulmonary diseases, cut into 8 cm in length and rinsed immediately in ice-17 cold Ca-Mg-free Hanks' balanced salt solution (HBSS) (PAA Laboratories, Pasching, Austria). Tracheal segments were opened longitudinally, rinsed again and the epithelium-18 19 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-20 Aldrich Chemie GmbH, Steinheim, Germany) for 2 h at 37° C in 95% O<sub>2</sub> : 5% CO<sub>2</sub> 21 22 atmosphere. Trypsin digestion was stopped by the addition of 20% foetal calf serum in 23 HBSS, and cell dissociation was enhanced by flushing the tissues. The cell suspension was 24 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 25  $\times$  g for 10 min and resultant pellet was resuspended in supplemented serum-free Airway 26

Epithelial Cell Growth Medium (AECGM) (PromoCell, Heidelberg, Germany). Cell 1 2 counting and viability (> 95%) test were carried out using trypan blue dye. The purity of 3 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 4 to  $10^6$  cells/ml in supplemented AECGM. Further, ETEC were cultured in supplemented 5 AECGM at a density of  $7 \times 10^4$  cells /cm<sup>2</sup>. At confluence of about 75%, cells were detached 6 using 0.1% trypsin/EDTA, counted and adjusted at a density of  $10^6$  cells/ml for binding 7 8 studies.

9

#### 10 2.3. Determination of β-AR density, affinity and subtypes

11 Receptor density and subtype distribution of β-AR were determined by using radioligand -[<sup>125</sup>I] (-)-iodocyanopindolol (ICYP) binding as described by Abraham et al. [20]. Suspensions 12 13 of freshly isolated or cultured primary equine tracheal epithelial cells (150,000 cells in 150 14 µl/assay) were incubated with six increasing ICYP concentrations (5 to 160 pM) in the 15 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 16 in the presence (i.e. non-specific binding) or absence (i.e. total binding) of 1  $\mu$ M (±)-CGP 17 12177, a non-selective  $\beta$ -adrenergic receptor antagonist. The incubation was terminated by 18 19 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 20 21 using Brandl cell harvester (Brandl Biomedical Research and Development, Gaithersburg, 22 USA). Each filter paper was washed with an additional 8 ml of the wash buffer. The filter 23 bound radioactivity was counted in Gamma Counter Wallac WIZARD 1470 (PerkinElmer Wallac GmbH, Freiburg, Germany) at an efficiency > 80%. Specific binding was defined as 24 the difference between total ICYP binding and non-specific binding. All assays were 25 performed in duplicate. 26

For determining the  $\beta$ -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<sup>-10</sup>- 10<sup>-4</sup>M) of the  $\beta_2$ -AR selective antagonist ICI 118.551, or  $\beta_1$ -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.

8

#### 9 **2.4. Western blot analysis**

The abundance of the  $\beta_2$ -AR in ETEC was determined by Western blot analysis using 10 subtype specific antiserum against  $\beta_2$ -AR (Santa Cruz Biotechnology, Santa Cruz, CA, 11 USA). ETEC were homogenized in 1 mM KHCO<sub>3</sub> and protein concentration was 12 determined by the method of Lowry. For immunoblotting, whole cell lysates were 13 dissolved with an equal volume of sample buffer (60 mM Tris-HCl, 25% glycerol, 2% 14 SDS, 14.4 mM mercaptoethanol, 0.1% bromophenol blue) and boiled for 5 min. Membrane 15 16 proteins (20 µg/lane) were subjected to 3% stacking and 12% resolving gels (v/v) SDS-17 polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [24] using a Mini Protean III apparatus (Bio-Rad Laboratories, Munich, Germany). Subsequently, proteins 18 19 were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany) using the wet transfer system (Bio-Rad Laboratories, Munich, 20 21 Germany). Non-specific binding was blocked by incubation of the nitrocellulose 22 membranes for 1 h at room temperature in TBS-T (50 mM Tris, pH 7.5, 2 mM MgCl<sub>2</sub>, 140 23 mM NaCl), containing 1% BSA and 0.1% Tween-20. Thereafter, blots were reacted for 1 h at room temperature with rabbit polyclonal  $\beta_2$ -AR antibody (H-73) (1:200) diluted in TBS-24 25 T. The blots were washed three times with TBS-T for 5 min each and finally incubated for

1 1h at room temperature with a secondary anti-rabbit IgG-alkaline phosphatase conjugate 2 (1:10000) (Vector Laboratories, Burlingame, CA, USA). After three times washing, antibody binding to  $\beta$ -AR was visualized and the specific signal was developed using 3 4 enhanced chemiluminescence western blot detection reagent (western blue®, Promega Corporation, Madison, WI, USA). Mouse brain tissue lysate was used as positive control 5 6 for  $\beta_2$ -adrenergic receptor expression and negative controls were similarly treated but no 7 primary antibody was added. To ascertain equal protein loading, transferred proteins were 8 routinely stained with mouse monoclonal antibody against  $\beta$ -actin and with Coomassie 9 blue.

10

#### 11 **2.5. Immunocytochemical staining**

12 Either cytocentrifuged acutely dissociated ETEC or on glass cover slips grown ETEC 13 (200,000 cells/well) were fixed with ice-cold acetone (-20°C for 5 min) and washed with 14 PBS. After neutralizing with ammonium chloride (50 mM) for 10 min at room temperature 15 and, washing with PBS, non-specific binding was blocked for 30 min in 3% BSA in PBS. 16 Cells were then incubated with 1:100 dilution of rabbit polyclonal  $\beta_2$ -adrenergic receptor 17 antibody (clone H-73) (Santa Cruz Biotechnology, CA, USA) for 1 hr at room temperature, 18 washed 3x5 min with PBS, and incubated with 1:200 dilution of FITC-labelled secondary 19 anti-rabbit IgG antibody (Sigma-Aldrich, Chemie GmbH, Munich, Germany ). Nuclear 20 staining was performed using DAPI at 5 µg/ml. After 3x5 min PBS washings, slides were 21 embedded in Fluorsave anti-fade reagent and immunofluorescent images were captured 22 using Olympus BX50 fluorescent microscope (Olympus Optical Co. Ltd, Tokyo, Japan).

23

#### 24 **2.6.** Cyclic AMP assay

25 Freshly dissociated or trypsinized ETEC were washed in PBS and were stimulated by  $\beta$ -AR

26 agonists (-)-isoproterenol, (-)-epinephrine or (-)-norepinephrine and cAMP accumulation

1 was determined as previously described [20]. In brief, cells were resuspended in PBS 2 containing 100 µM 3-isobutyl-1-methylxanthine (IBMX). Aliquots of 300 µl cell suspension (10<sup>6</sup> cells) were incubated at 37 °C for 15 min with either 30 µl PBS/IBMX 3 (basal cAMP) or 30 µl of increasing concentrations ( $10^{-9}$ - $10^{-5}$  M) of β-AR agonists in a 4 5 final volume of 330 µl. Isoproterenol-induced cAMP accumulation was also measured in 6 the presence or absence of highly selective  $\beta_2$ -adrenergic receptor antagonist ICI 118.551 7 (100 nM), or highly selective  $\beta_1$ -adrenergic receptor antagonist CGP 20712A (300 nM). 8 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 9 thawed aliquots using AlphaScreen<sup>®</sup>cAMP assay kit following the manufacturer's 10 directions in 384-well white opaque microplate at room temperature in triplicate at a total 11 12 volume of 25µl/well. The standard cAMP dilutions (0-5 µM) were prepared with PBS 13 buffer containing 100 µM IBMX. Then, 5 µl sample or 5 µl standard cAMP were added to 14 each well, followed by the addition of 0.1 µl anti-cAMP acceptor beads in 10 µl assay 15 buffer (5 mM HEPES, 1x HBSS, 0.1% BSA, pH 7.4). The microplate was incubated for 30 16 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% 17 18 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<sup>®</sup> Multilabel Reader (PerkinElmer, 19 20 Boston, MA, USA).

21

#### 22 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<sub>50</sub>

1 values) for antagonists were calculated from concentration-inhibition curves, and the 2 inhibition constants (K<sub>I</sub>) were calculated according to the method of Cheng and Prusoff 3 [26]. The accuracy of the fit to one- or two-site model was estimated by the F-test of the 4 sum of squares of residuals from the fittings. Data from cAMP experiments were calculated 5 by non-linear regression analysis using the equation for a sigmoid concentration response 6 curve with variable slope: 7 V. Bettern (Tag Bettern)/(1 + 10<sup>((log EC -x)\*Hill slope))</sup> her derivation

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

8  $X = cAMP [nM] = 10^{\log 10EC} \frac{(\log 10((Top-Bottom)/Y[cps] - Bottom))-1)}{50}$ 

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

#### 1 **3. Results**

#### 2 **3.1.** β-Adrenergic receptor density and subtype

3 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 4 5 at equilibrium was 13.86% of total binding for freshly dissociated ETEC, whereas this 6 value was higher (39.48%) in primary cultures. The density of  $\beta$ -adrenergic receptor 7 binding sites per cell (B<sub>max</sub>) obtained from saturation binding experiments was greater in 8 acutely dissociated than in cultured ETEC. Freshly isolated and cultured ETEC expressed  $12,727 \pm 883$  and  $3,730 \pm 212$   $\beta$ -ARs/cell, respectively (n = 7, p < 0.001). However, there 9 10 was no significant difference between ligand binding affinities (K<sub>D</sub>) for ICYP for both 11 types of ETEC preparations. Also, linear regression analysis of the specific ICYP binding 12 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 13 equine tracheal epithelial cells. B<sub>max</sub> and K<sub>D</sub> obtained here were similar to those obtained 14 15 from non-linear regression fit.

The percentage of  $\beta$ -AR subtypes ( $\beta_1$ -AR and  $\beta_2$ -AR) measured by competitive 16 displacement of ICYP binding was identical in both ETEC preparations (n = 7). In both cell 17 18 preparations, the concentration inhibition curves of the  $\beta_2$ -AR selective antagonist ICI 19 118.551 and the  $\beta_1$ -AR selective antagonist CGP 20712A (in freshly isolated cells), were 20 steep monophasic, indicating the presence of one population of receptors (Fig. 3). However, 21 ICI 118.551 was more potent (> 10,000) than CGP 20712A in displacing the ICYP binding. 22 These results indicate that the  $\beta_2$ -AR subtype was the predominant receptor subtype ( > 23 95%) expressed by both ETEC preparations, despite different inhibition affinities of the two ligands in various cell preparations (K<sub>I</sub> for ICI 118.551: 1.19  $\pm$  0.41 nM and 1.74  $\pm$ 24

1 0.15 nM in freshly isolated and primary cultures respectively;  $K_I$  for CGP 20712A: 17 ± 2 7.90  $\mu$ M in freshly isolated ETEC).

3

#### 4 **3.2.** β-Adrenergic receptor expression in ETEC

5 To affirm the cellular distribution of  $\beta_2$ -AR, we compared the expression of this receptor in freshly dissociated and cultured ETEC by western blot analysis and immunocytochemical 6 7 staining. As shown in Fig. 4A, using antibody against human  $\beta_2$ -adrenergic receptors, a protein band for  $\beta_2$ -AR was detected at molecular weight of about 72 kDa in both ETEC 8 preparations; indeed, higher  $\beta_2$ -AR expression was seen in freshly isolated ETEC. There 9 was no change at expression levels of  $\beta$ -actin in cultured and acutely dissociated ETEC 10 (Fig. 4B). Moreover, immunocytochemistry of  $\beta_2$ -AR in ETEC showed strong 11 immunoreactive signals, confirming the expression of the receptor in intact epithelial cells 12 13 (Fig. 4C). Negative controls, obtained without primary antibody, did not show any positive 14 signal.

15

#### 16 **3.3. β-Adrenergic receptor - cAMP system function**

17 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 18 increased with increasing concentration of these  $\beta$ -AR agonists in both cell preparations. 19 However, concentration of formed cAMP per cell number was greater in freshly isolated 20 21 ETEC than in cultured cells. In freshly isolated ETEC, the half-maximal effective concentration (EC<sub>50</sub>) of the  $\beta$ -AR agonist-response curves was 58 nM, 13.55  $\mu$ M and 430 22  $\mu M$  (n = 6) for isoproterenol, epinephrine and norepinephrine, respectively. cAMP 23 accumulation of these  $\beta$ -AR agonist at the highest concentration tested (10  $\mu$ M) was, 24 respectively, 50 nmol/ $10^6$  cells, 30 nmol/ $10^6$  cells and 18 nmol/ $10^6$  cells. At the same 25

1 agonist concentrations, in primary culture cells, isoproterenol, epinephrine and norepinephrine induced maximum cAMP accumulation (n = 6) with 27 nmol/10<sup>6</sup> cells, 20 2  $nmol/10^6$  cells and 17  $nmol/10^6$  cells, respectively. The agonists were able to increase 3 cAMP formation with an order of potency isoproterenol > epinephrine > norepinephrine, 4 which is characteristic for the existence of the  $\beta_2$ -AR subtype. Moreover, the isoproterenol-5 stimulated cAMP production in freshly isolated cells and primary cultures (Fig. 6) was 6 7 attenuated significantly (p < 0.05) by 100 nM of  $\beta_2$ -AR selective antagonist ICI 118.551 but not by the  $\beta_1$ -AR selective antagonist CGP 20712A, suggesting also the predominant 8 9 expression of the  $\beta_2$ -AR subtype in these cells.

#### 1 **4. Discussion**

2 The present investigation demonstrated for the first time that the  $\beta$ -adrenergic receptor-3 cAMP system is expressed in primary equine tracheal epithelial cells. Freshly dissociated primary tracheal epithelial cells appear to express larger number of  $\beta$ -ARs (12,727±883.6 4 binding sites/cell) than cultured ETEC (3,730±212 binding sites/cell). Indeed, competition 5 6 binding studies with  $\beta_1$ -AR and  $\beta_2$ -AR selective antagonists and cAMP assays indicated 7 that both cell preparations express predominantly (> 95%) the  $\beta_2$ -AR subtype. Moreover, 8 Western blot analysis and immunocytochemical studies indicated that the anti- $\beta_2$ -AR 9 antibody labels also surface ETEC  $\beta_2$ -ARs.

10 Throughout the mammalian respiratory system the  $\beta_2$ -AR is known to be expressed not only 11 in the airway smooth muscle but also on different airway epithelial cell types [27] and [17] whereby the density of  $\beta_2$ -ARs are greater in epithelial than in smooth muscle cells [28]. A 12 13 number of studies have demonstrated that the epithelial  $\beta$ -ARs are reduced in diseases such as 14 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 15 16 airway obstruction (RAO) [15]. However, the alteration of the  $\beta_2$ -AR system between 17 asthmatic and non-asthmatic whole lung in comparative studies could not always be 18 correlated. Since the partial shedding of the airway epithelial cell layer is associated with the 19 pathophysiology of obstructive respiratory diseases like RAO in horse [30], an in vitro cell 20 model should allow detailed study of the role of epithelial  $\beta$ -AR system in RAO and represent 21 an important target of the class of  $\beta_2$ -adrenergic drugs. However, no appropriate cell culture 22 models of the airway epithelium were available for the horse which expresses functional  $\beta_2$ -AR and thus, could give insight in the underlying mechanisms of  $\beta$ -adrenergic drug 23 24 responses.

1 Recently, in our laboratory freshly isolated and cultured ETEC were established for the first 2 time. In the current study these cells were characterized for their  $\beta$ -AR expression and 3 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  $\beta$ -AR. It was 4 saturable and reversible and of high affinity in both cell preparations (K<sub>D</sub> value in fresh and 5 cultured ETEC:  $31.78 \pm 6.57$  pM and  $15.26 \pm 3.37$  pM, respectively). Though not significant, 6 7 there was a difference in K<sub>D</sub> values between the two cell types. The relatively higher K<sub>D</sub> value 8 for ICYP binding was, however, in agreement with K<sub>D</sub> values found in freshly isolated human 9 tracheal epithelial cells (K<sub>D</sub>:  $35 \pm 14$  pM [17] and K<sub>D</sub>: 29.7 pM [31]. The K<sub>D</sub> for primary cultures was comparable with data from Davis et al. [17] who found  $K_D$  value of 25 ± 13 pM 10 11 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  $20 \pm 3 \text{ pM}$ 12 13 in cultured bovine tracheal submucosal gland cells.

With regard to the receptor density, greater number of  $\beta$ -AR binding sites were detected in 14 15 primary freshly isolated ETEC ( $12,727 \pm 883$  binding sites/cell) than in cultured ETEC (3,730 $\pm$  212 binding sites/cell). This discrepancy in  $\beta$ -AR expression between the two cell 16 preparations presumably appears to be explained by differences in epithelial cell 17 subpopulation, cell size and culturing of ETEC. Acutely isolated primary equine tracheal 18 19 epithelial cells, though we did not specify them, should contain mixed cells of basal, 20 columnar, goblet cells and ciliated cells which have different cell sizes and thus, it can 21 strongly be assumed that this may affect the expression of membrane-bound  $\beta$ -ARs [33], 22 since one would expect larger surface area correlates with higher receptor number. An 23 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. 24 Serum supplementation often affects cell differentiation and cellular protein expression. In 25 permanent airway epithelial cell lines which were cultured in serum-containing media,  $\beta$ -AR 26

binding sites were comparable with those of freshly isolated ETEC; high density of  $\beta_2$ -ARs 1 2 were obtained in Calu-3 (9,908  $\pm$  1127 binding sites/cell) and in 16HBE14o- cells (6,423  $\pm$ 3 895 binding sites/cell) [20]. This was also close to the numbers found in human tracheal epithelial cells  $(7,000 - 9,000 \beta$ -ARs/cell; Kelsen et al. [34]), in freshly isolated rabbit 4 tracheal epithelial cells (10,700 β-ARs/cell; Mardini et al. [35]) as well as in primary cultures 5 of bovine bronchial epithelial cells (13,000  $\beta$ -ARs/cell; Nogami et al. [36]). The low density 6 7 of binding sites observed in primary cultured ETEC were comparable with the  $\beta$ -AR density 8 found in primary cultures of human tracheobronchial epithelial cells  $(3.795 \pm 957)$  binding 9 sites/cell; Aksoy et al [37]).

Furthermore, the results from western blot analysis indicate expression of the protein band of 72 kDa. Mammalian pulmonary  $\beta_2$ -AR purified from several species show nearly identical relative molecular masses of 64 – 67 kDa [38] and [39]. The molecular mass of  $\beta_2$ -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  $\beta_2$ -AR on intact epithelial cells.

17 Competitive ICYP binding assays employing the  $\beta$ -AR selective antagonist ICI 118.551 ( $\beta_2$ ) 18 and CGP 20712A ( $\beta_1$ ) showed that in both ETEC preparations the  $\beta_2$ -AR subtype is 19 predominant (> 95%). The present results are in good agreement with reports in the literature 20 that the  $\beta_2$ -AR represents the mere binding sites as for both antagonists. Identical results were 21 found in for ICI 118.551 and betaxolol ( $\beta_1$ -selective) in BEAS-2B cells [37]. Percentages of  $\beta_{2}$ - and  $\beta_{1}$ -ARs were 80% and 20% in freshly isolated human airway epithelial cells [34] and 22 >90% in primary rat tracheal epithelial cells [33], which agrees with the present data obtained 23 24 from primary equine tracheal epithelial cells.

1 Finally, in both freshly isolated and cultured primary equine tracheal epithelial cells, 2 stimulation of  $\beta$ -AR system by  $\beta$ -AR agonists induced the formation of intracellular cAMP, suggesting the functional coupling of the adenylate cyclase to the receptor. Generally, in 3 4 both cell preparations, the isoproterenol-induced cAMP accumulation was larger than the 5 epinephrine and norepinephrine-induced cAMP, whereby in both cells, the agonist-induced cAMP production was diminished only by  $\beta_2$ -AR selective antagonist ICI 118.551, but not 6 by  $\beta_1$ -AR selective antagonist CGP 20712A, strongly suggesting the  $\beta_2$ -AR-mediated 7 8 cAMP formation in these cells. The less cAMP responses to  $\beta$ -AR agonists in cultured 9 ETEC suggest that the  $\beta$ -AR number as well as presumably the catalytic component of the 10 adenylate cyclase is lower in these cells. In both cells, however, the pattern of potency to 11 increase agonist-induced intracellular cAMP formation over basal activity was 12 isoproterenol > epinephrine > norepinephrine, a characteristic feature of the  $\beta_2$ -AR subtype [41]. The magnitude of the isoproterenol-stimulated cAMP response in ETEC was 13 comparable to that observed in primary airway epithelial cells [33] and airway epithelial 14 15 cell lines [20].

In conclusion, we have demonstrated that tracheal epithelial cells from the horse, freshly dissociated or cultured, express an intact, functional  $\beta_2$ -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.

21

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- 3

# 1 Legends

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3 Figure 1:

4	Saturation binding of ICYP to freshly isolated equine tracheal epithelial cells (ETEC). Cells
5	were incubated at 37°C for 90 min with 5–160 pM ICYP in the absence (total binding, TB) or
6	presence (non-specific binding, NSB) of 1 $\mu$ M (±)-CGP 12177. Bound radioactivity was
7	separated by a rapid vacuum filtration. Specific ICYP binding was calculated as the difference
8	between TB and NSB. Inset shows Scatchard transformations of the specific ICYP binding.
9	Values are mean $\pm$ SEM for 7 independent experiments performed in duplicate. BS = binding
10	sites, cpm = counts per minute, ICYP = iodocyanopindolol
11	
12	Figure 2:
13	Saturation binding of ICYP to primary cultures of equine tracheal epithelial cells (ETEC).
14	Cells were incubated at 37°C for 90 min with 5–160 pM ICYP in the absence (total binding,
15	TB) or presence (non-specific binding, NSB) of 1 $\mu$ M (±)-CGP 12177. Bound radioactivity
16	was separated by a rapid vacuum filtration. Specific ICYP binding was calculated as the
17	difference between total binding and non-specific binding. Scatchard transformations of the
18	specific ICYP binding are shown in the inset. Values are mean $\pm$ SEM for 6 separate
19	experiments performed in duplicate. BS = binding sites, cpm = counts per minute
20	
21	Figure 3:
22	Competitive displacement of ICYP binding by the $\beta_2$ -AR selective antagonist ICI 118.551
23	and $\beta_1$ -AR selective antagonist CGP 20712A in primary equine tracheal epithelial cells.
24	Values are mean $\pm$ SEM for 5-7 independent experiments performed in duplicate. In each
25	displacement experiment, the ICYP binding was best fit to a one-site model, and 100%

23

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

3

4 Figure 4:

Expression of  $\beta$ -adrenergic receptors in primary equine tracheal epithelial cells. A) Western 5 6 blot signals obtained with rabbit polyclonal  $\beta_2$ -AR antibody of human origin and B) the 7 corresponding  $\beta$ -actin staining which was used as control. Lane 1 = ladder, 2 and 3 = lysate 8 from primary cultured ETEC, 4 and 5 = lysate from freshly isolated ETEC, 6 and 7 mouse 9 brain tissue lysates (positive control); C) Immunofluorescence localization of the  $\beta_2$ -AR in 10 freshly isolated epithelial cells (a, b, c) and 7 day old primary cultures (d, e, f). Images a and 11 d show anti- $\beta_2$ -AR stained cells; c and f show corresponding DAPI nuclear staining; b and e 12 show overlays of DAPI stained nuclei and anti- $\beta_2$ -AR stained cell membranes. Positive FITC signal is shown green, and DAPI staining nuclei is blue. Data shown are representative of 13 14 three independent experiments. 15

16 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.

22

Figure 6:

24 The effect of ICI 118.551 ( $\beta_2$ -AR antagonist) and CGP 20712A ( $\beta_1$ -AR antagonist) on

25 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

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

### 1 Figure 1



#### ACCEPTED MANUSCRIPT Figure 2 Total binding Specific binding -Non-specific binding ICYP binding (cpm/150,000 cells) 10000-8000 $B_{max} = 3820 \text{ BS/cell}$ 6000-0.25 0.20-0.20-0.15-0.10-0.05- $K_{\rm D} = 17.40 \ \rm pM$ 4000-2000 $r^2 = 0.9$ 0 0.00-175 50 75 100 125 150 25 1000 2000 3000 4000 0 0 Bound(BS/cell) ICYP [pM]

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1 Figure 4



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# 1 Figure 5





## 1 Figure 6

