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Pharmacological and biochemical characterization of the beta-adrenergic signal transduction pathway in different segments of the respiratory tract

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Abstract

Although in the respiratory system there is great therapeutic interest in manipulating and understanding the β-adrenoceptor–G-protein–adenylate cyclase (AC) signal transduction pathway, little is known on segmental differences among lung, bronchus, and trachea with regard to the receptor concentration and interaction to G-proteins and coupling to AC. In this study, patterns of distribution and absolute quantities of β-adrenoceptor subtypes β_1 and β_2 were determined in membranes of equine lung parenchyma, bronchial and tracheal epithelium with the underlying smooth muscle by saturation and competition binding assays using the radioligand (−)-[^{125}I]-iodocyanopindolol (ICYP). Additionally, the functional coupling of β-adrenoceptors to G-proteins (assessed by β-agonist competition binding in the presence and absence of GTP) as well as the coupling efficiency and biochemical activities of AC was investigated in each region. The specific ICYP binding was rapid, reversible, saturable with time and of high affinity. The radioligand binding identified more total β-adrenoceptors in the lung than in bronchus or trachea (428 ± 19, 162.4 ± 4.8, 75.6 ± 1.2 fmol/mg protein, respectively) with about 40% of receptors in the high affinity state. The β_2-adrenoceptor subtype predominated in all segments (approximately 74–80%), as the highly selective β_2-adrenoceptor antagonist ICI 118,551 was about 10,000 times more potent in inhibiting ICYP binding than was the β_1-selective adrenoceptor antagonist CGP 20712A, and β-adrenoceptor agonists inhibited ICYP binding with an order of potency: (−)-isoprenaline > (−)-adrenaline > (−)-noradrenaline. The dissociation constant (K_d) was higher in the trachea than in bronchus or lung (13.0 ± 0.9 pM vs. 20.0 ± 2.3 pM vs. 30.8 ± 4.4 pM, $P < 0.05$, respectively). The β_2-adrenoceptor-mediated AC response was tissue-dependent; stimulants acting on β-adrenoceptor (isoproterenol), G-protein (GTP, NaF) and AC (forskolin, Mn^{2+}) enhanced AC responses in all three regions, but the AC activity was higher in tracheal crude membranes than in bronchus or lung (trachea ⋙ bronchus > lung), hence, the number of β_2-adrenoceptors correlated inversely with the amount of AC. We conclude that (1) the stoichiometry of components within the pulmonary β-adrenoceptor–G-protein complex is segment-dependent, and (2) the receptor number or AC activity is possibly the rate-limiting factor in the β-adrenoceptor–G-protein–AC-mediated physiological responses. Thus, it is speculated that this could have important therapeutic consequences in β-adrenoceptor agonist-induced receptor regulation in bronchial asthma.

Keywords: β-Adrenoceptor; Radioligand binding; ICYP; Adenylate cyclase; Cyclic AMP; Equine; Lung; Bronchus; Trachea

1. Introduction

β-Adrenoceptors, mainly of the β_2-subtype, are known to mediate various important physiological functions in the respiratory system in man and animals, including smooth muscle relaxation, clearance of alveolar fluid, influence of ion fluxes, modulating the release of bronchodilating mediators [1]. For the assessment of the regulation of pulmonary responses to drugs the classical pharmacological preparation which widely used is human and non-human

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Abbreviations: AC, adenylate cyclase; cAMP, cyclic adenosine 3′,5′-monophosphate; (±)-CGP 12177, (±)-4-(3-tertiarybutylamino-2-hydroxy-propoxy)-benzimidazole-2-ol; CGP 20712A, 1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-imidazolyl)-phenoxy]-2-propanol; GTP, guanosine 5′-triphosphate; GPCRs, G-protein-coupled receptors; ICI 118,551, erythro-(+/−)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol; ICYP, (−)[^{125}I]-iodocyanopindolol; PKA, protein kinase A.

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airway smooth muscle, particularly of tracheal smooth muscle, and less frequently bronchial smooth muscle. However, especially small and bronchial airway smooth muscles are therapeutic targets in human and non-human broncho-obstructive disorders. Activation of airway smooth muscle relaxation or contraction involves all known forms of transmembrane signaling through GPCRs [2]. It is well known that multiple GPCRs are expressed in airways smooth muscle, including β-adrenoceptors, mainly of the β_2-subtype which, upon stimulation, can reduce smooth muscle tone [3]. Airway epithelium which masks the underlying smooth muscle is not only known as a physiological defense barrier against exogenous noxious substances but also to release anti- or pro-inflammatory mediators, and other factors which regulate in such a way the smooth muscle tone [4]. Mediator release from airway epithelial cells is known to be mediated by β_2-adrenergic receptor–G-protein–AC system [4].

It is generally well established that these GPCRs exert their effects by coupling to the heterotrimeric G-protein G_S and stimulation of the AC activity. The activity of this effector protein in smooth muscle induces the synthesis of the second messenger cAMP, which, via activation of PKA, alters intracellular Ca^{2+} dynamics and contractile function by phosphorylating calcium channels, Ca^{2+}-ATPases, and myosin light-chain kinase [5–7]. In addition, β-adrenergic agonists which are widely used as bronchodilator seem to induce relaxation via a cAMP- and PKA-independent mechanism in smooth muscle from some tissues [8–10].

In addition, many studies have shown that in most animal species as well as in humans predominantly G_S-coupled β_2-adrenoceptor subtypes are identified in peripheral lung [11–13], in tracheal [14] and bronchial smooth muscle [15]. Topographical differences existed in the number of receptors and functional responses. However, studies which assess quantification of the signaling components, in whole, β-adrenoceptors and subtypes, G-protein (receptor affinity), AC in equine peripheral lung and proximal, in bronchial and tracheal epithelium with the underlying smooth muscle are still missing. Moreover, it is unclear, whether intra-pulmonal and segmental differences exist in the interaction between the signaling components. To investigate the efficacy based on second messenger production, research has been conducted in murine lymphoma cells [16] in rat cardiomyocytes [17] and NG 108-15 cells [18].

Thus, the present study should systematically examine, in equine lung parenchyma, in bronchial and tracheal epithelium with the underlying smooth muscle, the β-adrenoceptor–G-protein–AC signal transduction system, and in particular, whether regional differences exist in receptor concentration, pattern of distribution, affinity of the receptor to G-protein coupling, and receptor–G-protein-mediated activation of AC. We contrasted three respiratory segments with regard to β-adrenoceptor-mediated stimulation of AC with response to stimulants acting at the level of the receptor or G-proteins or AC itself. Distal to proximal in the respiratory segments, our study revealed that β-adrenergic concentration was inversely correlated with AC activity with no remarkable change in the receptor affinity state.

2. Materials and methods

2.1. Chemicals

ICYP (specific activity: 2200 Ci/mmol) and [α-^{32}P]-ATP (specific activity: 30 Ci/mmol) were purchased from Perkin-Elmer Life Sciences. [^{3}H]-Cyclic AMP (specific activity: 44.5 Ci/mmol) was obtained from Amersham Biosciences. ICI 118,551, CGP 20712A, (±)-CGP 12177, (−)-Isoproterenol bitartrate, (−)-adrenaline hydrochloride, (−)-noradrenaline hydrochloride, (−)-propranolol hydrochloride, (+)-propranolol hydrochloride, GTP, forskolin, NaF, ATP, $MnCl_2$, and bovine serum albumin were from Sigma Chemical Co. cAMP, creatine, phosphocreatine and creatine kinase were bought from Boehringer. All other chemicals were of analytical grade commercially available.

2.2. Animals

Eight adult thoroughbred horses of 12–20 years of age were killed at a local abattoir after clinical examination was ahead. The horses received hay and water *ad libitum*. The horses in this study had not previously received at least 3 months prior to slaughter any medications except routine vaccination. Within 1 hr after death, fresh lung parenchyma, secondary and small bronchi and tracheas were obtained and kept on ice during transport to the laboratory. Only lungs and airway segments without any pathological alterations were used for this study. The whole trachea, secondary and small bronchus were dissected free of excess connective tissues and the epithelium with the underlying smooth muscle was removed from cartilage. Peripheral lung tissue was prepared free of pleura, large blood vessels, large airways and connective tissue. These samples, approximately 1 g, were snap frozen in liquid nitrogen and stored at −70°.

2.3. Membrane preparation

For the assessment of β-adrenoceptor binding, frozen peripheral lung which contained no visible airway structures, secondary and small bronchial as well as tracheal epithelium with the underlying smooth muscle were thawed on ice in 20 vol. (w/v) of ice-cold lysis buffer (20 mM $NaHCO_3$) over approximately 1 hr, and were finely minced with scissors. The slurry was homogenized with an Ultra-Turrax tissue homogenizer (Janke & Kunkel, Staufen, Germany) two times for 10 s at half maximal

speed in 1-min interval. The homogenate was centrifuged at 500 *g* for 10 min at 4°. The pellets were discarded and the supernatant was filtered through four layers of cheesecloth and then centrifuged at 40,000 *g* (Beckman UltrA–Centrifuge) for 30 min at 4°. The resulting pellets were washed once in buffer by gentle homogenization with five strokes of a Potter-Elvehjem homogenizer and recentrifuged at the same speed. The final pellets were resuspended and dispersed (homogenizer) in incubation buffer (10 mM Tris buffer, pH 7.4, 154 mM NaCl, containing 0.55 mM ascorbic acid). For the determination of AC activity, crude membranes were also prepared from peripheral lung, main bronchial and tracheal epithelium with the underlying smooth muscle after homogenizing with Ultra-Turrax tissue homogenizer three times 15 s at full speed. Membrane pellets were obtained after centrifugation at 500 *g* for 10 min at 4°. Both membrane preparations were frozen at −70° at protein concentration of 0.05–0.1 mg/mL.

2.4. Protein determination

Membrane protein concentration was determined according to the method of Lowry *et al.* [19] (assessed with Folin reagent) using bovine serum albumin as the standard.

2.5. ICYP β-adrenoceptor binding experiments

The density of β-adrenoceptors in membranes of peripheral lung, bronchial and tracheal epithelium with the underlying smooth muscle was determined by the radioligand binding using the radiolabeled β-adrenoceptor antagonist ICYP [20] as described recently [21] with 10 μg of protein from each tissue preparation. Saturation binding experiments were carried out in duplicate with six different increasing concentrations of ICYP ranging from 5 to 200 pM in 10 mM Tris buffer, pH 7.4, containing 154 mM NaCl, 0.55 mM ascorbic acid at a total volume of 250 μL. Incubations were performed at 37° for 90 min in a shaking water bath set at 120 rpm. Non-specific binding was defined as ICYP binding at various concentrations to membranes, which could not be displaced by a high concentration of the non-selective hydrophilic β-adrenoceptor antagonist (±)-CGP 12177 (1 μmol/L). Non-specific binding of ICYP amounted usually less than 30% of total ICYP binding even at highest radioligand concentration (200 pM). Specific binding was determined as the difference between ICYP binding in the absence and presence of the (±)-CGP 12177. The reaction was terminated by diluting the entire reaction mixture with 10 mL of ice-cold buffer of 10 mM Tris buffer, pH 7.4, and 154 mM NaCl followed by a rapid filtration over Whatman GF/C glass fiber filters. Each filter was washed with an additional 10 mL of the buffer. The radioactivity was determined with a gamma counter (1470 WIZARD Automatic Gamma Counter, Perkin-Elmer Life Sciences) at a counting efficiency of about 80%.

2.6. Competition binding studies

To characterize and determine the presence and the ratio of β-adrenoceptor subtypes expressed in membranes of peripheral lung, bronchial and tracheal epithelium with the underlying smooth muscle we undertaken competitive binding studies concentration-dependent with selective and non-selective β-adrenoceptor agonists and antagonists. In brief, to assess the potency of β-adrenergic agonist drugs, purified membrane protein (10 μg/tissue and tube) were incubated with fixed ICYP concentration (80 pM) and increasing concentrations (about 10–22 concentrations: 10^{-10} to 10^{-4} M) of β-adrenergic agonists ((−)-isoproterenol, (−)-adrenaline, (−)-noradrenaline), whereas the stereospecificity of ICYP binding was assessed with non-selective β-adrenergic antagonist drugs ((−)-propranolol, (+)-propranolol). Further, to evaluate the relative amount of β_1- and β_2-adreneoceptors, membrane suspension was pre-treated with subtype selective competing antagonists, highly β_1-selective adrenoceptor antagonist (CGP 20712A) and highly β_2-selective adrenoceptor antagonist (ICI 118,551) in concentrations between 10^{-10} and 10^{-4} M. Non-specific binding was determined as described above. Binding isotherm and separation of free radioactivity were performed as formerly described.

2.7. GTP-induced shift in agonist binding affinity

To proof the receptor-mediated modulation of G-protein function that G-proteins exert a reciprocal modulatory action on the function of their corresponding receptors, as the ''guanine nucleotide shift'', of the receptor to an altered affinity state, i.e. to determine the number of receptors in the high affinity state in membranes from the three tissues, we assayed GTP effects on the inhibition of ICYP binding with the β-agonist isoproterenol. Membrane suspensions were treated either with vehicle or with 100 μM GTP. ICYP (80 pM) binding to these membranes was then inhibited with increasing concentrations of (−)-isoproterenol (16 concentrations: 0.1 nM to 100 μM), measured in duplicates. The amount of specific ICYP binding was determined as described above.

2.8. Adenylate cyclase activity

Aliquots of frozen crude membrane preparation (lung, bronchus, trachea) were thawed on ice and re-centrifuged at 2000 *g* for 10 min at 4°. Pellets were resuspended in the incubation buffer containing final concentrations of 40 mM HEPES, pH 7.4, 5 mM $MgCl_2$ and 1 mM EDTA (Buffer A). AC activity was determined according to the method of Salomon *et al.* [22] with some modifications.

Assays were carried out in triplicates and at a total volume of 100 μL. To assess the contributions of G-protein-coupled processes to AC, several experimental procedures have been employed. First, basal AC activity was determined in 30–40 μg protein suspensions in the incubation buffer (see above) containing final concentrations of 0.5 mM [α-^{32}P]-ATP (about 200 000 cpm/tube), 0.1 mM cAMP and ATP regenerating system (Buffer B: 5 mM phosphocreatine and 50 U/mL creatine phosphokinase) in the presence or absence of 10 μM GTP. Second, β-adrenoceptor stimulation of AC activity via G_S-protein was determined as the assay tubes contained membranes in Buffer A and 10 μM (−)-isoproterenol in the presence of GTP. Third, we determined the response of AC in membranes to 10 μM forskolin in Buffer B without GTP. Forskolin requires association of G-proteins with AC for maximal effect [23]. Fourth, for direct AC activation membranes were incubated in the presence of 10 mM $MnCl_2$ in Buffer B without GTP and Mg^{2+} to reduce the possible influence of G_S-protein on AC activity [24]. Mn^{2+} activates AC by replacing magnesium at the active site [25]. Finally, membranes were incubated with 10 mM NaF in Buffer B without GTP to determine the net G-protein-coupled response to AC activity with maximal activation of all G-proteins [26]. All incubations were started by the addition of the crude membranes at 37° and stopped after 10 min by diluting the mixture with 100 μL of a buffer (containing cyclic [^{3}H]-AMP (8500 cpm), 70 mM SDS, 40 mM ATP, 1.4 mM non-labeled cAMP and 50 mM Tris–HCl) and 800 μL distilled water. Cyclic [^{32}P]-AMP was separated from [α-^{32}P]-ATP by elution of [α-^{32}P]-AMP with 5 mL of imidazoline by using Dowex AG 50W-X4 and neutral alumina columns. The converted cyclic [^{32}P]-AMP was determined by liquid scintillation counting with 50% counting efficiency (Tri-Carb liquid scintillation analyzer 2250 CA, Packard) using Lumasafe scintillation fluid. Less than 5% of the added [α-^{32}P]-ATP was converted to cyclic [^{32}P]-AMP in whole experiments.

2.9. Data analysis and statistics

Results are expressed as means ± SEM of N experiments. Binding data were analyzed by computer-supported non-linear regression analysis using a computer program GraphPad Prism (GraphPad Software). The maximal number of ICYP binding sites (B_{max}) and the equilibrium dissociation constant (K_d) were calculated from saturation curves by iterative non-linear least-square curve fittings for one- and two-site models. For convenience, data were also ananlyzed from Scatchard plots [27]. Similar B_{max} and K_d were obtained.

Agonist and antagonist displacement curves were analyzed using the iterative non-linear least-square curve fitting program GraphPad Prism (GraphPad Software). For statistical analysis the F-ratio test was carried out to determine the goodness of fit of the concentration–inhibition curves for either one- or two-site competition for ICYP binding sites. The inhibition concentrations (IC_{50}-values) for inhibition of binding by competing ligands were calculated from concentration–inhibition curves, and the inhibition constants (K_i) were calculated from the equation of Cheng and Prusoff [28]:

$$K_i = \frac{IC_{50}}{[L]/K_d} + 1$$

where IC_{50} is defined as the concentration of competing agonist and antagonist agents required to inhibit 50% of the specific ICYP binding, [L] is the concentration of ICYP in the assay. The K_d values for ICYP were independently estimated from saturation studies.

Statistical significance of differences was estimated by non-paired two-tailed Student's t-test or, if appropriate, by repeated measures of analysis of variance followed by the t-test using Bonferoni corrections for multiple comparisons. Significance was accepted at a level of the P-value smaller than 0.05. All statistical calculations were performed with Instat program (GraphPad Software).

3. Results

3.1. β-Adrenoceptor binding

Membranes were prepared from equine peripheral lung, secondary and small bronchial as well as tracheal epithelium with the underlying smooth muscle, and the β-adrenoceptor concentration level was determined by saturation binding studies with the labeled β-adrenoceptor antagonist ICYP. The data for ICYP binding to these membranes are shown in Fig. 1 and mean values for the three respiratory segments are summarized in Table 1. In the lung, bronchus and trachea, the specific ICYP binding was rapid, saturable, reversible and displayed high affinity with a dissociation constant (K_d) of 30.8 ± 4.4, 20.0 ± 2.3 and 13.0 ± 0.9 pM, respectively ($P < 0.05$, lung vs. bronchus,

Table 1
β-Adrenoceptor ICYP binding to membrane obtained from peripheral lung, secondary and small bronchial and tracheal epithelium with the underlying smooth muscle

	Lung	Bronchus	Trachea
B_{max} (fmol/mg protein) (N = 6)	428.2 ± 19.0	162.4 ± 4.8**	75.9 ± 1.2***,a
K_d (pM) (N = 6)	30.8 ± 4.4	20.0 ± 2.3*	13.0 ± 0.9**
Ratio β_1:β_2-subtypes (N = 6)	21:79 ± 1.5	24:76 ± 2.8	24:76 ± 3.9

Data are means ± SEM, number of experiments in parenthesis performed in duplicates.
[a] $P < 0.01$, bronchus vs. trachea.
* $P < 0.05$ vs. lung.
** $P < 0.01$ vs. lung.
*** $P < 0.001$ vs. lung.

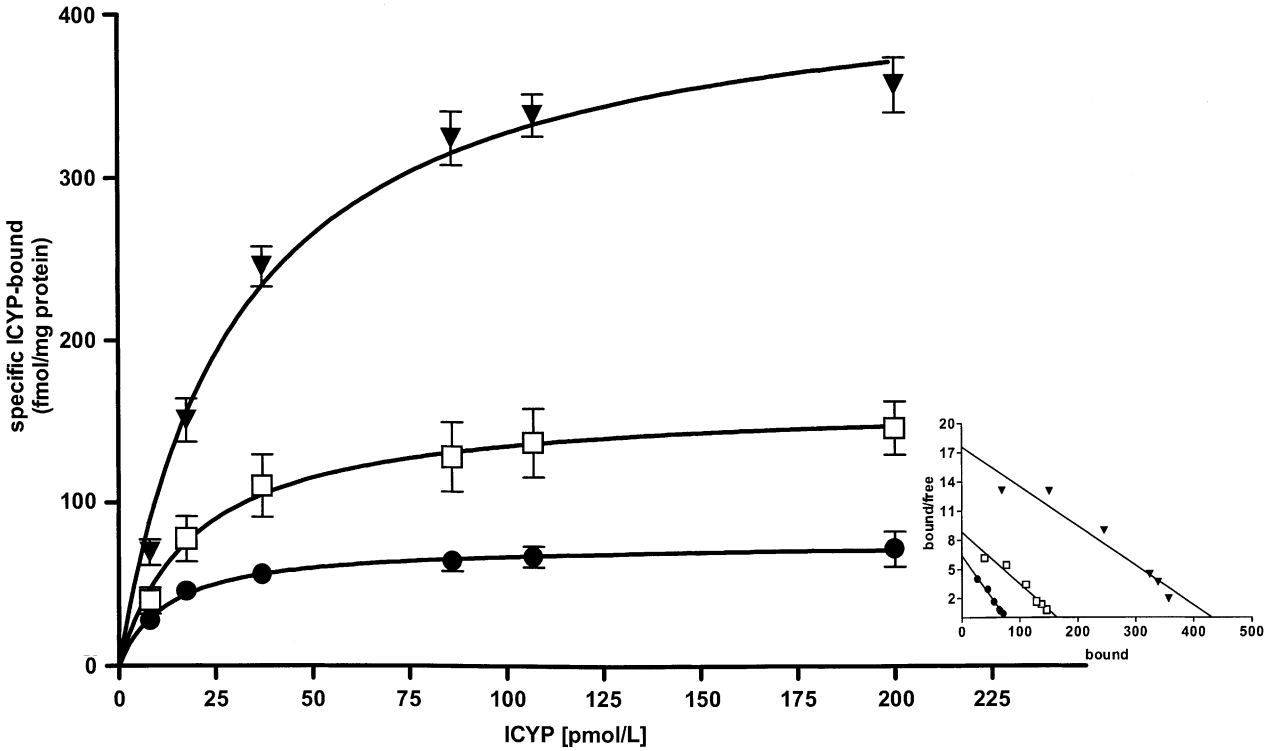

Fig. 1. Saturation binding curves of increasing ICYP concentrations for membranes of equine peripheral lung (▼), bronchial (□) and tracheal (●) epithelium with the underlying smooth muscle. ICYP binding was performed as described in Section 2. Data shown are the means ± SEM of six experiments each performed in duplicates. Maximal receptor density (B_{max}) and the dissociation constant (K_d) for the specific ICYP binding were analyzed by non-linear regression for best fit to one-site binding model. *Inset*, the corresponding Scatchard analysis of specific ICYP binding. The ratio *B*/*F*: specifically bound ICYP (*B*: fmol/mg protein; N = 6)/free ICYP concentrations (pM) vs. *B*: specifically bound ICYP (fmol/mg protein; N = 6). Both ways of analysis gave identical results. The results of this analysis are summerized in Table 1.

and bronchus vs. trachea; $P < 0.01$, lung vs. trachea; Table 1). The difference in the affinity to ICYP binding can possibly be attributed to regional differences in the fraction of receptors in the high affinity state (see result part 3, Table 3). The increase in the affinity (K_d) from proximal to distal (trachea down to the lung) was associated with the increase in the maximal binding capacity (B_{max}) between different regions of the respiratory tract, even though the saturation binding isotherm for all examined tissues was obtained at concentrations between 80 and 200 pM. Non-specific binding represented about 20% at 200 pM, and about 5% at 50 pM of ICYP concentration. The characteristic ICYP binding pattern suggests the presence of apparently a homogeneous population of high affinity binding sites in each of the three preparations. Figure 1 (inset) illustrates data of the specific ICYP binding plotted according to Scatchard [27], and resulted in a linear line indicating that ICYP labels a single class of binding sites. The density of total β-adrenoceptors was determined by saturation binding experiments. In membranes of peripheral lung, the extrapolated number of maximal binding sites (B_{max}) was 2.5-fold significantly higher when compared to secondary and small bronchial epithelium with the underlying smooth muscle ($P < 0.01$, lung vs. bronchus, 428.2 ± 19.0 vs. 162.4 ± 4.8 fmol/mg protein; Fig. 1 and Table 1). In the trachea with the epithelium and underlying smooth muscle, the receptor concentration was 5.6-fold lower than in the lung and 2.1-fold lower than in the bronchus (75.9 ± 1.2 fmol/mg protein; $P < 0.001$ and $P < 0.01$, respectively; Fig. 1 and Table 1). The gradient for β-adrenoceptor density was in the respiratory tract as follows: lung ⋙ bronchus ⩾ trachea.

3.2. Characteristics of β-adrenoceptors in the lung, bronchus and trachea

In order to characterize and determine the ratio of the β-adrenoceptor subtypes present in the membranes of peripheral lung, secondary and small bronchial and tracheal epithelium with the underlying smooth muscle, we assessed inhibition of ICYP binding (fixed concentration: 80 pM) by increasing concentrations of β-adrenoceptor agonists and antagonists. Table 2 summarizes the apparent binding affinities (inhibition constants; K_i-values) for (−)-isoproterenol, (−)-adrenaline, (−)-noradrenaline, (+)-propranolol, (−)-propranolol, the β_1-adrenoceptor subtype-selective antagonist (CGP 20712A) and β_2-adrenoceptor subtype-selective antagonist (ICI 118,551) calculated from the 50% inhibition concentration (IC_{50}). Figure 2 displays the competitive ICYP displacement curves of (+)- and (−)-isomers of propranolol. The non-selective (−)-propranolol displaced ICYP in a characteristic stereospecific

Table 2
Characteristics of agonist and antagonist binding to β-adrenoceptors in membrane preparations of peripheral lung, bronchial and tracheal epithelium with the underlying smooth muscle

	N	Lung	Bronchus	Trachea
Agonists				
(−)-Isoproterenol				
K_{iH} (nM)	8	26.3 ± 6.9	6.7 ± 1.1	2.2 ± 1.3
K_{iL} (nM)	8	1405.1 ± 331.6	1403.9 ± 389.4	887.5 ± 329.4
(−)-Adrenaline				
K_{iH} (nN)	8	108.8 ± 22.1	15.6 ± 13.5	64.7 ± 21.7
K_{iL} (nM)	8	15853.5 ± 4886.8	13994.0 ± 2469.7	9359.2 ± 2922.7
(−)-Noradrenaline				
K_{iH} (nM)	8	308.7 ± 108	77.5 ± 72.7	66.2 ± 26.7
K_{iL} (nM)	8	53507 ± 12292	12381 ± 10248	39095 ± 5662
Antagonists				
(−)-Propranolol				
K_i (nM)	6	1.3 ± 0.2	1.0 ± 0.1	0.8 ± 0.2
(+)-Propranolol				
K_i (nM)	6	636.5 ± 481	130.6 ± 30	72.8 ± 21.7
ICI 118,551				
K_{iH} (nM)	6	3.3 ± 0.5	2.4 ± 1.2	1.9 ± 0.1
K_{iL} (nM)	6	496 ± 37	193 ± 15	249 ± 13
Ratio β_1:β_2	6	21:79 ± 2.7	27:73 ± 13.9	15:85 ± 3.5
CGP 20712A				
K_{iH} (nM)	6	2.6 ± 0.3	4.2 ± 0.8	4.8 ± 1.3
K_{iL} (nM)	6	31028 ± 13745	39840 ± 14760	41007 ± 13455
Ratio β_1:β_2	6	21:79 ± 1.5	23:77 ± 3.9	23:77 ± 4.6

IC_{50} values were calculated from the mean inhibition curves and transformed into K_i-values, as described in Section 2. Data given are mean ± SEM of N experiments performed in duplicates.

and concentration-dependent competitive manner for β-adrenoceptors from its binding sites with 100 times higher affinity than (+)-propranolol. The concentration–inhibition curves were steep and monophasic. The Hill-coefficient (n_H-value) was not significantly different from 1.0, and displacement potencies were, with in the range of variation, comparable among all three preparations (Fig. 2A–C and Table 2).

The highly selective β_2-adrenoceptor antagonist ICI 118,551 [29] inhibited ICYP binding in all tissues in a concentration-dependent manner with about 10,000 times more potency than the highly selective β_1-adrenoceptor antagonist CGP 20712A [30] (Fig. 3, Table 2). In the three tissues, displacement curves for both ICI 118,551 and CGP 20712A were fitted significantly better to a two-site model than to a one-site model (F-test: $P < 0.01$; Fig. 3A–C). The K_i-values for ICI 118,551 at the high affinity site were in the lung (3.3 ± 0.5 nM), bronchus (2.4 ± 1.2 nM) and trachea (1.9 ± 0.1 nM); and the corresponding values at low-affinity site were 496 ± 37 nM (lung), 193 ± 15 nM (bronchus), 248 ± 13 nM (trachea) (Table 2). We obtained the ratio of β_2:β_1 = 80:20 binding sites in the lung, 75:25 in the bronchus and 75:25 in the trachea.

Similarly, CGP 20712A inhibited ICYP binding in all tissues with shallow concentration–inhibition curves which were fitted significantly better to a two-site model than to a one-site model (F-test: $P < 0.05$; Fig. 3A–C). The K_i-values for CGP 20712A at the high affinity site were in the lung (2.6 ± 0.3 nM), bronchus (4.2 ± 0.8 nM) and trachea (4.8 ± 1.3 nM); and for the low-affinity site were 31028 ± 13745 nM (lung), 39840 ± 14760 nM (bronchus) and 41007 ± 13455 nM (trachea) as summarized in Table 2. The ratio of β_1:β_2 binding sites were in the lung 21%:79%, in bronchus 23%:77% and the trachea 23%:77%. Accordingly, the data from both selective β-adrenergic antagonists showed in upper to lower segments of the respiratory tract the predominance of β-adrenoceptor of the β_2-subtype, despite the reciprocal receptor concentration in these regions.

Further, to subclassify β-adrenoceptors in the membranes of peripheral lung, secondary and small bronchial and tracheal epithelium with the underlying smooth muscle, we investigated the β-adrenoceptor agonist potency by concentration-dependently inhibiting the ICYP binding with isoproterenol, adrenaline and noradrenaline. The binding affinities of these three compounds for the β_2-adrenoceptor are listed in Table 2. Agonists inhibited ICYP binding with the rank order of potency isdproterenol > adrenaline > noradrenaline (Fig. 4A–C) with K_i-values in the same range, which is typical for β_2-adrenoceptors [31]. Agonists competition curves were shallow with n_H-values of 0.40 suggesting binding to different affinity states of the receptor. Non-linear regression analysis of the concentration–inhibition curves of (−)-isoproterenol revealed that

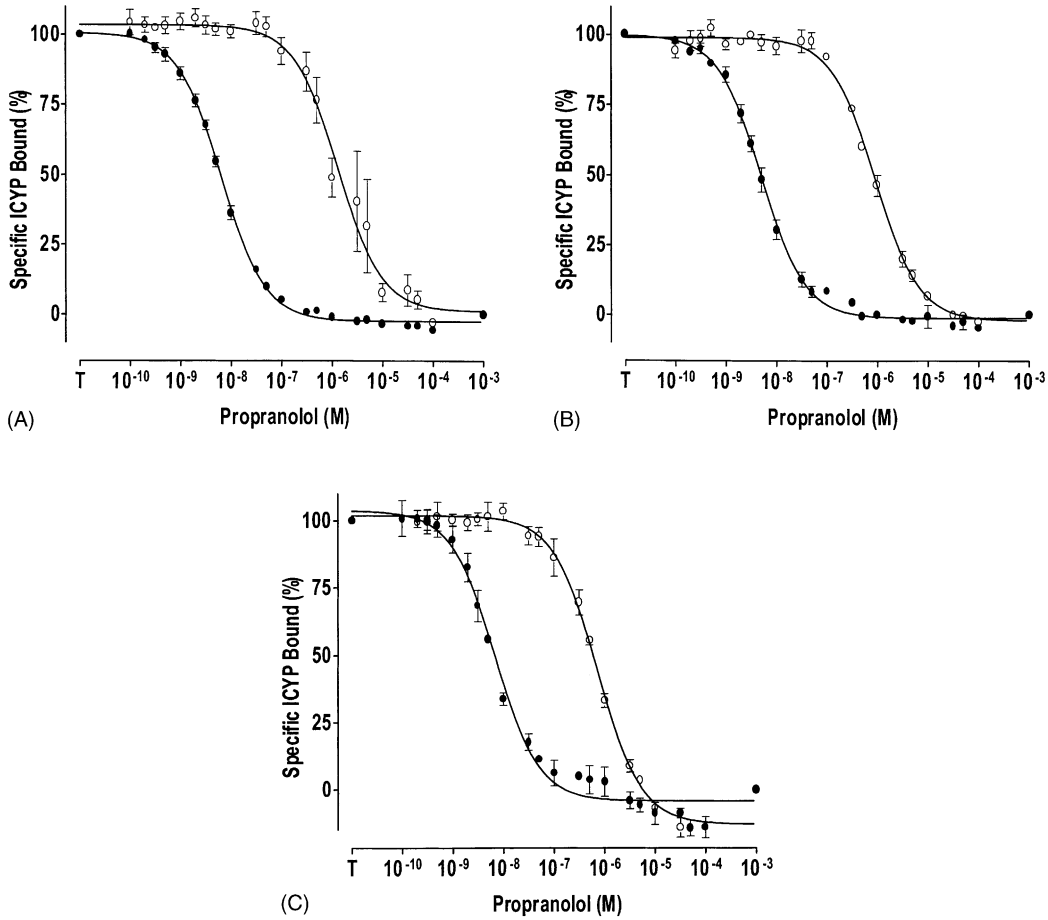

Fig. 2. Inhibition of specific ICYP binding by (−)-propranolol (●) and (+)-propranolol (○) in (A) equine lung, (B) bronchus and (C) trachea. Membrane proteins (10 μg/tube) were incubated with ICYP (~80,000 cpm; 80 pM) in the presence or absence of 18–22 increasing concentrations of propranolol (10^{-10} to 10^{-4}), and specific binding was determined as described in Section 2. One hundred percent binding represents the specific ICYP binding detected with 1 μmol/L (±)-CGP 12177 in the absence of (+)- and (−)-propranolol. The data plotted are the means ± SEM of duplicate determinations from six separate experiments and represent the percent of specific binding at concentration of the added isomers of propranolol. Data were analyzed by non-linear regression for best fit to one-site binding model. Results are summerized in Table 2.

about 34–37% (Fig. 5, Table 3) of the β-adrenoceptors in the three regions of the respiratory tract are in the high affinity state.

Table 3
High- and low-affinity binding properties for isoproterenol displacement ICYP binding in the presence or absence of GTP (100 μM) for β-adrenoceptors of peripheral lung, bronchial and tracheal epithelium with the underlying smooth muscle

	K_{iH} (nM)	K_{iL} (nM)	R_H (%)	R_L (%)
(−)-Isoproterenol				
Lung	2.6 ± 1.5	1405 ± 332	34.7 ± 2.3	65.3
Bronchus	6.7 ± 1.1	1404 ± 389	33.7 ± 3.1	66.3
Trachea	2.2 ± 1.3	888 ± 329	36.9 ± 2.4	63.1
(−)-Isoproterenol + GTP (100 μM)				
Lung		943 ± 208		100
Bronchus		1355 ± 457		100
Trachea		1296 ± 303		100

Data presented are means ± SEM of four separate experiments performed in duplicates. K_{iH} and K_{iL} are high- and low-affinity constants, respectively, obtained in the absence of GTP. R_H and R_L (%) are percentage of receptors in the high- and low-affinity state (for details see Section 2).

3.3. β-Adrenoceptor coupling to G-protein

The above results of the agonist binding suggested that the high affinity agonist binding observed in displacement experiments in the absence of guanine nucleotides might be due to the formation of a tight complex binding of ligand–receptor and nucleotide free G-proteins [32]. We next evaluated the number of β-adrenoceptors in the high-affinity state which couple to G_S-protein in response to β-adrenergic stimulation. With the high affinity state the receptors are coupled to G-protein regulatory processes. Isoproterenol (adrenaline and noradrenaline, for these, data not shown) displaced ICYP binding in a dose-dependent manner with two binding sites of high- and low-affinity state of the β-adrenoceptors, and the curves were significantly fitted better for two-site binding model (F-ratio: $P < 0.05$, Fig. 5A–C). As shown in Table 3, from these curves it could be calculated a percentage of high affinity β-adrenoceptors approximately 35% in lung, 34% in bronchus and in 37% trachea; the K_i-values for isoproterenol at the high affinity state were 2–7 nM, at the low-affinity

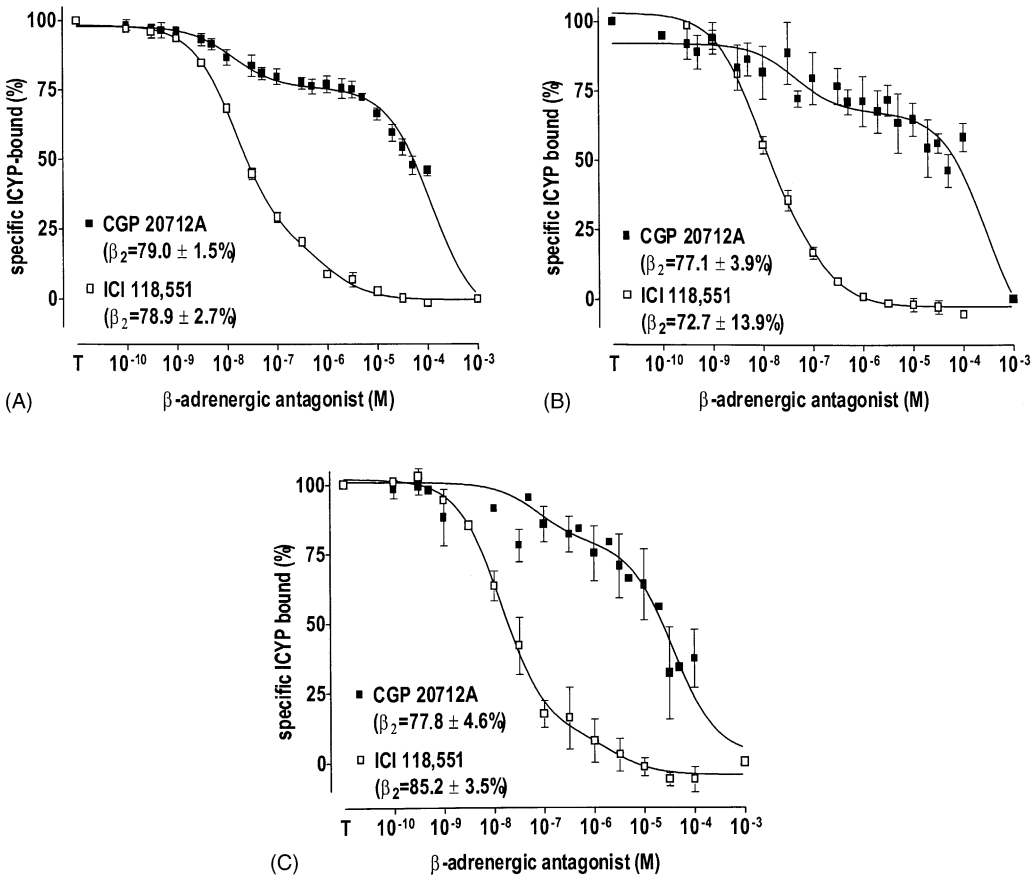

Fig. 3. Inhibition of specific ICYP binding by ICI 118,551 (■) and CGP 20712A (□) in (A) equine lung, (B) bronchus and (C) trachea. Membrane proteins (10 μg/tube) were incubated with ICYP (~80,000 cpm; 80 pM) in the presence or absence of 18–22 increasing concentrations of ICI 118,551 and CGP 20712A or (10^{-10} to 10^{-4}); specific ICYP binding was determined as described in Section 2. One hundred percent binding represents the specific ICYP binding detected with 1 μmol/L (±)-CGP 12177 in the absence of ICI 118,551 and CGP 20712A. The data plotted are the means ± SEM of duplicate determinations from six separate experiments and represent the percent of specific binding at concentration of added selective antagonists. Data were analyzed by non-linear regression for best fit to two-site binding model. Results are summarized in Table 2.

state 800–1400 nM. This data would provide evidence the tendency for the high affinity of the β-adrenoceptors for the radioligand ICYP in the tracheal epithelium with the underlying smooth muscle than in bronchus or lung parenchyma as described above. The addition of 100 μM GTP to the incubation mixture converted the biphasic-inhibition curves of isoproterenol into a steepened monophasic ones resulting thus in a loss of the number of β-adrenoceptors in the high affinity state and a shift of the concentration–inhibition curves to the right to the low-affinity state (K_i-values: 900–1300 nM, Fig. 5A–C, Table 3).

3.4. Pulmonary adenylate cyclase activity

We finally performed pharmacological characterization of AC in membranes of the peripheral lung, bronchus and trachea. As shown in Fig. 6 and Table 4, the basal enzyme activity levels were identical in all tissue preparations (in the lung: 29.9 ± 7.4, bronchus: 29.5 ± 3.3 and trachea: 36.1 ± 5.1 pmol/mg protein/min). However, stimulants of either the β-adrenoceptors or G-proteins or directly of AC gave a major difference between the three regions (Table 4). In this context, the β-adrenergic agonist isoproterenol acting through β-adrenoceptors (10 μM) in the presence of GTP (10 μM) led to a 3.3-fold (peripheral lung), 4.2-fold (secondary and small bronchial epithelium with the underlying smooth muscle) and 5.7-fold (tracheal epithelium with the underlying smooth muscle) increase in the AC activity. However, it was worthwhile to note that when we compared the activity level among tissue samples, the isoproterenol-stimulated increase was significantly higher in the trachea ($P < 0.01$; vs. lung, and bronchus; Fig. 6D and Table 4). The AC was coupled to G-proteins in all membrane preparations, as the response upon the addition of 10 μM of GTP (which activates G-proteins), though being lower, 1.6, three times above the basal values in the peripheral lung, bronchial and tracheal epithelium with the underlying smooth muscle, respectively. Again, the GTP-stimulated response was significantly higher in the trachea ($P < 0.05$; vs. lung; Fig. 6D and Table 4). The addition of 10 mM NaF, which causes maximal activation of all G-proteins, induced a significant stimulation of AC activity in all regions with increases of 388% in the trachea ($P < 0.001$, N = 6, vs. basal) and 334% in the bronchus

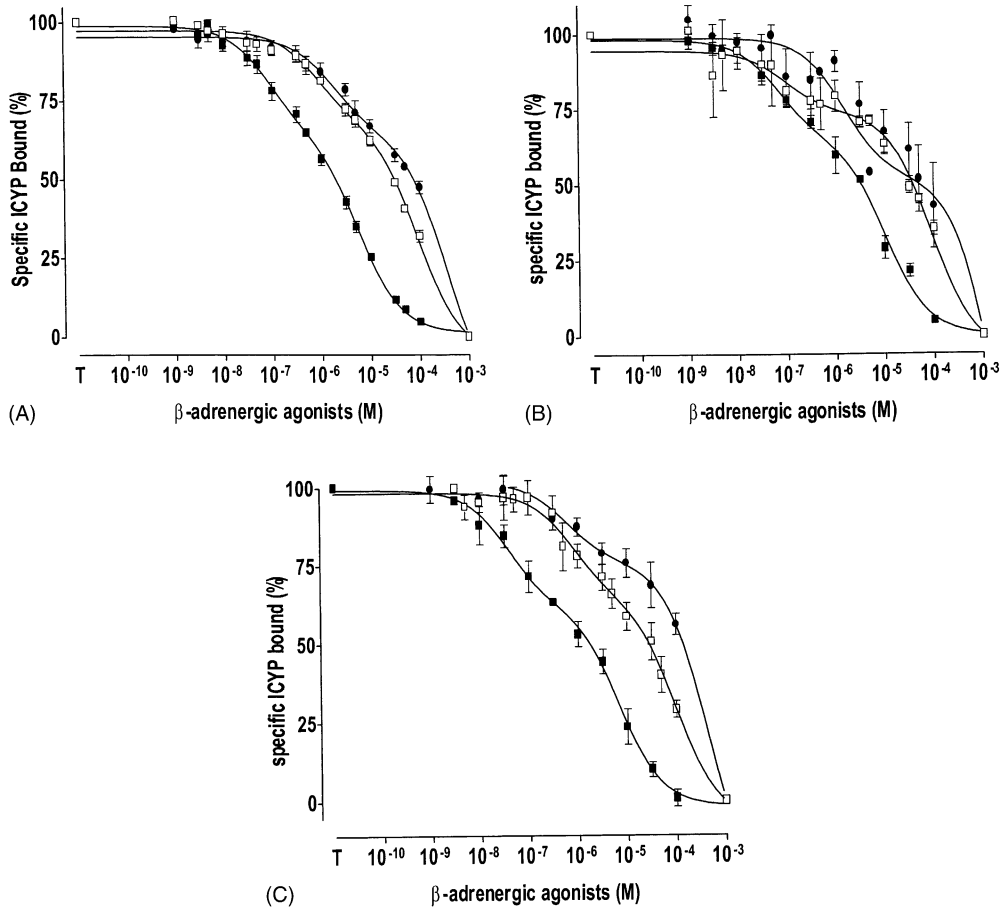

Fig. 4. Inhibition of specific ICYP binding by isoproterenol (■), adrenaline (□) and noradrenaline (●) in (A) equine lung, (B) bronchus and (C) trachea. Membrane proteins (10 μg/tube) were incubated with ICYP (~80,000 cpm; 80 pM) in the presence or absence of 18–22 increasing concentrations of isoproterenol, adrenaline or noradrenaline (10^{-10} to 10^{-4}); specific ICYP binding was determined as described in Section 2. One hundred percent binding represents the specific ICYP binding detected with l μmol/L (±)-CGP 12177 in the absence of isoproterenol, adrenaline or noradrenaline. The data plotted are the means ± SEM of duplicate determinations from eight separate experiments and represent the percent of specific binding at concentration of added agonists. Data were analyzed by non-linear regression for best fit to two-site (isoproterenol and adrenaline) and one-site (noradrenaline) binding model. Results are summarized in Table 2.

($P < 0.001$, N = 6, vs. basal), but to a lesser extent of 220% in the lung ($P < 0.001$, N = 6, vs. basal). The pattern of fluoride stimulated activity was similar to that of the isoproterenol-response. Moreover, substances which directly activate the catalytic units of the enzyme induced substantial increases in the AC activity. Thus, the addition of forskolin much significantly increased the net AC activity in the bronchial (8 times) and tracheal (12.5 times) epithelium with the underlying smooth muscle ($P < 0.001$, bronchus vs. basal; $P < 0.0001$, trachea vs. basal; Fig. 6A–D, Table 4), while the stimulatory effect was much lower in the lung membranes (2.3 times), albeit statistically significant ($P < 0.05$). Using Mn^{2+} to directly activate the catalytic unit of the AC, in contrast to forskolin, only a slightly increased, but a significant response, in the lung and bronchus could be observed ($P < 0.01$, lung vs. basal; $P < 0.05$, bronchus vs. basal, N = 6). In all three regions of the respiratory tract tested, we observed statistically significant stimulatory responses by all G-protein-dependent measures as well as by all AC measures. However, the stimulatory responses were much larger in the tracheal epithelium with the underlying smooth muscle than in the bronchial epithelium with the underlying smooth muscle followed by an even a lesser increase in the AC activity in the peripheral lung.

4. Discussion

In this study, we investigated the pharmacological properties of β-adrenoceptors and AC activity in different segments of the respiratory tract, in lung parenchyma, bronchial and tracheal epithelium with the underlying smooth muscle with respect to regional differences in receptor concentration, pattern of distribution, affinity of the receptor to G-protein coupling, and receptor–G-protein-mediated activation of AC. Comparative studies, showing a direct correlation between β-adrenoceptor density, affinity states and AC activity and the function of these components which in turn determine the β-adrenergic

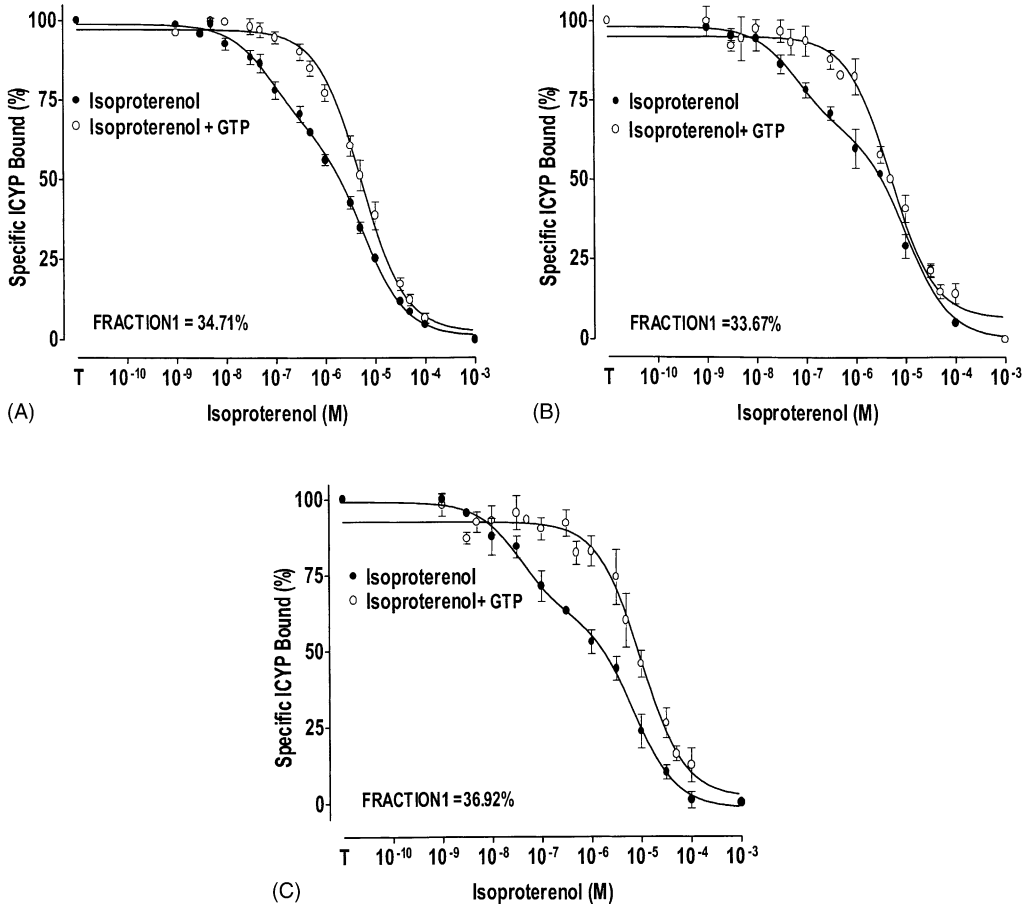

Fig. 5. Isoproterenol competition ICYP binding in the presence (●) or absence (○) of 100 μM GTP in (A) equine lung, (B) bronchus and (C) trachea. Membranes (10 μg) were incubated with ICYP (~80,000 cpm; 80 pM) and 18–22 increasing concentrations of isoproterenol (10^{-10} to 10^{-4}) in the presence or absence of GTP (100 μM); specific ICYP binding was determined as described in Section 2. One hundred percent binding represents the specific ICYP binding detected with l μmol/L (±)-CGP 12177 in the absence isoproterenol. The data plotted are the means ± SEM of duplicate determinations from four separate experiments and represent the percent of specific binding at concentration of added agonists. Data were analyzed by non-linear regression for best fit to two-site (only isoproterenol) and one-site (in the presence of GTP) binding model. Results are summarized in Table 3.

signal transduction pathway, have not been conducted to date in membranes of intact tissues of the respiratory tract. It is still unclear to what extent the concentration of the receptors, G-protein and AC favor rapid, high fidelity interactions between these components which are required for the signaling pathways in cells and membranes. Here, we attempted to provide at least indirect but suggestive evidence for the interrelationship between the receptor density, state of the receptor affinity to G-proteins, and stimulant responses of basal signaling of either β-adrenoceptor-, G-protein-mediated or direct activated AC in pulmonary preparations.

The main finding of the present study was that in the respiratory tract distinct regional differences exist in β-adrenoceptor concentration and AC responses. β-Adrenoceptors were largely overexpressed in the peripheral lung when compared to their density in the bronchial or in the tracheal epithelium with the underlying smooth muscle. In contrast, the stimulatory AC activity showed an inverse distinct regional hierarchy with the greatest response in the trachea, followed by the bronchus and then by the lung parenchyma. On the functional level, the proportion of the high- and low-affinity states of the β-adrenoceptors obtained from concentration–inhibition curves of (−)-isoproterenol was comparable among the three segments of the respiratory tract.

In line with our findings, previous autoradiographic and radioligand binding studies have shown, in the human and non-human lung [5,11,20], bronchus and trachea [14,33], the co-existence of both β_1- and β_2-adrenoceptor. The non-selective β-adrenoceptor antagonist ICYP, used in this study to characterize β-adrenoceptors, was bound to a homogeneous population of β-adrenoceptors in the equine peripheral lung, bronchial and tracheal epithelium with the underlying smooth muscle, with affinities comparable to those found in human airways [11] and airway preparations of other species [20]. In the present study, ICYP binding was rapid, reversible, saturable and of high affinity in all regions examined, although ICYP exhibited higher affinity for tracheal sites (K_d: 13.0 ± 0.9 pM) and slightly higher affinity for bronchial sites (K_d: 20.0 ± 2.3 pM) than for the lung (30.8 ± 4.4 pM), suggesting therefore, the difference

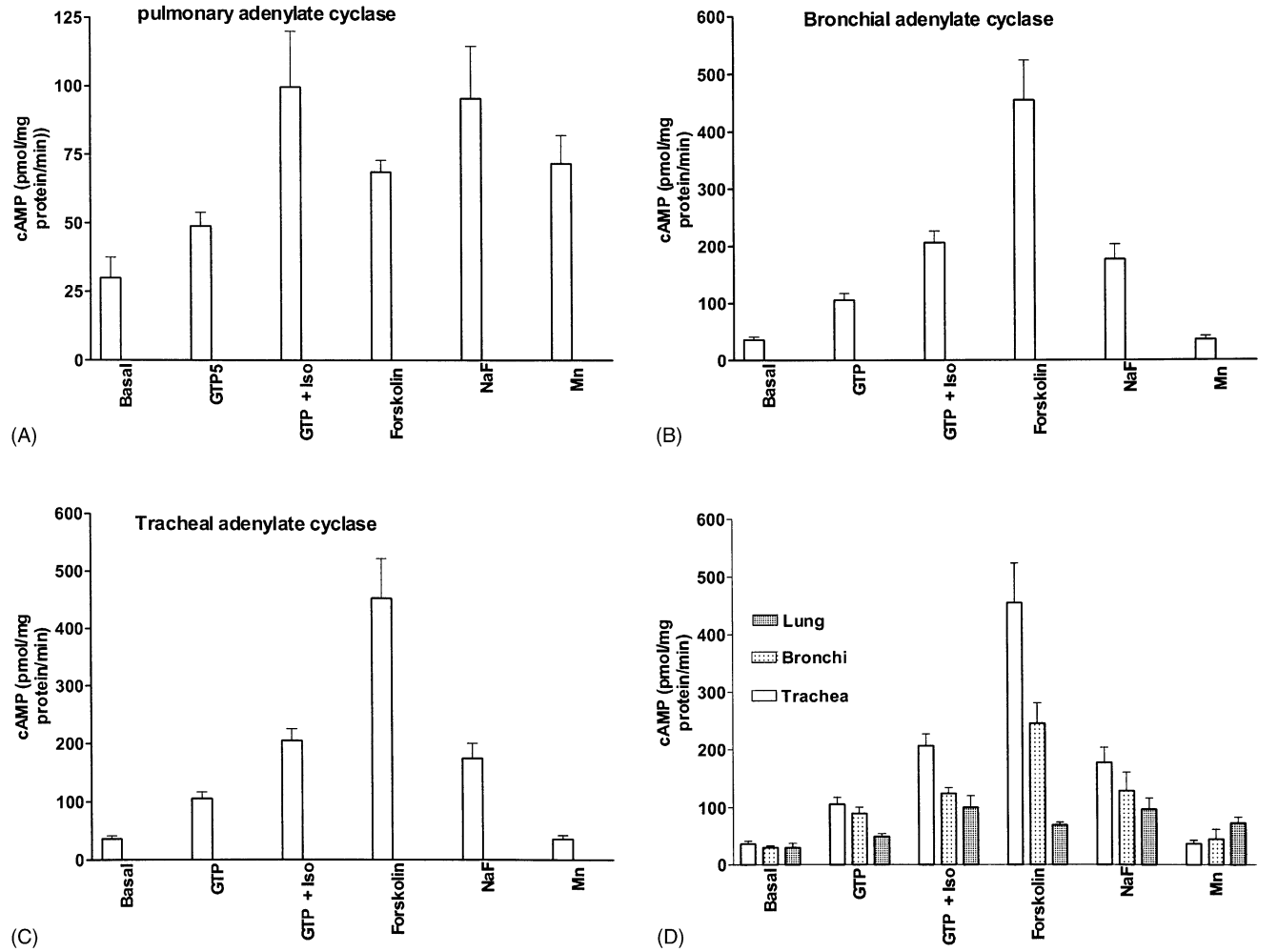

Fig. 6. Stimulation of adenylate cyclase activity in (A) equine lung, (B) bronchus, (C) trachea and all three tissues (D) The response was measured under basal conditions and in the presence of 10 μM GTP, 10 μM ISO, 10 μM forskolin, 10 mM NaF or 10 mM Mn^{2+}. Data represent the means ± SEM of six determinations in triplicates. Results and statistics are summarized in Table 4.

in the high affinity state (displays high receptor–G_S-protein coupling) might contribute to the prevailing differences in the ICYP binding capacity to β-adrenoceptors in the three tissue membranes. The maximal ICYP binding sites, calculated from the specific saturation curves either by non-linear regression or Scatchard analysis, were inversely correlated with 428.2 ± 19.0 fmol/mg protein in peripheral lung, which were 2.6- or 5.7-fold higher when compared to binding sites in the bronchus, and in the trachea, respectively. The density of β-adrenoceptors in the peripheral lung was, within the range, but slightly higher than that found by other investigators in peripheral lung of other mammalian species including man [34–36], whereas the densities in bronchus and trachea were similar to values previously reported in humans [37] and dogs [14]. Despite the difference in ICYP affinity, however, there is a limited number of comparable studies which have investigated the receptor heterogeneity in different regions of the respiratory tract. The high density in total β-adrenoceptor subtypes in the peripheral lung could at least partly be explained by the occurrence of heterogeneous epithelial cell types (about 40) in the lung parenchyma which differently express β-adrenoceptors. On the other hand, our data confirm in more detail the data from earlier investigations in guinea-pigs, showing that β-adrenoceptors are unevenly distributed throughout the airways with the highest density in the lung and the lowest density in the trachea [15,38].

In earlier and recent studies it was unanimously demonstrated that in most mammalian species, the β_2-adrenoceptors as well as β_2-adrenoceptor mRNA both predominate in peripheral lung, including airway smooth muscle with the ratio of about 80:20 (β_2:β_1) [12,39], and in airway epithelium [4]. However, there is still a long-standing debate to what extent functional responses (relaxation) to β-adrenergic agonists or to obstructive disease states in the respiratory tract are mediated in relation to the β-adrenergic receptor subtype concentration and the subsequent β-adrenergic signal transduction [40]. Since no information is available with regard to the equine airways, in the current study, we explicitly characterized β-adrenoceptor subtype properties in pulmonary preparations using competition binding studies. In membrane preparations of lung parenchyma,

Table 4
Adenylate cyclase activity in equine peripheral lung, secondary and small bronchial and tracheal epithelium with the underlying smooth muscle

	Lung	Bronchus	Trachea
Basal	29.9 ± 3.3	29.5 ± 7.4	36.1 ± 5.1
GTP (10 μM)	48.9 ± 4.9**	89.5 ± 10.8***	105.5 ± 11.4***,a
Stimulatory effect (% basal)	66	103	192
GTP + ISO (10 μM)	99.7 ± 20.2**	124 ± 10.2***	206.0 ± 20.5***,b
Stimulatory effect (%)	233	320	470
Forskolin (10 μM)	68.7 ± 4.3**	244.8 ± 34.9***,c	454.6 ± 68.7***,d,e
Stimulatory effect (%)	130	730	1157
NaF (10 mM)	95.8 ± 18.8**	128.1 ± 12***	176.4 ± 25.8***,f
Stimulatory effect (%)	220	334	388
Mn^{2+} (10 mM)	71.9 ± 10.3**,g	44.3 ± 16.9*	36.2 ± 6.2
Stimulatory effect (%)	140	50	0.30

The enzyme activity is expressed in cAMP pmol/mg protein/min, data are means ± SEM six experiments performed in triplicates.
[a] $P < 0.05$ vs. lung.
[b] $P < 0.01$ vs. lung and bronchus.
[c] $P < 0.001$ vs. lung.
[d] $P < 0.001$ vs. lung.
[e] $P < 0.05$ vs. bronchus.
[f] $P < 0.05$ vs. lung.
[g] $P < 0.05$ vs. trachea.
* $P < 0.05$ vs. basal adenylate cyclase activity.
** $P < 0.01$ vs. basal adenylate cyclase activity.
*** $P < 0.001$ vs. basal adenylate cyclase activity.

bronchial and tracheal epithelium with the underlying smooth muscle, the non-selective β-adrenoceptor antagonist (−)-propranolol displaced ICYP from its binding sites with 100 times more affinity than its (+)-isomer. By the data of the present study and from the literature, a straightforward explanations for the results concerning the stereoslectivity of β-adrenoceptor binding properties could be made in all three regions. In accordance to previous results and by the use of the highly selective β_2-adrenoceptor antagonist ICI 118,551 [29] and β_1-adrenoceptor antagonist CGP 20712A [30], we found that ICI 118,551 inhibited ICYP binding approximately >10,000 times more potent than the β_1-adrenoceptor antagonist in all three respiratory segments. Both ligands displaced ICYP from two binding sites in the lung, bronchus or trachea with concentration–inhibition curves, which fitted significantly better to a two-site model than to a one-site model. These biphasic and shallow inhibition curves favor the presence of two β-adrenoceptor subtypes; the β_2-subtype selectively charecterized by ICI 118,551 and the β_1-subtype by CGP 20712A, which both provided a ratio of β_2:β_1 = 80:20 in lung, 76:24 in bronchus and 76:24 in trachea. The present results demonstrate in good agreement with reports in the literature [12,39] that the β_2-adrenoceptors represent the vast majority of binding sites. The proportion of the β_2-subtype concentration in the peripheral lung was slightly higher, though not statistically significant, than that in the bronchus and trachea. In accordance with our data, membranes of the bronchus and trachea exhibited similar ratio of β_2-adrenoceptors [14,37]. Thus, the larger total β-adrenoceptor density in the lung parenchyma and bronchus might be due to the higher concentration of the β_2-subtype and hence, merits consideration in view of agonist-dependent changes in the receptor number and functional responsiveness.

The conclusions of prevalence of β_2-adrenoceptors through the equine airways were supported by the fact that, in all three segments, lung, bronchus and trachea, the binding patterns of catecholamine agonists for β-adrenoceptors were identical, with the rank order of potency, (−)-isoproterenol > (−)-adrenaline > noradrenaline, as expected from previous findings for the β_2-subtype [31]. However, as a classical picture of GPCR (−)-isoproterenol, (−)-adrenaline and (−)-noradrenaline inhibited ICYP binding with concentration–inhibition curves that better fitted to a two-site than one-site binding model comprising β-adrenoceptors in the 'high-affinity state' (∼34–37%, i.e. that conformation of the receptor that is essential for coupling stimulated receptor to the G_S-protein–AC system [41]) and 'low-affinity state' correlating to receptor states coupled and uncoupled to the heterotrimeric G-protein, respectively. Thus, difference in the affinity of the ICYP binding to β-adrenoceptors of studied membranes can, it appears, possibly be associated to regional differences in the fraction of these receptors with high affinity state which efficiently couple to G_S-protein. It has long been proposed that ligand, receptor and G-protein form a high-affinity ternary complex in the absence of guanine nucleotides, and that the complex is transient in the presence of guanine nucleotides [42]. Accordingly, our data demonstrate that the addition of GTP triggered reduced high-affinity binding sites of (−)-isoproterenol with the concomitant

increase in low-affinity state, as indicated by a rightward-shift of the displacement curves to higher agonist concentration. Therefore, it could be demonstrated that in various segments of the equine respiratory tract, β-adrenoceptors are coupled to G-protein.

Finally, in the present study we have correlated the functional coupling efficiency of the β-adrenoceptors to the stimulatory AC cascade in membranes from all three segments of the respiratory tract, an enzyme which catalysis the formation of the second messenger cAMP. Accordingly, in the face of signal transduction across β-adrenoceptor–G-protein–effector system, we assessed the AC response: (a) by stimulating the β-adrenoceptors (mediated by the β-adrenoceptor agonist isoproterenol), (b) by non-receptor-mediated activation of AC response by GTP (acting on G_S- and G_I-protein), by forskolin (activate predominantly the catalytic unit of the AC, but partly also G_S [43]), by NaF (stimulates G_S [44]) and by Mn^{2+} (activates also the catalytic unit of the AC [25]). Activity of AC increased in response to all activators in membranes of all three lung regions, but they showed different degrees and patterns of cAMP generation, with a larger increment in the trachea (except for Mn^{2+}-mediated responses) than in the bronchi and lung parenchyma. These data document that in crude membranes of the lung, bronchus and trachea AC was not only stimulated by agents acting on β-adrenoceptors (isoproterenol), reflecting that AC is functionally coupled and recruitable through β-adrenoceptor stimulation [45], but also to agents which bypass the receptor and acting on post-receptor level (GTP, forskolin, NaF, Mn^{2+}), indicating AC response can be enhanced by stimulating G_S-protein coupling and directly by augmenting the activity of the catalytic unit of AC [46]. Among the direct activators of AC unlike forskolin which induced the most prominent response, indicating an increase in the catalytic activity of the enzyme, Mn^{2+} induced a much lower response in membranes of all segments (in the trachea no AC activation) in line with cardiac membranes [47], whereas other studies showed much larger Mn^{2+}-mediated enhancement in other *in vivo* models [48]. The discrepancies for this was recently suggested that the two stimulants act upon different regions of the AC molecule, with forskolin binding to the catalytic core [49], whereas Mn^{2+} replaces Mg^{2+} at metal binding sites [49]. Such studies have led to the conclusion that these different catalytic properties of AC could be resolved by adding GTP [43], since forskolin-induced activity should be enhanced by G_S–AC association, whereas Mn^{2+} probably interferes with Mg^{2+} at the catalytic unit of AC and, presumably with G-protein interaction, leading to a loss of receptor-mediated AC response [25].

Furthermore, despite lower numbers of cell surface receptors in the tracheal epithelium with the underlying smooth muscle (∼76 fmol/mg protein) than in the bronchus (∼162 fmol/mg protein) and in the lung (∼428 fmol/mg protein), and similar coupling efficiency of β-adrenoceptors to G_S-protein in all three regions, we were able to measure maximal AC responses through β-adrenoceptor as well as AC stimulation in the trachea. Data on connection between the receptor density and AC response, and at all as a subject to regulation, in either tissues of the respiratory tract of human or animal origin, are equally scarce, but deserve comment. Some functional studies conducted in the bronchus and trachea have shown that topographical difference does exist in β-adrenergic signaling pathway in airways, estimated to be a greater isoproterenol-induced relaxation responses in the trachea than in the bronchus [40,50], despite lower β-adrenoceptor density. Here, it appears, β-adrenoceptor concentrations are presumably responsible for the tissue-specific differences what we also uniquely saw in the patterns of receptor-mediated AC responses. On the other hand, studies in cardiac myocytes and other cells have suggested that receptor number or effector protein AC might be a limiting factor for the β-adrenoceptor-induced transmembrane signaling [16,17]. However, studies in the heart have shown that overexpressing β-adrenoceptor leads to a less net effect to increase agonist-mediated cAMP generation [51]. In contrast, overexpressing AC in cardiac myocytes increased β-adrenoceptor-mediated cAMP responses [52]. Together, these results in keeping with our data led the authors to speculate that the highest AC response in crude membranes, albeit lower β-adrenoceptor density such as in the trachea, would increase substantial β-adrenoceptor-mediated physiological responses. However, this can be answered by functional studies. As to the function and regulation of these receptors, Barnes [3], in an attempt to pharmacological clarity, reviewed the discrepancies between β-adrenoceptor protein as well as number and β-agonist-mediated responsiveness in human airways. Accordingly, in airway smooth muscle where the density of β-adrenoceptors (cf. Table 1) was low, the β-adrenoceptor mRNA level was increased, suggesting high rate of receptor synthesis or rapid turnover, or high stability of the transcripts. This has led to explain the difficulty to show a phenomenon of β-agonist-induced down-regulation of β-adrenoceptor in airway smooth muscle. On the contrary, lung parenchyma with high number of β-adrenoceptors (cf. Table 1) and low mRNA level with low receptor turnover was associated with rapid down-regulation. The present study served to characterize β-adrenoceptor–G-protein–AC system in the bronchial tree of horses, and would provide future insight to assess the therapeutic influence of β-adrenergic, glucocorticoids and other drugs on β-adrenergic system of the bronchial tree.

In conclusion, the data of the present study show that in the intrapulmonary branches of the equine respiratory tract the number of β_2-adrenoceptors and the amount of AC are segment-dependent. The density of β_2-adrenoceptors showed distinct regional differences, with highest number in the lung followed by the bronchus and then by the

trachea. In contrast, AC response was more pronounced in the trachea, than in the bronchus and in the lung. Our finding likely provide new information regarding the relationship between receptor density and G_S–AC coupling efficiency in membranes of different segments of the respiratory tract that regulate smooth muscle function. Thus, this discrepancies may limit the transmembrane β-adrenergic signaling, and therefore, has to be considered in pharmacological studies depending on the drug targeting strategies.

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