



HAL
open science

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

Getu Abraham, Workineh Shibeshi, Fritz R. Ungemach

► **To cite this version:**

Getu Abraham, Workineh Shibeshi, Fritz R. Ungemach. Identification and characterization of β -adrenergic receptors in isolated primary equine tracheal epithelial cells. *Pulmonary Pharmacology & Therapeutics*, 2011, 24 (1), pp.174. 10.1016/j.pupt.2010.06.003 . hal-00721580

HAL Id: hal-00721580

<https://hal.science/hal-00721580>

Submitted on 28 Jul 2012

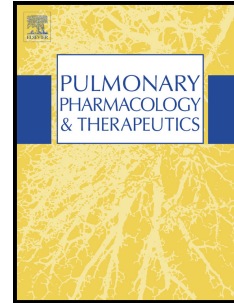
HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

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

Authors: Getu Abraham, Workineh Shibeshi, Fritz R. Ungemach



PII: S1094-5539(10)00076-3

DOI: [10.1016/j.pupt.2010.06.003](https://doi.org/10.1016/j.pupt.2010.06.003)

Reference: YPUPT 1017

To appear in: *Pulmonary Pharmacology & Therapeutics*

Received Date: 7 October 2009

Revised Date: 4 June 2010

Accepted Date: 18 June 2010

Please cite this article as: Abraham G, Shibeshi W, Ungemach FR. Identification and characterization of β -adrenergic receptors in isolated primary equine tracheal epithelial cells, *Pulmonary Pharmacology & Therapeutics* (2010), doi: 10.1016/j.pupt.2010.06.003

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

3

4 Getu Abraham*, Workineh Shibeshi, Fritz R. Ungemach

5

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

8

9 **Running Title:** equine tracheal epithelial cell β -adrenergic receptors

10

11 *Corresponding author:

12 Getu Abraham, Ph.D.

13 University of Leipzig

14 Institute of Pharmacology, Pharmacy and Toxicology

15 An den Tierkliniken 15

16 04103 Leipzig

17 Germany

18 Phone: +49 341 9738 134

19 Fax: +49 341 9738 149

20 Email: gabraham@rz.uni-leipzig.de

21

1 Abstract

2 Responses and functions of airway epithelial cells are stimulated by β_2 -agonists via the β_2 -
3 adrenergic receptors (β_2 -ARs)-G_s-protein-cAMP-system, thus, affecting airway
4 inflammation such as in asthma and equine recurrent airway obstruction (RAO). Though
5 horses can be used as large animal model for human asthma, evaluation of the expression
6 and functions of the β -AR system in primary equine airway epithelial cells has not been yet
7 carried out. Thus, for the first time, we determined the β -AR density and subtype
8 distribution by [¹²⁵I]-iodocyanopindolol (ICYP) binding, examined β -AR function by
9 cAMP-assay as well as their expression by western blot analysis and immunocytochemical
10 staining in primary equine tracheal epithelial cells (ETEC). Cells were collected from 19
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 β -
13 ARs were $12,727 \pm 883$ binding sites/cell and 31.78 ± 6.57 pM, respectively, and in
14 cultured ETEC $3,730 \pm 212$ binding sites/cell and 15.26 ± 3.37 pM, respectively. The β -AR
15 subtype assessed by β_1 -selective (CGP 20712A) and β_2 -selective (ICI 118.551) adrenergic
16 receptor antagonists demonstrated that the β_2 -AR subtype predominated (> 95%) in both
17 cell populations ($p < 0.001$). The β -AR agonists increased cAMP formation with a rank
18 order of potency: isoproterenol > epinephrine > norepinephrine. ICI 118.551 (100 nM)
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 β_2 -AR subtype in both cell preparations. Our data indicate
22 that in acutely dissociated and primary cultured ETEC the β_2 -AR-AC system is expressed,
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 β -adrenergic receptor (β -ARs)-G-protein-adenylate cyclase system; thus, epithelial
12 damage in airway disorders and altered β -AR function lead to airway hyper-responsiveness
13 [10] and [11]. Epithelial β -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 β -AR-G-protein-adenylate cyclase system [12], [13],
16 [14] and [15], bronchodilator drugs (β -AR agonists) are, therefore, beneficial by acting on
17 β -AR located mainly on smooth muscle cells and on airway epithelial cells [16]. The
18 expression and function of the β -AR system of the airway epithelial cells from asthmatic
19 human or animals might differ from that of normal individuals, thus, information with
20 regard to this receptor system should provide the physiological, pathological and
21 pharmacological importance of airway epithelial cells.

22 The expression of functional β -ARs has been described in airway epithelial cells obtained
23 from humans and several other laboratory animals [17], [18], [19] and [20]. Even though
24 horses with RAO, a disease which is similar to human bronchial asthma, can be used as
25 large animal model for the human asthma [21] and [22], the extent to which the β -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 β -ARs and cAMP accumulation in freshly isolated and
4 primary cultures of equine tracheal epithelial cells (ETEC) from adult horses.

ACCEPTED MANUSCRIPT

1 2. Materials and Method

2 2.1. Chemicals

3 (-)-[¹²⁵I]-iodocyanopindolol (specific activity: 2200 Ci/mmol) and AlphaScreen cAMP
4 assay kit were purchased from PerkinElmer (Boston, MA, USA). ICI118.551 (erythro-
5 (+/-)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol), (±)-CGP12177 [(±)-4-(3-
6 tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on], CGP20712A (1-[2-((3-
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.

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,
18 Austria). Tracheal segments were opened longitudinally, rinsed again and the epithelium-
19 mucosal layer was removed and about 500 mg manually minced epithelial tissue was
20 digested with 10 ml HBSS containing 0.25% porcine trypsin and 0.6 mM EDTA (Sigma-
21 Aldrich Chemie GmbH, Steinheim, Germany) for 2 h at 37° C in 95% O₂ : 5% CO₂
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
25 strainers (BD Biosciences, Heidelberg, Germany). Cells were then centrifuged (4°C) at 200
26 × g for 10 min and resultant pellet was resuspended in supplemented serum-free Airway

1 Epithelial Cell Growth Medium (AECGM) (PromoCell, Heidelberg, Germany). Cell
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
4 marker cytokeratin. For binding studies in freshly isolated ETEC, cell density was adjusted
5 to 10^6 cells/ml in supplemented AECGM. Further, ETEC were cultured in supplemented
6 AECGM at a density of 7×10^4 cells /cm². At confluence of about 75%, cells were detached
7 using 0.1% trypsin/EDTA, counted and adjusted at a density of 10^6 cells/ml for binding
8 studies.

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 -
12 [¹²⁵I] (-)-iodocyanopindolol (ICYP) binding as described by Abraham et al. [20]. Suspensions
13 of freshly isolated or cultured primary equine tracheal epithelial cells (150,000 cells in 150
14 μ l/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
16 90 min at 37°C in a shaking water bath in a total volume of 250 μ l. The assay was performed
17 in the presence (i.e. non-specific binding) or absence (i.e. total binding) of 1 μ M (\pm)-CGP
18 12177, a non-selective β -adrenergic receptor antagonist. The incubation was terminated by
19 the addition of 8 ml of ice-cold wash buffer (10 mM Tris-base and 154 mM NaCl, pH 7.4)
20 followed by a rapid vacuum filtration through pre-soaked Whatman GF/C glass fibre filters
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
24 Wallac GmbH, Freiburg, Germany) at an efficiency > 80%. Specific binding was defined as
25 the difference between total ICYP binding and non-specific binding. All assays were
26 performed in duplicate.

1 For determining the β -AR subtype distribution, intact freshly isolated or cultured ETEC
2 (150,000 cells/assay) were incubated with fixed ICYP concentration (40-45pM) in the
3 presence or absence of increasing concentrations (10^{-10} - 10^{-4} M) of the β_2 -AR selective
4 antagonist ICI 118.551, or β_1 -AR selective antagonist CGP 20712A for 90 min at 37°C in
5 incubation buffer (10 mM Tris-base, 154 mM NaCl and 0.55 mM ascorbic acid, pH 7.4).
6 Determination of non-specific binding, definition of specific binding and termination of the
7 binding procedures were performed as described above for saturation binding.

8

9 **2.4. Western blot analysis**

10 The abundance of the β_2 -AR in ETEC was determined by Western blot analysis using
11 subtype specific antiserum against β_2 -AR (Santa Cruz Biotechnology, Santa Cruz, CA,
12 USA). ETEC were homogenized in 1 mM KHCO_3 and protein concentration was
13 determined by the method of Lowry. For immunoblotting, whole cell lysates were
14 dissolved with an equal volume of sample buffer (60 mM Tris-HCl, 25% glycerol, 2%
15 SDS, 14.4 mM mercaptoethanol, 0.1% bromophenol blue) and boiled for 5 min. Membrane
16 proteins (20 $\mu\text{g}/\text{lane}$) 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
18 Protean III apparatus (Bio-Rad Laboratories, Munich, Germany). Subsequently, proteins
19 were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences,
20 Freiburg, Germany) using the wet transfer system (Bio-Rad Laboratories, Munich,
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_2 , 140
23 mM NaCl), containing 1% BSA and 0.1% Tween-20. Thereafter, blots were reacted for 1 h
24 at room temperature with rabbit polyclonal β_2 -AR antibody (H-73) (1:200) diluted in TBS-
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,
3 antibody binding to β -AR was visualized and the specific signal was developed using
4 enhanced chemiluminescence western blot detection reagent (western blue®, Promega
5 Corporation, Madison, WI, USA). Mouse brain tissue lysate was used as positive control
6 for β_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 β -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 β_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 μ g/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 β -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 μ M 3-isobutyl-1-methylxanthine (IBMX). Aliquots of 300 μ l cell
3 suspension (10^6 cells) were incubated at 37 $^{\circ}$ C for 15 min with either 30 μ l PBS/IBMX
4 (basal cAMP) or 30 μ l of increasing concentrations (10^{-9} - 10^{-5} M) of β -AR agonists in a
5 final volume of 330 μ l. Isoproterenol-induced cAMP accumulation was also measured in
6 the presence or absence of highly selective β_2 -adrenergic receptor antagonist ICI 118.551
7 (100 nM), or highly selective β_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.
9 Supernatants were collected and kept at -20 $^{\circ}$ C until analysis. The cAMP was determined in
10 thawed aliquots using AlphaScreen[®] cAMP assay kit following the manufacturer's
11 directions in 384-well white opaque microplate at room temperature in triplicate at a total
12 volume of 25 μ l/well. The standard cAMP dilutions (0-5 μ M) were prepared with PBS
13 buffer containing 100 μ M IBMX. Then, 5 μ l sample or 5 μ l standard cAMP were added to
14 each well, followed by the addition of 0.1 μ l anti-cAMP acceptor beads in 10 μ l 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 μ l of streptavidin donor
17 beads and 0.0075 μ l of biotinylated-cAMP in 10 μ l of lysis buffer (5 mM HEPES, 0.1%
18 BSA, 0.3% Tween-20, pH 7.4) was added to each well and incubated for 1 hr. Finally, the
19 AlphaScreen signals were measured by Fusion-Alpha[®] Multilabel Reader (PerkinElmer,
20 Boston, MA, USA).

21

22 **2.7. Data analyses**

23 All binding data were analyzed using an iterative, non-linear curve fitting computer
24 program GraphPad Prism (GraphPad Software, San Diego, CA). The maximal number of
25 ICYP binding sites (B_{max}) and the equilibrium dissociation constant (K_D) were calculated
26 from saturation curves, also by Scatchard analysis [25]. The inhibition concentrations (IC_{50}

1 values) for antagonists were calculated from concentration-inhibition curves, and the
2 inhibition constants (K_I) 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:

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

$$8 \quad X = \text{cAMP [nM]} = 10^{\log_{10} EC_{50} - (\log_{10}((\text{Top} - \text{Bottom}) / Y[\text{cps}] - \text{Bottom}) - 1) / \text{hill slope}}$$

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
4 ICYP binding sites was observed between 60-150 pM (Fig. 1 and 2). Non-specific binding
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 β -adrenergic receptor
7 binding sites per cell (B_{\max}) obtained from saturation binding experiments was greater in
8 acutely dissociated than in cultured ETEC. Freshly isolated and cultured ETEC expressed
9 $12,727 \pm 883$ and $3,730 \pm 212$ β -ARs/cell, respectively ($n = 7$, $p < 0.001$). However, there
10 was no significant difference between ligand binding affinities (K_D) 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
13 line ($r^2 > 0.9$) indicating the presence of one class of specific binding sites in primary
14 equine tracheal epithelial cells. B_{\max} and K_D obtained here were similar to those obtained
15 from non-linear regression fit.

16 The percentage of β -AR subtypes (β_1 -AR and β_2 -AR) measured by competitive
17 displacement of ICYP binding was identical in both ETEC preparations ($n = 7$). In both cell
18 preparations, the concentration inhibition curves of the β_2 -AR selective antagonist ICI
19 118.551 and the β_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 β_2 -AR subtype was the predominant receptor subtype ($>$
23 95%) expressed by both ETEC preparations, despite different inhibition affinities of the
24 two ligands in various cell preparations (K_I for ICI 118.551: 1.19 ± 0.41 nM and $1.74 \pm$

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

3

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

5 To affirm the cellular distribution of β_2 -AR, we compared the expression of this receptor in
6 freshly dissociated and cultured ETEC by western blot analysis and immunocytochemical
7 staining. As shown in Fig. 4A, using antibody against human β_2 -adrenergic receptors, a
8 protein band for β_2 -AR was detected at molecular weight of about 72 kDa in both ETEC
9 preparations; indeed, higher β_2 -AR expression was seen in freshly isolated ETEC. There
10 was no change at expression levels of β -actin in cultured and acutely dissociated ETEC
11 (Fig. 4B). Moreover, immunocytochemistry of β_2 -AR in ETEC showed strong
12 immunoreactive signals, confirming the expression of the receptor in intact epithelial cells
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
18 and norepinephrine stimulation for 15 min are shown in Fig. 5. The cAMP accumulation
19 increased with increasing concentration of these β -AR agonists in both cell preparations.
20 However, concentration of formed cAMP per cell number was greater in freshly isolated
21 ETEC than in cultured cells. In freshly isolated ETEC, the half-maximal effective
22 concentration (EC_{50}) of the β -AR agonist-response curves was 58 nM, 13.55 μ M and 430
23 μ M ($n = 6$) for isoproterenol, epinephrine and norepinephrine, respectively. cAMP
24 accumulation of these β -AR agonist at the highest concentration tested (10 μ M) was,
25 respectively, 50 nmol/ 10^6 cells, 30 nmol/ 10^6 cells and 18 nmol/ 10^6 cells. At the same

1 agonist concentrations, in primary culture cells, isoproterenol, epinephrine and
2 norepinephrine induced maximum cAMP accumulation (n = 6) with 27 nmol/10⁶ cells, 20
3 nmol/10⁶ cells and 17 nmol/10⁶ cells, respectively. The agonists were able to increase
4 cAMP formation with an order of potency isoproterenol > epinephrine > norepinephrine,
5 which is characteristic for the existence of the β_2 -AR subtype. Moreover, the isoproterenol-
6 stimulated cAMP production in freshly isolated cells and primary cultures (Fig. 6) was
7 attenuated significantly (p < 0.05) by 100 nM of β_2 -AR selective antagonist ICI 118.551
8 but not by the β_1 -AR selective antagonist CGP 20712A, suggesting also the predominant
9 expression of the β_2 -AR subtype in these cells.

1 4. Discussion

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

10 Throughout the mammalian respiratory system the β_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]
12 whereby the density of β_2 -ARs are greater in epithelial than in smooth muscle cells [28]. A
13 number of studies have demonstrated that the epithelial β -ARs are reduced in diseases such as
14 asthma, chronic obstructive lung diseases and cystic fibrosis [29]. A similar reduction in β -
15 AR-G-protein-adenylate cyclase system has been observed in airways of horses with recurrent
16 airway obstruction (RAO) [15]. However, the alteration of the β_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 β -AR system in RAO and represent
21 an important target of the class of β_2 -adrenergic drugs. However, no appropriate cell culture
22 models of the airway epithelium were available for the horse which expresses functional β_2 -
23 AR and thus, could give insight in the underlying mechanisms of β -adrenergic drug
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 β -AR expression and
3 function. It could be demonstrated that in both freshly isolated and cultured ETEC, ICYP
4 binding clearly showed the criteria expected of ligand binding to cell surface β -AR. It was
5 saturable and reversible and of high affinity in both cell preparations (K_D value in fresh and
6 cultured ETEC: 31.78 ± 6.57 pM and 15.26 ± 3.37 pM, respectively). Though not significant,
7 there was a difference in K_D values between the two cell types. The relatively higher K_D value
8 for ICYP binding was, however, in agreement with K_D values found in freshly isolated human
9 tracheal epithelial cells (K_D : 35 ± 14 pM [17] and K_D : 29.7 pM [31]). The K_D for primary
10 cultures was comparable with data from Davis et al. [17] who found K_D value of 25 ± 13 pM
11 for cultured primary human tracheal epithelial cells, more similarity exists with studies from
12 Madison et al. [32] who determined from radioligand binding studies a K_D value of 20 ± 3 pM
13 in cultured bovine tracheal submucosal gland cells.

14 With regard to the receptor density, greater number of β -AR binding sites were detected in
15 primary freshly isolated ETEC ($12,727 \pm 883$ binding sites/cell) than in cultured ETEC ($3,730$
16 ± 212 binding sites/cell). This discrepancy in β -AR expression between the two cell
17 preparations presumably appears to be explained by differences in epithelial cell
18 subpopulation, cell size and culturing of ETEC. Acutely isolated primary equine tracheal
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 β -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
24 carried out in distinct serum-free but supplemented airway epithelial cell growth medium.
25 Serum supplementation often affects cell differentiation and cellular protein expression. In
26 permanent airway epithelial cell lines which were cultured in serum-containing media, β -AR

1 binding sites were comparable with those of freshly isolated ETEC; high density of β_2 -ARs
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
4 epithelial cells ($7,000 - 9,000$ β -ARs/cell; Kelsen et al. [34]), in freshly isolated rabbit
5 tracheal epithelial cells ($10,700$ β -ARs/cell; Mardini et al. [35]) as well as in primary cultures
6 of bovine bronchial epithelial cells ($13,000$ β -ARs/cell; Nogami et al. [36]). The low density
7 of binding sites observed in primary cultured ETEC were comparable with the β -AR density
8 found in primary cultures of human tracheobronchial epithelial cells ($3,795 \pm 957$ binding
9 sites/cell; Aksoy et al [37]).

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

17 Competitive ICYP binding assays employing the β -AR selective antagonist ICI 118.551 (β_2)
18 and CGP 20712A (β_1) showed that in both ETEC preparations the β_2 -AR subtype is
19 predominant ($> 95\%$). The present results are in good agreement with reports in the literature
20 that the β_2 -AR represents the mere binding sites as for both antagonists. Identical results were
21 found in for ICI 118.551 and betaxolol (β_1 -selective) in BEAS-2B cells [37]. Percentages of
22 β_2 - and β_1 -ARs were 80% and 20% in freshly isolated human airway epithelial cells [34] and
23 $> 90\%$ in primary rat tracheal epithelial cells [33], which agrees with the present data obtained
24 from primary equine tracheal epithelial cells.

1 Finally, in both freshly isolated and cultured primary equine tracheal epithelial cells,
2 stimulation of β -AR system by β -AR agonists induced the formation of intracellular cAMP,
3 suggesting the functional coupling of the adenylate cyclase to the receptor. Generally, in
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
6 cAMP production was diminished only by β_2 -AR selective antagonist ICI 118.551, but not
7 by β_1 -AR selective antagonist CGP 20712A, strongly suggesting the β_2 -AR-mediated
8 cAMP formation in these cells. The less cAMP responses to β -AR agonists in cultured
9 ETEC suggest that the β -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 β_2 -AR subtype
13 [41]. The magnitude of the isoproterenol-stimulated cAMP response in ETEC was
14 comparable to that observed in primary airway epithelial cells [33] and airway epithelial
15 cell lines [20].

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

22 **Acknowledgment**

23 This work was supported financially by the Ernst von Weber Stiftung (project no.
24 25140196). Workineh Shibeshi received a PhD scholarship from the German Academic
25 Exchange Service (DAAD). We thank Dr. Sascha Kretzing for editing assistance.

1 **References**

- 2 [1] Rogers DF. Physiology of airway mucus secretion and pathophysiology of
3 Hypersecretion. *Respir Care* 2007; 52:1134-46.
- 4 [2] Blouquit-Laye S, Chinet T. Ion and liquid transport across the bronchiolar epithelium.
5 *Respir Physiol Neurobiol* 2007; 159:278-82.
- 6 [3] Davidson DJ, Gray MA, Kilanowski FM, Tarran R, Randell SH, Sheppard DN,
7 Argent BE, Dorin JR. Murine epithelial cells: isolation and culture. *J Cyst Fibros*
8 2004; 3:59-62.
- 9 [4] Ninomiya H, Yu XY, Uchida Y, Hasegawa S, Spannhake EW. Specific binding of
10 endothelin-1 to canine tracheal epithelial cells in culture. *Am J Physiol* 1995;
11 268:L424-31.
- 12 [5] Wilkens JH, Becker A, Wilkens H, Emura M, Riebe-Imre M, Plein K, Schöber S,
13 Tsikas D, Gutzki FM, Frölich JC. Bioassay of a tracheal smooth muscle-constricting
14 factor released by respiratory epithelial cells. *Am J Physiol* 1992; 263:L137-41.
- 15 [6] Polito AJ, Proud D. Epithelia cells as regulators of airway inflammation. *J Allergy*
16 *Clin Immunol* 1998; 102:714-18.
- 17 [7] Forrest IA, Murphy DM, Ward C, Jones D, Johnson GE, Archer L, Gould FK,
18 Cawston TE, Lordan JL, Corris PA. Primary airway epithelial cell culture from lung
19 transplant recipients. *Eur Respir J* 2005; 26:1080-5.
- 20 [8] Levine SJ. Bronchial epithelial cell-cytokine interactions in airway inflammation. *J*
21 *Investig Med* 1995; 43:241-9.
- 22 [9] Radi ZA, Ackermann MR. Growth of differentiated ovine tracheal epithelial cells in
23 vitro. *J Vet Med A Physiol Pathol Clin Med* 2004; 51:167-70.
- 24 [10] Nijkamp FP, Engels F, Henricks PA, Van Oosterhout AJ. Mechanisms of beta-
25 adrenergic receptor regulation in lungs and its implications for physiological
26 responses. *Physiol Rev* 1992; 72:323-67.

- 1 [11] Folkerts G, Nijkamp F.P. Airway epithelium: more than just a barrier, *Trends*
2 *Pharmacol Sci* 1998; 19:334-41.
- 3 [12] Goldie RG, Spina D, Henry PJ, Lulich KM, Paterson JW. In vitro responsiveness of
4 human asthmatic bronchus to carbachol, histamine, beta-adrenoceptor agonists and
5 theophylline. *Br J Clin Pharmacol* 1986; 22:669-76.
- 6 [13] Bai TR. Abnormalities in airway smooth muscle in fatal asthma. *Am Rev Respir Dis*
7 1990; 41:552-7.
- 8 [14] Gosens R, Zaagsma J, Meurs H, Halayko AJ. Muscarinic receptor signaling in the
9 pathophysiology of asthma and COPD. *Respir Res* 2006; 7:73.
- 10 [15] Abraham G, Kottke C, Dhein S, Ungemach FR. Agonist-independent alteration in
11 beta-adrenoceptor-G-protein-adenylate cyclase system in an equine model of
12 recurrent airway obstruction. *Pulm Pharmacol Ther* 2006; 19:218-29.
- 13 [16] McGraw DW, Forbes SL, Mak JC, Witte DP, Carrigan PE, Leikauf GD, Liggett SB.
14 Transgenic overexpression of beta (2)-adrenergic receptors in airway epithelial cells
15 decreases bronchoconstriction. *Am J Physiol* 2000; 279: L379-89.
- 16 [17] Davis PB, Silski CL, Kercksmar CM, Infeld M. Beta-adrenergic receptors on human
17 tracheal epithelial cells in primary culture. *Am J Physiol* 1990; 258:C71-C76.
- 18 [18] Kelsen SG, Higgins NC, Zhou S, Mardini IA, Benovic JL., Expression and function
19 of the beta-adrenergic receptor coupled-adenylyl cyclase system on human airway
20 epithelial cells. *Am J Respir Crit Care Med* 1995; 152:1774-83.
- 21 [19] Giembycz MA, Newton R. Beyond the dogma: novel beta2-adrenoceptor signalling in
22 the airways. *Eur Respir J* 2006; 27:1286-1306.
- 23 [20] Abraham G, Kneuer C, Ehrhardt C, Honscha W, Ungemach FR. Expression of
24 functional beta2-adrenergic receptors in the lung epithelial cell lines 16HBE14o(-),
25 Calu-3 and A549. *Biochim Biophys Acta* 2004;1691:169-79.

- 1 [21] Turlej RK, Fiévez L, Sandersen CF, Dogné S, Kirschvink N, Lekeux P, Bureau F.
2 Enhanced survival of lung granulocytes in an animal model of asthma: evidence for
3 role of GM-CSF activated STAT5 signalling pathway. *Thorax* 2001; 56:696-702.
- 4 [22] Hein WR, Griebel PJ. A road less travelled: large animal models in immunological
5 research. *Nat Rev Immunol* 2003; 3:79-84.
- 6 [23] Shibeshi W, Abraham G, Kneuer C, Ellenberger C, Seeger J, Schoon HA, Ungemach
7 FR. Isolation and culture of primary equine tracheal epithelial cells. *In Vitro Cell Dev*
8 *Biol Anim* 2008; 44:179-84.
- 9 [24] Laemmli U.K. Cleavage of structural proteins during the assembly of the head of
10 bacteriophage T₄. *Nature* 1970; 227:680-85.
- 11 [25] Scatchard G. The interaction of proteins for molecules and ions. *Ann NY Acad Sci*
12 1949; 51:660–72.
- 13 [26] Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the
14 concentration of inhibitor which causes 50 percent inhibition (IC₅₀) of an enzymatic
15 reaction. *Biochem. Pharmacol* 1973; 22:3099-108.
- 16 [27] Carstairs JR, Nimmo AJ, Barnes PJ. Autoradiographic visualization of beta-
17 adrenoceptor subtypes in human lung. *Am Rev Respir Dis* 1985; 132:541-47.
- 18 [28] Agrawal DK, Schugel JW, Townley RG. Comparison of beta adrenoceptors in bovine
19 airway epithelium and smooth muscle cells. *Biochem Biophys Res Commun* 1987;
20 148:178-83.
- 21 [29] Sharma RK, Jeffery PK. Airway beta-adrenoceptor number in cystic fibrosis and asthma
22 *Clin Sci* 1990; 78:409-17.
- 23 [30] Marinkovic D, Aleksic-Kovacevic S, Plamenac P. Cellular basis of chronic
24 obstructive pulmonary disease in horses. *Int Rev Cytol* 2007; 257:213-47.

- 1 [31] Kelsen SG, Ciccolella D, Brennan K. Brush biopsy and culture of airway epithelial
2 cells: β -adrenoceptor system function. *Methods in Molecular Medicine in Asthma*
3 *mechanisms and protocols* 2000; 44:41-52.
- 4 [32] Madison JM, Basbaum CB, Brown JK, Finkbeiner WE. Characterization of beta-
5 adrenergic receptors in cultured bovine tracheal gland cells. *Am J Physiol* 1989;
6 256:C310-14.
- 7 [33] Kelsen SG, Zhou S, Anakwe O, Mardini I, Higgins N, Benovic JL. Expression of the
8 beta-adrenergic receptor-adenylyl cyclase system in basal and columnar airway
9 epithelial cells. *Am J Physiol* 1994; 267:L456-63.
- 10 [34] Kelsen SG, Higgins NC, Zhou S, Mardini IA, Benovic JL. Expression and function of
11 the beta-adrenergic receptor coupled-adenylyl cyclase system on human airway
12 epithelial cells. *Am J Respir Crit Care Med* 1995; 152:1774-83.
- 13 [35] Mardini IA, Higgins NC, Zhou S, Benovic JL, Kelsen SG. Functional behavior of the
14 beta-adrenergic receptor-adenylyl cyclase system in rabbit airway epithelium. *Am J*
15 *Respir Cell Mol Biol* 1994; **11**:287-95.
- 16 [36] Nogami M, Romberger DJ, Rennard SI, Toews ML. Agonist-induced desensitization
17 of beta-adrenoceptors of bovine bronchial epithelial cells. *Clin Sci* 1993; 85:651-57.
- 18 [37] Aksoy MO, Mardini IA, Yang Y, Bin W, Zhou S, Kelsen SG. Glucocorticoid effects
19 on the beta-adrenergic receptor-adenylyl cyclase system of human airway epithelium.
20 *J Allergy Clin Immunol* 2002; 109:491-97.
- 21 [38] Insoft R, Sawutz DG, Homcy CJ. Purification and characterization of the beta 2
22 adrenergic receptor from calf lung. *Biochim Biophys Acta* 1986; 861:345-52.
- 23 [39] Benovic JL, Shorr RG, Caron MG, Lefkowitz RJ. The mammalian beta 2-adrenergic
24 receptor: purification and characterization. *Biochemistry* 1984; 23:4510-8.
- 25 [40] George ST, Ruoho AE, Malbon CC. N-glycosylation in expression and function of
26 beta-adrenergic receptors. *Biol Chem* 1986; 261:16559-64.

1 [41] Lands AM, Arnold A, McAuliff JP, Luduena FP, Braun TG. Differentiation of
2 receptor systems activated by sympathomimetic amines. *Nature* 1967; 214:597-98.

3

ACCEPTED MANUSCRIPT

1 **Legends**

2

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 μ M (\pm)-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 μ M (\pm)-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 β_2 -AR selective antagonist ICI 118.551
23 and β_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%

1 binding represents the specific ICYP binding detected in the presence of 1 μ M (\pm)-CGP
2 12177 and in the absence competing ligands.

3

4 Figure 4:

5 Expression of β -adrenergic receptors in primary equine tracheal epithelial cells. A) Western
6 blot signals obtained with rabbit polyclonal β_2 -AR antibody of human origin and B) the
7 corresponding β -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 β_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- β_2 -AR stained cells; c and f show corresponding DAPI nuclear staining; b and e
12 show overlays of DAPI stained nuclei and anti- β_2 -AR stained cell membranes. Positive FITC
13 signal is shown green, and DAPI staining nuclei is blue. Data shown are representative of
14 three independent experiments.

15

16 Figure 5:

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

22

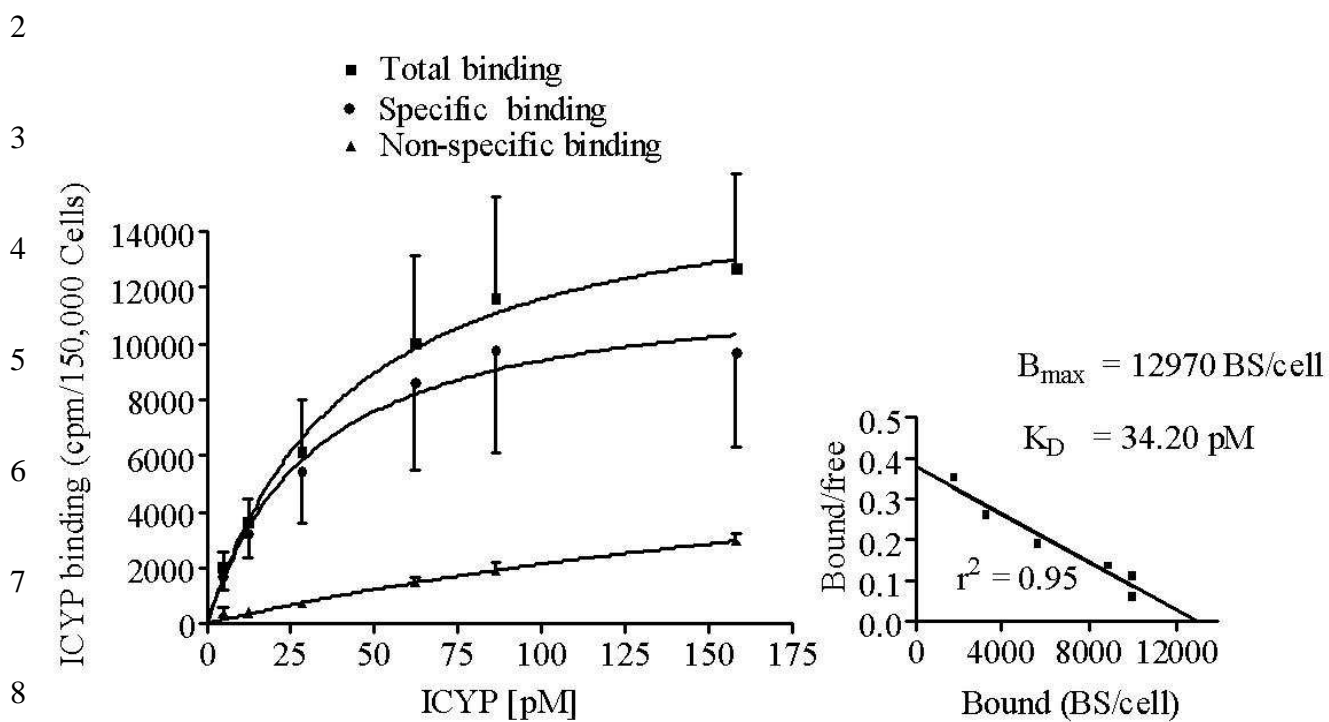
23 Figure 6:

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

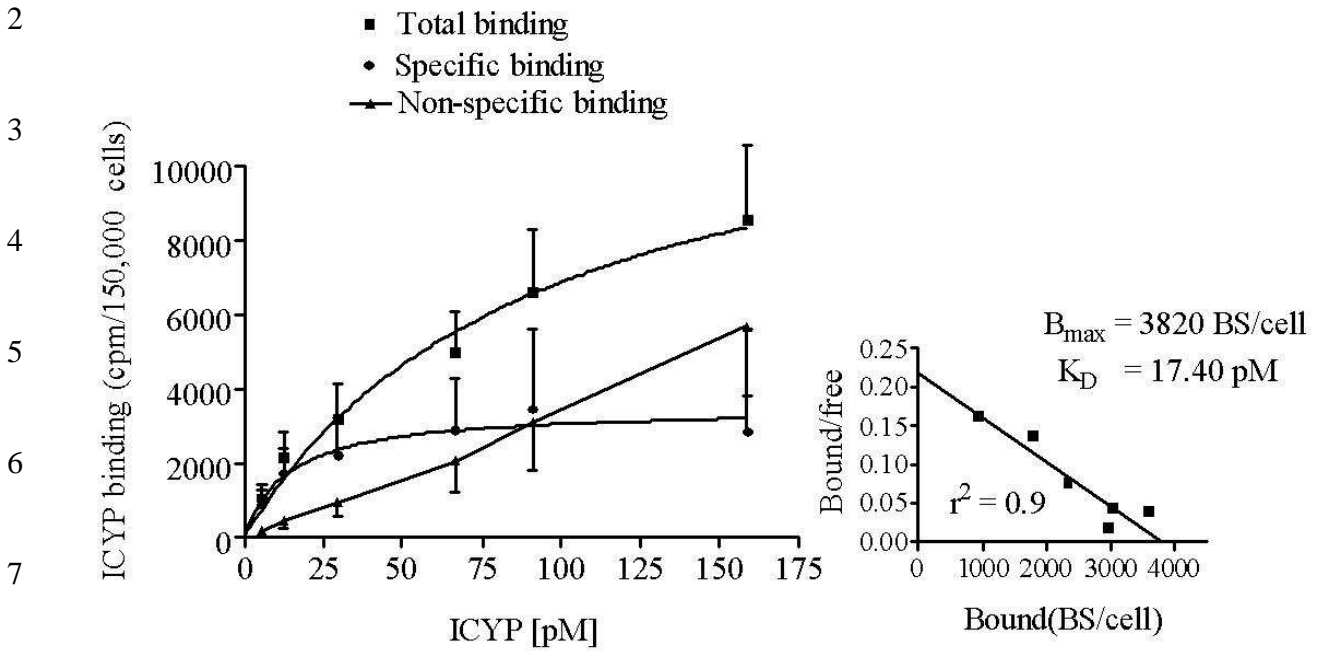
- 1 in the presence of or 300 nM of CGP 20712A. Values are mean \pm SEM for 5 independent
- 2 experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

ACCEPTED MANUSCRIPT

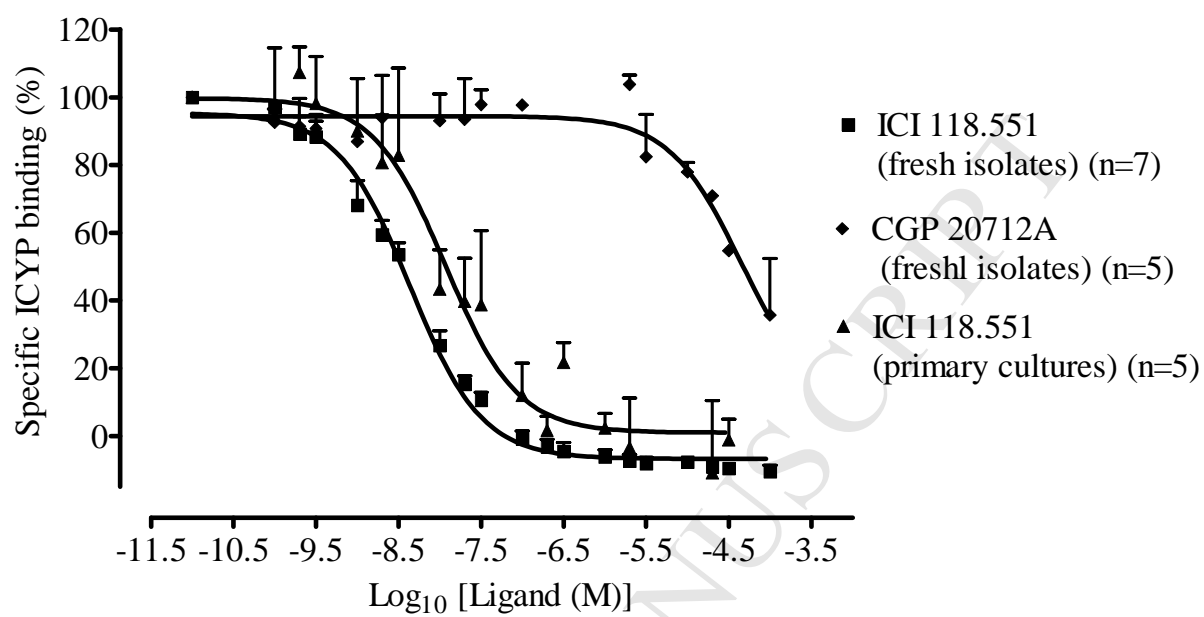
1 Figure 1



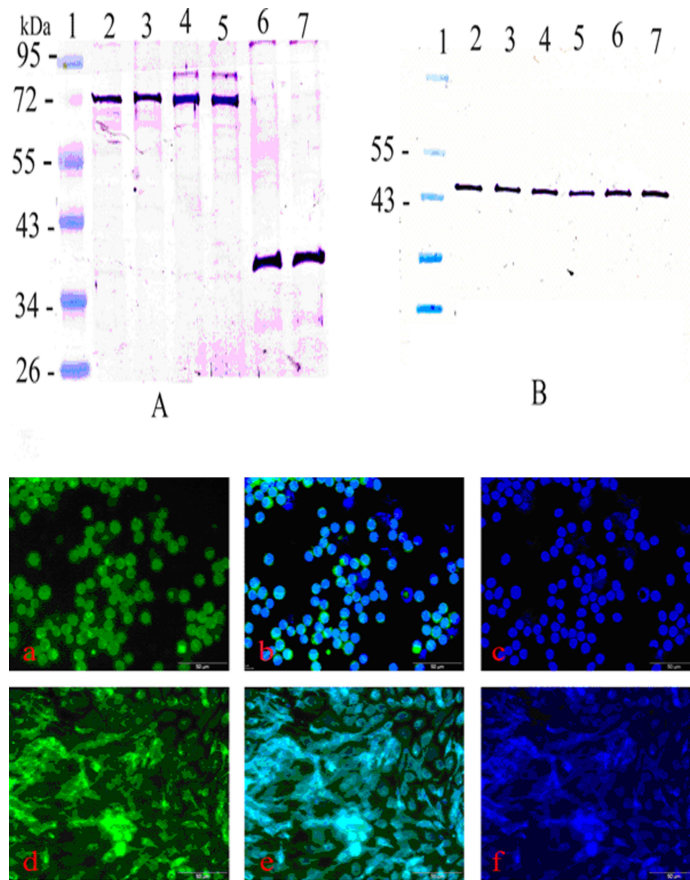
1 Figure 2



1 Figure 3

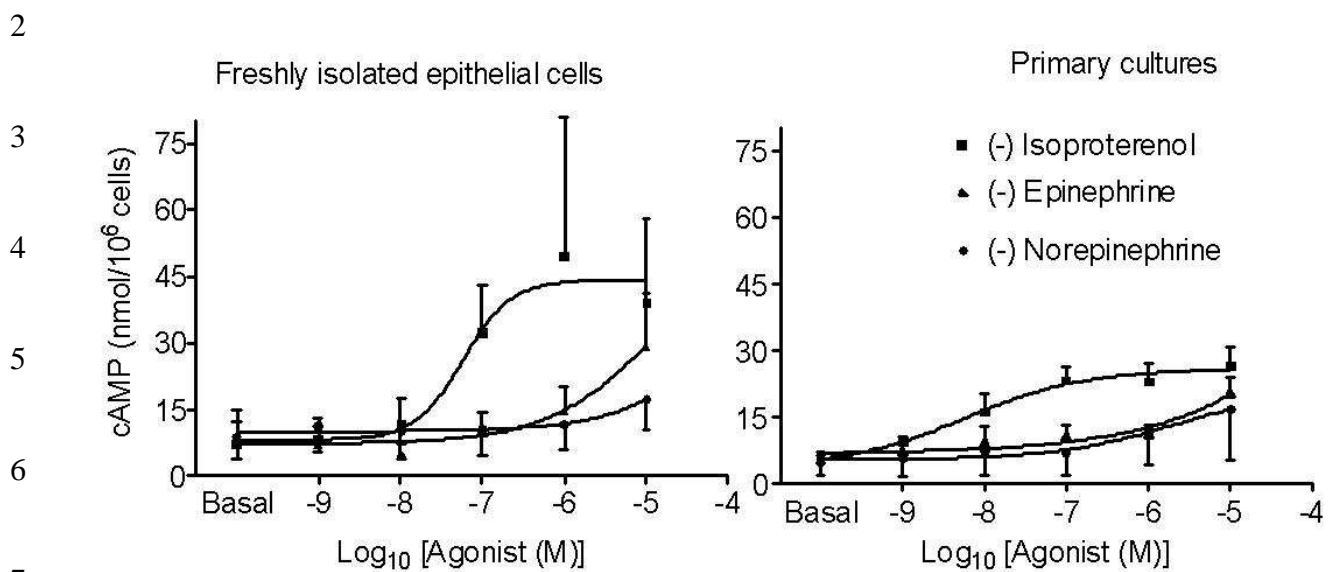


1 Figure 4



2

1 Figure 5



1 Figure 6

