A comparison between clenbuterol, salbutamol and terbutaline in relation to receptor binding and *in vitro* relaxation of equine tracheal muscle

K. TÖRNEKE
C. INGVAST LARSSON &
L.-E. APPELGREN

Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, BMC, Box 573, SE-751 23 Uppsala, Sweden

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Beta$_2$-adrenoceptor agonists are used as bronchodilators in both humans and horses. Of these drugs, clenbuterol is the one most frequently used when treating chronic obstructive pulmonary disease in the horse, while salbutamol and terbutaline are used in the treatment of human asthma. Little is known of the properties of the latter two drugs in equine medicine.

We have compared salbutamol and terbutaline with clenbuterol in relation to their ability to relax muscle strips from equine tracheal muscle, precontracted with 40 nM carbachol, in tissue chambers. The affinities of these drugs to the β_2-adrenoceptors in homogenates of the same muscle tissue were also examined. These experiments were performed with radioligand binding studies using the very potent β-adrenoceptor antagonist ^{125}I-cyanopindolol.

The three drugs were almost equipotent in relaxing the muscle strips. The EC_{50}-values for salbutamol, terbutaline and clenbuterol were 5.6, 13.8 and 2.1 nM, respectively, and all three drugs relaxed the preparations completely. In the competitive binding study, however, the K_d-value of clenbuterol was much lower (24 nM) than that of salbutamol and terbutaline (1100 nM and 3900 nM, respectively). The amount of receptors bound at the EC_{50}-value of clenbuterol was 8% compared to less than 1% for salbutamol and terbutaline. This indicates a lower intrinsic efficacy of clenbuterol than of the other two drugs. The β-adrenoceptor density was 45 $\pm$ 14.3 fmol/mg protein (mean $\pm$ SD) and the K_d-value of ^{125}I-cyanopindolol was 11.4 $\pm$ 3.3 pM.

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Karolina Törneke, Department of Pharmacology and Toxicology, SLU, Box 573, SE-751 23 Uppsala, Sweden.

INTRODUCTION

Beta$_2$-adrenoceptor agonists are often used in the treatment of obstructive pulmonary diseases. These substances are used to mediate a relaxation of the airway smooth muscles in asthmatic patients with acute bronchial spasm. They are used either locally (as aerosols) or systemically (Rang *et al.*, 1995). In equine medicine, the drug most commonly used is clenbuterol, which is approved in some countries for systemic use as a bronchodilating agent in horses suffering from chronic obstructive pulmonary disease (COPD, heaves).

Salbutamol and terbutaline are two β_2-stimulating drugs which are commonly used for the treatment of human asthma. The experience of the use of these substances in equine practice is limited. They have been used clinically, but varying dose regimes have been suggested and no pharmacokinetic data are available (Plumb, 1995). *In vitro*, salbutamol and terbutaline inhibited the contraction of equine tracheal muscle induced by 500 nM carbachol to 80%. Salbutamol was slightly more potent than terbutaline (Hanna & Eyre, 1980).

Clenbuterol is known to have low clinical efficacy in horses (Derksen *et al.*, 1987; Erichsen *et al.*, 1994). This may be due to the fact that clenbuterol is a partial agonist. In a study on clenbuterol *in vitro* we investigated the efficacy of the drug relaxing muscle strips contracted by 500 nM carbachol. Only 50% of the preparations responded with relaxation when clenbuterol was added to the tissue chambers (Ingvast Larsson, 1991). We have also studied the ability of clenbuterol and several other agonists to relax muscle strips contracted by 130 nM carbachol in tissue chambers. In this study the preparations responded with relaxation to a clenbuterol concentration which was comparable to plasma concentrations achieved after recommended dosage. The efficacy of clenbuterol was rather high, 90% of that of isoprenaline (Törneke *et al.*, 1997).

The aim of the present study was to investigate the *in vitro* potency and efficacy of salbutamol and terbutaline and to compare these substances with clenbuterol.

A preliminary report of this work was presented at the 15th annual symposium of the Comparative Respiratory Society, Liège, Oct. 24–25 1997.

MATERIALS AND METHODS

Preparations

Six to eight rings of the trachea close to the carina were taken from nine horses, slaughtered at a local abattoir. The horses were of both sexes, varying age and breed and suffered from no apparent disease. The material was rinsed and transported to the laboratory in ice cold Ringer-glucose solution (Kabi Pharmacia, Uppsala, Sweden) immediately after slaughter.

The pieces of trachea taken from the horses were divided into three parts where the middle part was stored in ice cold Krebs solution to be used later in a tissue chamber study. The tracheal smooth muscle of the other two parts was dissected from the underlying cartilage and was completely removed from the airway epithelium using blunt dissection. The smooth muscle ($\approx$ 10 g) was then minced with scissors and homogenised with an Ultra Turrax homogeniser (Janke & Kunkel, Stauffen, Germany) for 3 $\times$ 10 s. Tris buffer (50 mM Tris, 0.15 M NaCl, pH 7.4) was added during the procedure to a final volume of $\approx$ 10 times the weight of the muscle. This slurry was then centrifuged at 1700 $\times$ ***g*** for 10 min. The supernatant was filtered through gauze dressing and centrifuged at 40000 $\times$ ***g*** for 15 min. The resulting pellet was washed once in Tris buffer, the centrifugation was repeated, and the resulting pellet was resuspended in Tris buffer to a final volume of 10 mL. The material was kept in 4°C during the preparation procedure. Finally, the suspension was divided into 10 aliquots, 'snap-frozen' in liquid nitrogen, and stored at -70°C for up to 2 months. The protein content was determined using the method of Lowry *et al.* (1951).

Dose–response studies in tissue chambers

Preparations for the tissue chambers were made immediately before use, 24 h after slaughter. The smooth muscle layer was dissected from the cartilage and the airway epithelium was carefully removed. Thin strips of muscle (measuring $\approx$ 2 $\times$ 20 mm) were prepared and secured with silk ties in 50-mL High Tech Tissue Chambers (Radnoti Glass Technology Inc, Monrova, CA, USA) between a stationary glass holder and a high sensitivity isometric force transducer (Kent Scientific Corp., Lichfield, CT, USA). The signals were recorded on compact flatbed recorders (SE120, Asea Brown Boveri, Vienna, Austria).

In the tissue chambers, the preparations were put under a preload of 5 g which gave a final preload of $\approx$ 2 g after equilibration for 30 min. The preparations were kept in Krebs solution, gassed with 93.5% O_2 and 6.5% CO_2. The pH was 7.4 and the temperature 37°C. Two strips were taken from each horse and the results were calculated from the mean values.

The preparations were contracted with 1 mM acetylcholine to establish the level of 100% contraction and then washed. The study was performed at a contraction level of 60% of the maximum contraction. This was obtained by $\approx$ 40 nM carbachol.

To relax this contraction clenbuterol, salbutamol or terbutaline was added cumulatively until 100% relaxation was achieved. All of the three agonists were tested on each horse.

Binding assay

The membrane homogenate was thawed and diluted in Tris buffer. From this membrane suspension aliquots of 150 µL (40 µg protein) were added to test tubes containing a solution of the non selective β-adrenoceptor antagonist ^{125}I-cyanopindolol (ICYP) and a solution of an unlabelled drug or the vehicle only to a final volume of 200 µL. All drugs were dissolved in a vehicle containing 20 mL ethanol and 0.2 mL 5 M HCl per 1000 mL H_2O.

The samples were incubated in a water bath for 1 h at 37°C and then harvested through Whatman GF/B filters in a 12-well cell harvester (Skatron, Lier, Norway). The filters were then washed with Tris buffer. Finally the γ-radiation from the filters was recorded in a gamma spectrophotometer (Packard Cobra, Canberra, Australia).

In the saturation study six different concentrations of ICYP were used, ranging from 5 to 200 pM. Binding in the presence of 1 µM of the non selective β-adrenoceptor antagonist propranolol was defined as non specific binding.

The affinities of clenbuterol, salbutamol and terbutaline were studied in competition binding studies. Eleven different concentrations of each drug, ranging from 0.1 nM to 10 µM (clenbuterol) or 1 nM to 100 µM (salbutamol and terbutaline), competed for the binding sites with 20–25 pM ICYP.

Calculations

Data recorded from the tissue chambers and the binding studies were analysed using the computer program Ultrafit for Macintosh (Biosoft, Ferguson, MO, USA). The binding maximum (B_{max}) and the dissociation constant (K_d) for the radioligand were determined with non linear regression using the equation:

$$B = B_{max} \cdot [C]/([C] + K_d)$$

This equation was also used when the percentages of receptor occupancy were determined.

In the competition studies, binding in absence of any competing drug was defined as 100%. Non-specific binding was defined as 0%. The level of 50% inhibition in the competition binding studies (IC_{50}) was also determined with non linear regression, using the negative logarithms of the concentrations for the calculations. The equation used was the classic logistic sigmoid function:

$$\text{Inhibition} = 100/1 + \exp(-K(-\log[C] + \log IC_{50}))$$

where K is a constant determining the shape of the curve. The level of 50% effect in the dose/response studies (EC_{50}) was calculated using the same equation:

$$\text{Effect} = 100/1 + \exp(-K(-\log[C] + \log EC_{50}))$$

The K_d-values of the competitors were calculated from the IC_{50}-values using the equation:

$$K_d = IC_{50}/(1 + ([C]^*/K_d^*))$$

where [C]* and Kd* refer to the radioligand.

Drugs and chemicals

Racemic clenbuterol was obtained from Boehringer Ingelheim vet medica GmbH (D-55216 Ingelheim, Rhine, Germany) and terbutaline hemisulfate, salbutamol hemisulfate, propranolol hydrochloride (racemate), acetylcholine chloride and carbamylcholine chloride from Sigma (St. Louis, MO, USA). ^{125}I-cyanopindolol (2000 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK).

The composition of the Krebs solution was: NaCl 118.1 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 24.9 mM, KH_2PO_4 1.2 mM, and glucose 5.6 mM.

RESULTS

Salbutamol and terbutaline were almost as potent as clenbuterol at relaxing equine tracheal muscle *in vitro* (Table 1). The muscle strips, precontracted by 40 nM carbachol, were completely relaxed by all the three drugs (Fig. 1). However, the affinity of clenbuterol to the receptors was much higher than that of salbutamol and terbutaline (Fig. 2, Table 1). The proportion of the total number of receptors which was bound when the effect was obtained was much larger for clenbuterol than for the other two drugs. At EC_{50} for each drug less than 1% of the receptors were occupied by salbutamol and terbutaline compared to 8% occupancy for clenbuterol.

The density of β-adrenoceptors in the tracheal muscles of these horses was 45 ± 14.3 fmol/mg (mean ± SD, $n = 9$) and the K_d-value of ICYP was 11.4 ± 3.3 pM. The nonspecific binding was very low, less than 10% of the total binding at the K_d-value. The negative logarithms of the EC_{50}-values for the three drugs were also plotted as a function of binding maximum of ICYP. No correlations were seen between the β-adrenoceptor density and the potency of any of the agonists.

DISCUSSION

The potency and efficacy of functional agonists in tissue chamber systems are dependent not only on the characteristics of the drugs involved but also on the kind of assay used. A partial agonist can appear to be either a full agonist or an antagonist depending on how much amplification there is in the assay (Black, 1996). Clenbuterol is a partial agonist (Cohen *et al.*, 1982) and we have shown in two earlier studies that the maximum relaxing response from clenbuterol is dependent on the concentration of carbachol used for the precontraction of equine muscle strips (Ingvast Larsson, 1991; Törneke *et al.*, 1997). In the present study we have shown that the affinity of clenbuterol to the β-adrenoceptors of equine tracheal muscle is much higher than the affinity of salbutamol and terbutaline, even though they are almost equally potent agonists. This indicates that clenbuterol has antagonistic properties and gives further evidence to the fact that this drug has low efficacy acting on equine tracheal muscle.

Table 1. A comparison between muscle relaxation in the tissue chambers (EC_{50}, mean values, $n = 9$) and receptor binding (K_d, mean values, $n = 9$)

Drug	EC_{50}-value (95% conf. int.)	K_d-value (95% conf. int.)
Salbutamol	5.6 nM (3.2–10.2)	1100 nM (780–1400)
Terbutaline	13.8 nM (6.8–28.2)	3900 nM (2600–5800)
Clenbuterol	2.1 nM (0.9–6.8)	24 nM (6.8–31)

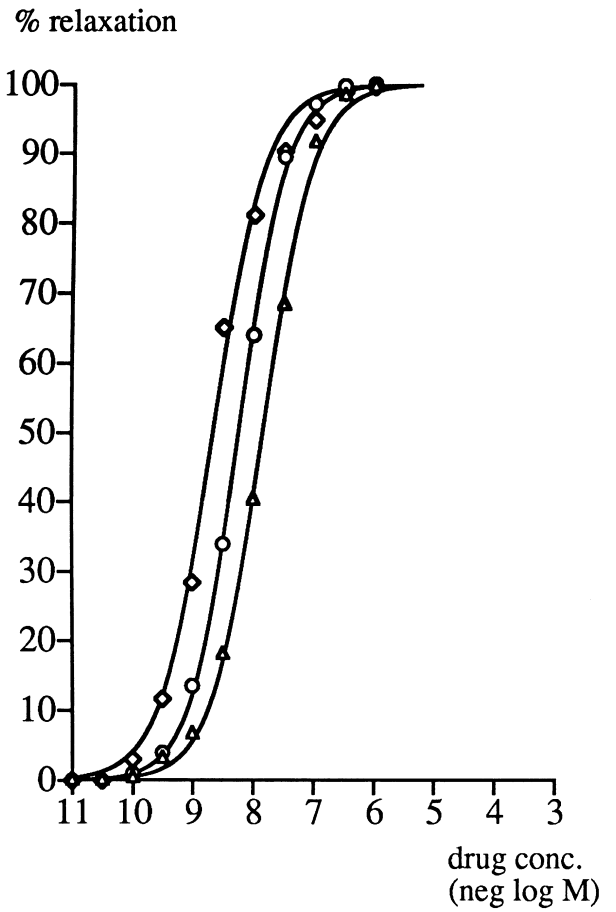

Fig. 1. The relative potency of salbutamol (○), terbutaline (△) and (◇) clenbuterol relaxing muscle strips contracted by 40 nM carbachol in tissue chambers. Each point shows the mean value of duplicates from 9 horses.

The affinities of salbutamol and terbutaline were low in comparison to their potencies. At drug concentrations corresponding to the EC_{50}-values, less than 1% of the receptors were occupied by the ligands. The K_d-value of terbutaline was in agreement with earlier reports and corresponded to the low affinity state of the receptor (Nerme *et al.*, 1989). The high affinity state was not seen in this study as magnesium had not been added to the buffer (Williams *et al.*, 1978).

We investigated β_2-adrenoceptor agonists which functionally inhibited the muscle contraction obtained by 40 nM carbachol. As the concentration of carbachol was low the maximum response obtained by clenbuterol was 100%. Clenbuterol is known to be a partial agonist but this property could not be distinguished in this tissue chamber assay.

On the other hand, the EC_{50}-value of clenbuterol (2.1 nM) corresponded well to the plasma concentrations obtained during

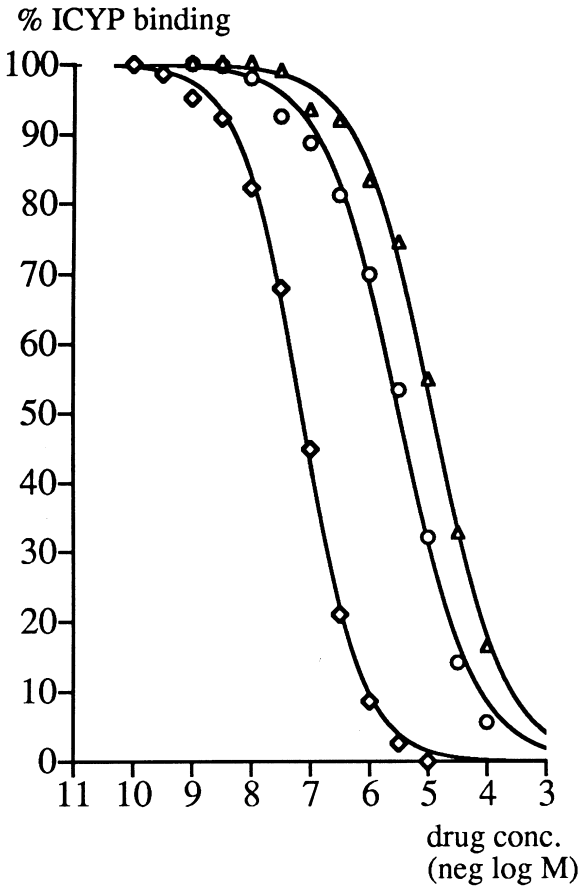

Fig. 2. Displacement of ICYP binding by salbutamol (○), terbutaline (△) and (◇) clenbuterol. Each point shows the mean value of duplicates from 9 horses.

treatment with the drug at the recommended dosage of 0.8 μg/mg. The plasma concentrations obtained after a dosage of 0.8 μg/kg twice daily were 1.6–2.7 nM (Kallings *et al.*, 1991). In humans the therapeutic plasma concentration is ≈ 1.8 nM (Zimmer & Bücheler, 1976). Thus this assay does not give a correct picture of the efficacy but seems to resemble the *in vivo* potency of the drugs. It could be assumed that a dose of salbutamol or terbutaline which gives plasma concentrations similar to the EC_{50}-values obtained in this study would be sufficient for therapy. Currently no data of therapeutic plasma concentrations are available for these drugs in horses. In humans the plasma concentration after recommended dosage of terbutaline (5 mg) is ≈ 11 nM. If the EC_{50}-value for terbutaline of 13.8 nM resembles the *in vivo* potency in horses this drug is equipotent in horses and humans. The plasma concentration after recommended dosage (4 mg) of salbutamol is ≈ 35 nM, which is six times higher than our EC_{50}-value (Hochhaus & Mollmann, 1992). The protein binding of salbutamol is rather low in humans, so this cannot explain the difference. Therefore, this drug seems to be more potent acting on equine tissue than human.

It is well known that there is a large interindividual variation in response to β-adrenoceptor ligands *in vivo*. It has been shown that response to propranolol can be correlated to β-adrenoceptor density on human lymphocytes (Zhou *et al.*, 1989). In a study of canine tracheal muscle, a correlation between relaxation caused by isoprenaline in tissue chambers and β-adrenoceptor density was found (Minneman *et al.*, 1983). It has also been shown that the density of β-adrenoceptors down-regulates upon chronic and excessive stimulation (Fraser *et al.*, 1981). In our study no correlation was observed between individual EC_{50}-values for clenbuterol and β-receptor density, probably because of the low concentration of carbachol used. Otherwise such a correlation should be evident for a partial agonist with high affinity for the receptors, such as clenbuterol.

The clinical efficacy needed to relax contracted airways in a horse *in vivo* is very difficult to estimate. Therefore, we can only speculate about whether or not the antagonistic properties of clenbuterol are too strong to make this drug suitable for therapy. Furthermore, the response to the drug will differ among individuals due to receptor density, level of functional antagonism and other interindividual differences. To use a partial β-adrenoceptor agonist in therapy could be an advantage. There might be less adverse effects as the drug would not be efficacious acting on β_1-adrenoceptors or acting in tissues where the β-adrenoceptor density is low (Waldeck *et al.*, 1986). However, the lack of response to clenbuterol is evident *in vivo* (Derksen *et al.*, 1987; Erichsen *et al.*, 1994) and it is reasonable to believe that this is due to insufficient intrinsic efficacy. Therefore, if it is possible to find an appropriate dosage based on pharmacokinetic data and clinical studies, at least in severe cases of airway obstruction, it might be preferable to use salbutamol or terbutaline instead of clenbuterol to provide adequate bronchodilation in acute cases of equine COPD.

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