Relaxing effects of clenbuterol, ritodrine, salbutamol and fenoterol on the contractions of horse isolated bronchi induced by different stimuli

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"Titbit" slaughterhouse (Bagnolo in Piano, Reggio Emilia, Italy)

ABSTRACT

β_2-adrenoceptor agonists are considered the most effective drugs to counteract bronchoconstriction in horses with asthma, but only clenbuterol is commonly employed in clinical practice. We evaluated the effects of different selective β_2 agonists: clenbuterol, ritodrine, salbutamol, and fenoterol on the contractions of isolated bronchial muscle of horses induced by electrical field stimulation (EFS), carbachol, histamine, and KCl. All β_2 agonists reduced the amplitude of contraction induced by the different stimuli but with variable efficacy and potency. Fenoterol and salbutamol were more effective than clenbuterol in relaxing the bronchial contractions induced by EFS and histamine, and were able to completely abolish carbachol-induced contractions, unlike clenbuterol and ritodrine. The respective potency values (pEC_{50}) of clenbuterol, ritodrine, salbutamol, and fenoterol were 7.74±0.20, 7.77±0.17, 7.30±0.23, 8.01±0.13, for EFS-induced contractions; 8.39±0.26, 5.49±0.28, 6.63±0.14, 7.68±0.11, for carbachol-induced contraction; 7.39±0.27, 7.04±0.28, 6.45±0.34, 7.34±0.22, for histamine-induced contraction; 7.15±0.06, 6.07±0.20, 6.48±0.14, 6.70±0.18, for KCl-induced contraction. Salbutamol and fenoterol showed a higher efficacy than clenbuterol in relaxing horse bronchial muscle pre-contracted by most stimuli. Clenbuterol displayed a good potency but a rather low efficacy, and this may be due to its partial agonist nature; ritodrine showed lower or not significantly different efficacy and potency compared to the other agonists. An evaluation of the clinical efficacy by fenoterol and salbutamol in horses with asthma could be of great interest to assess if they could represent more effective bronchodilators compared to clenbuterol.

Keywords:

Clenbuterol

Ritodrine

Salbutamol

Fenoterol

Horse

Bronchi

1. Introduction

Equine asthma is a common chronic disease of respiratory system which affects horses, inducing recurrent bronchoconstriction, variable airflow reduction, decreased performance, bronchial hyper-responsiveness, and inflammation (Leclere et al., 2011; Léguillette, 2003). Although this disease shares some features with human chronic obstructive respiratory disease (COPD) (Calzetta et al., 2017a), it is now considered more similar to human asthma, especially for its multifactorial pathogenesis, characterised by an interplay between genetic predisposition and environmental triggering factors (Bond et al., 2018; Robinson et al., 1996); the term "equine asthma" is therefore currently preferred in place of recurrent airway obstruction (RAO), COPD or heaves,. Since the exposure to specific airborne antigens in susceptible subjects is the spark that ignites the immune reaction leading to bronchospasm and inflammation of the airways, the most effective way to treat this disease would be environmental control. An effective and thorough removal of antigens is however often impossible, and thus the management of clinical signs in asthmatic horses relies heavily on the use of bronchodilator and anti-inflammatory drugs, such as β_2-adrenoreceptor (AR) agonists, antimuscarinic agents, and corticosteroids. Other drugs have been proposed for the therapy of this disease, and clinically tested, such as methylxanthines, and mast-cell stabilisers with variable results (Pirie, 2014), but a recent meta-analysis study on horses treated for asthma concluded that β_2 agonists and corticosteroids are the most effective drugs, and should be considered the paramount therapeutic tools against this disease (Calzetta et al., 2017b). Among β_2-AR selective agonists, clenbuterol is the most frequently administered to horses with asthma, while small clinical trials were conducted with other drugs such as terbutaline, fenoterol, salbutamol, or salmeterol (Erichsen et al., 1994; Henrikson and Rush, 2001; Matera et al., 2011; Murphy et al., 1980; Tesarowski et al., 1994). Although being effective therapeutic agents to counteract excessive bronchoconstriction, these drugs may cause several adverse effects such as tachycardia, arrhythmia, sweating, muscular tremors, hyperglycaemia, excitement and alterations of gastrointestinal motility (Derksen et al., 1987; Pearson

and Riebold, 1989; Thomson and McPherson, 1983). Most of these unwanted side effects are due to the activation of β_2-ARs in organs outside the respiratory system; moreover, even though these drugs display a selectivity for β_2-ARs, some degree of binding and stimulation of β_1-ARs may also occur, increasing the risk of inducing cardiac adverse effects. Despite its frequent usage, the clinical efficacy of clenbuterol in horses with obstructive pulmonary disease is rather controversial (Cohen et al., 1982; Erichsen et al., 1994; Kearns and McKeever, 2009; Torneke et al., 1998), at least at usually suggested dosage, and one of the reasons could be that this drug seems to behave as a partial agonist at β_2-ARs (Cohen et al., 1982; Törneke et al., 1998). Moreover, different drugs have diverse degrees of selectivity for β_2-ARs, and this may influence the incidence of side effects; indeed, clenbuterol was shown to possess a selectivity ratio for β_2 versus β_1 receptors five times lower compared to fenoterol (Baker, 2010), thus possibly posing a higher risk of causing adverse effects due to β_1-AR activation compared to more selective drugs. Salmeterol and formoterol are highly selective β_2 agonists, which induce long-lasting bronchodilation, and are currently prescribed for the treatment of asthma in human patients, but not in horses; aerosolised fenoterol and salmeterol have been tested in two small studies on horses with RAO (Henrikson and Rush, 2001; Tesarowski et al., 1994), whereas formoterol has not been, until now, investigated for its clinical efficacy against equine asthma.

The purpose of this study was to assess the effects of selective β_2-AR agonists clenbuterol, ritodrine, salbutamol, and fenoterol on the contractions of isolated bronchial muscle of the horse induced by different stimuli.

2. Materials and methods

2.1. Preparations of tissues

Samples of lung were collected from 28 male horses, slaughtered at a private abattoir; the experiments were performed between September 2017 and October 2018. The samples were excised from the apical lobes of lungs and collected tissues were stored in ice-cooled (4 °C) modified Krebs-Henseleit Solution (KHS) of the following composition (mM): NaCl 113.0, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 2H_2O$ 1.8, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and dextrose 11.2, for the 10-min transport from the slaughterhouse to the laboratory. Bronchial segments were then isolated from the surrounding parenchyma, and bronchial rings of 0.7-1 cm diameter (8 from each horse) were obtained to be used in motility experiments. Each ring was put into a 10 mL organ bath at 37 °C, containing the solution above described, gassed with 95% O_2 and 5% CO_2 (pH 7.4). The bronchial samples were left to stabilize for 60 min and then the mechanical activity was measured by means of an isotonic transducer (Ugo Basile) connected to the preparation, developing a passive stretch of 1 g throughout the entire experiment. Pilot experiments were performed to establish the optimum load to get the best contractile activity. The viability of bronchial rings was assessed by the ability of acetylcholine (ACh) 10^{-7} M to evoke a contractile response (> 0.1 cm shortening).

2.2. Motility experiments

The effects induced by different β_2 agonists were studied on the contractions of bronchial smooth muscle induced by electrical field stimulation (EFS), carbachol (nonselective muscarinic agonist), histamine, and potassium chloride (KCl). EFS was applied by means of two coaxial platinum electrodes positioned 10 mm from the longitudinal axis of the preparation and used to deliver trains

of square wave pulses (1 ms duration, 50 V amplitude) every 90 s to the tissue, at a frequency of 15 Hz (Ugo Basile). Phasic contractions of constant amplitude were obtained, and for each experiment, the intensity was adjusted to a level giving 70-80% of the maximum tissue response (usually 250-300 mA) in order to detect both a decrease and an increase of contraction amplitude. For the assessment of the effects of β_2 agonists on the sustained contractions induced by carbachol, histamine, or KCl, either a single aliquot of 10^{-7} M carbachol, 10^{-6} M histamine or $2x10^{-2}$ M KCl was added into the organ bath solution, and the effect was observed until a constant plateau of contraction was achieved. The EFS parameters and the concentrations of carbachol and histamine were selected because, according to preliminary experiments, they were able to induce stable contractions, suitable to evaluate the effects of different β_2 agonists. For each experiment, concentration-response curves of the agonists were constructed, by adding cumulatively each drug into the bath solution in 1-log unit increments of concentration. Each concentration-response curve was then fitted to nonlinear regression with variable slope using a commercial software (GraphPad Prism ver. 7.0a, GraphPad Inc.). The effects of β_2 agonists were expressed as the percentage of variation of pre-drug amplitude of contraction, assumed as 100%. Agonist efficacy was expressed by calculating the maximum effect obtained for each drug (E_{max}). The potency of each agonist was expressed by the concentration giving 50% of maximum effect (EC_{50}) from individual fitted concentration-response curves and expressed with pEC_{50} value (-Log EC_{50}).

In a separate set of experiments, the effects some of the β_2-AR agonists on carbachol-induced contraction were also evaluated in presence of ICI 118,551 a selective β_2-AR antagonist. From each horse, one bronchial ring was used for the concentration-response curve of the agonist alone, while two rings were employed for the curve of the agonist in presence of the antagonist. When the antagonist was used, a single concentration of the ICI 118,551 was left to incubate for twenty minutes in the organ bath solution, and the concentration-response curve with each agonist was constructed. In these experiments, antagonistic potency was expressed by pA_2 value (-log K_B, K_B being the equilibrium constant of the antagonist).

147 *2.3. Drugs*

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149 ACh chloride, carbachol chloride, histamine hydrochloride, KCl, selective β_2-AR agonists
150 clenbuterol hydrochloride, ritodrine hydrochloride, salbutamol hemisulphate, fenoterol
151 hydrobromide, and selective β_2-AR antagonist, ICI 118,551, were purchased by Sigma Aldrich
152 (Sigma Chemical Co.). All drugs were dissolved in distilled water; the solutions were freshly
153 prepared before each experiment, and proper aliquots (10-100 μL) were added to the organ baths to
154 obtain the desired molarity.

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156 *2.4. Statistical analysis*

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158 All data are expressed as mean±SEM from 8 experiments. Differences among groups of data were
159 evaluated by ANOVA followed by Turkey's post hoc test. $P<0.05$ was considered statistically
160 significant. All calculations were performed using GraphPad Prism software.

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163 **3. Results**

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165 *3.1. EFS-induced contractions*

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167 All β_2 agonists induced a concentration-dependent decrease of contraction amplitude of horse
168 bronchial smooth muscle (Fig. 1), with the following efficacy values (E_{max}): clenbuterol (-
169 53.62±5.21%), ritodrine (-68.55±5.26%), salbutamol (-75.94±6.95%), and fenoterol (-86.60
170 ±10.08%), (Fig. 1; Table 1). Fenoterol and salbutamol resulted significantly ($P < 0.05$) more
171 effective compared to clenbuterol (Table 1).

The potency values (pEC_{50}) calculated against EFS-evoked contractions were 7.74±0.20, 7.77±0.17, 7.30±0.23, and 8.01±0.13 for clenbuterol, ritodrine, salbutamol, and fenoterol, respectively (Table 2).

3.2. Carbachol-induced contraction

The amplitude of carbachol-induced sustained contraction was reduced by all β_2 agonists in a concentration-related fashion (Fig. 2). Fenoterol was able to completely abolish carbachol-induced contraction (E_{max} = 100%) at the concentration of 10^{-7} M; salbutamol was also effective in reducing the contraction by 100% but only at 100-fold higher concentration (10^{-5} M). By contrast, clenbuterol and ritodrine had a maximal efficacy of -83.80±11.52% and -88.30±11.70%, respectively (Fig. 2; Table 1). Clenbuterol was however the most potent (pEC_{50} = 8.39±0.26), followed by fenoterol (pEC_{50} = 7.68±0.11), salbutamol (pEC_{50} = 6.63±0.14), and ritodrine (pEC_{50} = 5.49±0.28) (Table 2).

3.3. Histamine-induced contraction

The amplitude of histamine-induced plateau of contraction of horse bronchi was reduced by all β_2 agonists in a concentration-related fashion (Fig. 3), even though with substantial differences in efficacy. Salbutamol and fenoterol were almost equally effective, and showed significantly higher values of E_{max} (-95.44±3.51%, and -91.63±6.24%, respectively), compared to clenbuterol and ritodrine (-47.17±10.92% and -24.14±6.21%, respectively) as shown in Table 1. Clenbuterol and fenoterol had similar potency values (pEC_{50} = 7.39±0.27, and 7.34±0.22, respectively), whereas pEC_{50} for ritodrine and salbutamol were 7.04±0.28 and 6.45±0.34, respectively (Table 2).

3.4. KCl-induced contraction

The sustained contraction of horse isolated bronchi induced by KCl was only partially inhibited by β_2 selective agonists (Fig. 4). The efficacy values of the agonists for clenbuterol, ritodrine, salbutamol, and fenoterol were: -29.02±6.46%, -29.36±8.92%, -55.25±9.97%, and -52.66±12.95%, respectively (Fig. 4; Table 1). Clenbuterol and fenoterol had significantly higher pEC_{50} values (7.15±0.06 and 6.70±0.18, respectively) compared to ritodrine (6.07±0.20, $P < 0.001$ and $P < 0.05$) (Table 2). Calculated pEC50 for salbutamol was 6.48±014.

3.5. Effects of clenbuterol and fenoterol on carbachol-induced contraction in presence of ICI 118,551

Selective β_2-AR antagonist, compound ICI 118,551 (10^{-8} and 10^{-7} M), competitively antagonised the relaxing effects of both salbutamol and fenoterol (Fig. 5). Calculated pA_2 values for ICI 118,551 were 8.86±0.29 and 8.91±0.16 for salbutamol and fenoterol, respectively.

4. Discussion

All tested β_2-AR agonists were able to reduce the contractions of isolated horse bronchi induced by different stimuli. Among β_2 agonists, only clenbuterol, however, has been so far approved for the use in the horse, while very few studies are available regarding the effects induced by other selective β_2-agonists on airway smooth muscle in this species.

ACh, released from parasympathetic nerve terminals, is the main neurotransmitter responsible for bronchoconstriction in most species, including the horse, and an excessive response of muscarinic receptors and/or an enhanced release of this substance have been involved in the pathogenesis of asthma both in horses and humans (Wang et al., 1995; Hashimoto et al., 1996; Zhang et al., 1999). In vitro assessment of the ability of β_2-AR agonists to relax bronchial smooth muscle pre-contracted with ACh or other muscarinic agonists (such as carbachol) is therefore of great utility for the screening of potential new drugs to be used clinically in asthma-affected horses. In our study, each of the four tested selective β_2 agonists inhibited the phasic contractions evoked by EFS in concentration-related manner; since these contractions were previously shown to be induced by the release of endogenous ACh (Menozzi et al., 2014), these effects closely relates to the ability of drugs to dilate bronchial muscle contracted by parasympathetic stimulation, as it occurs in horses with asthma. Although none of the β_2 agonists were able to completely abolish EFS-induced contractions, fenoterol and salbutamol resulted significantly more effective with respect to clenbuterol. These data are in agreement with previous findings of a higher efficacy in activating β_2-ARs by fenoterol and salbutamol compared to clenbuterol (Baker, 2010); moreover, clenbuterol was shown to possess the lowest intrinsic efficacy among different selective β_2 agonists in relaxing equine tracheal muscle pre-contracted with carbachol (Torneke et al., 1998). No significant differences in potency were found among the four selective β_2 agonists in relaxing bronchial muscle pre-contracted by EFS. This result is not in accordance with previous evidence of clenbuterol being the most potent among all four agonists at human β_2-AR, as well as in motility studies on horse tracheal muscle and human bronchi (Baker, 2010; Nials et al., 1993; Torneke et al., 1998). None of these studies, anyway, evaluated the relaxant properties of β_2 agonists against EFS-induced contractions, and this might be one reason for such discrepancy.

The results regarding the efficacy of the different β_2 agonists in relaxing carbachol-induced contraction are particularly interesting since the activation of muscarinic receptors plays a major role

246 in asthma-related bronchoconstriction in the horse (Broadstone et al., 1988); indeed, clenbuterol was
247 the most potent in reducing this type of sustained contraction, as previously found in isolated equine
248 tracheal muscle (Torneke et al., 1998), followed by fenoterol, salbutamol, and ritodrine. Fenoterol
249 was superior in potency compared to salbutamol in relaxing bronchial smooth muscle pre-contracted
250 by carbachol, confirming what was already observed in human bronchial muscle, since fenoterol was
251 up to 24 times more potent than salbutamol, in reducing airway inherent tone (Linden et al., 1996;
252 Nials et al., 1993).
253 However, clenbuterol and ritodrine , reached a maximal effect of -83.80% and -88.30%, respectively,
254 whereas fenoterol and salbutamol relaxed bronchial preparations completely; in addition, fenoterol
255 was able to abolish carbachol-induced contraction at a concentration (10^{-7} M) 100 times lower with
256 respect to salbutamol. By contrast, in a previous study, clenbuterol abolished completely the
257 contraction caused by carbachol (Torneke et al., 1998); in our experiments, however, we used a
258 concentration of carbachol which was more than two-fold with respect to that employed in that study,
259 and this may explain this difference. Since clenbuterol has a low intrinsic efficacy (Torneke et al.,
260 1998), its inability to completely relax bronchial smooth muscle contracted by a higher concentration
261 of carbachol is not surprising.
262 The relaxing effects of salbutamol and fenoterol on carbachol-induced contraction were
263 antagonised by selective β_2-AR antagonist ICI 118,551 in a competitive fashion, and pA_2 values
264 determined for this drug are in accordance with those previously found for this compound (Goldie et
265 al., 1984; Janssen and Daniel, 1991); since very few data are available about salbutamol in horse
266 isolated bronchi, and fenoterol has never been studied before in this tissue, our results seem to
267 confirm that these two agonists relax bronchial smooth muscle by activating adrenoceptors of the β_2
268 subtype.
269 Even though ACh is the main neurotransmitter responsible for the contraction of bronchial smooth
270 muscle in most species, other endogenous mediators exert a contractile effect on airway muscle, and
271 play a role in the pathogenesis of asthma, such as histamine. Histamine, released from mast-cells,

was shown to promote the release of ACh from parasympathetic nerve terminals, and to sensitize smooth muscle cells to ACh-induced contraction (Marinkovic et al., 2007); moreover, histamine directly contracts airway muscle by the activation of H_1 receptors. In the present study, selective β_2 agonists were effective in relaxing horse bronchial muscle preparations pre-contracted with 10^{-6} M histamine. However, only fenoterol and salbutamol were able to reduce the amplitude of contraction almost completely, whereas clenbuterol and ritodrine behaved as partial agonists with an efficacy of only -47.17% and -24.14%, respectively. These results seem to confirm the partial agonist nature of clenbuterol and highlight important differences in the efficacy of selective β_2 agonists against histamine-induced contraction of horse bronchial muscle. Indeed, in a previous study, clenbuterol was ineffective against airway narrowing caused by inhaled histamine in ponies (Derksen et al., 1987), whereas a low dose of fenoterol resulted effective in reducing bronchoconstriction in asthmatic human patients (Magnussen and Rabe, 1992).

The contraction of equine bronchial muscle caused by KCl resulted overall more resistant to the relaxant effect of β_2 agonists, since the maximal efficacy obtained among all drugs was of -55.25% only. This may be probably due to the fact that KCl-induced contraction depends on the influx of extracellular calcium (Bolton 1979), whereas β_2 agonists influence mostly the intracellular content of the ion (Barisione et al., 2010), like it occurs when muscarinic receptors are activated (Gosens et al., 2006).

5. Conclusions

The results of the present study showed that salbutamol and fenoterol were able to relax the bronchial smooth muscle of horses contracted by muscarinic receptor stimulation and by histamine with a higher efficacy compared to clenbuterol. Moreover, the partial efficacy displayed by

clenbuterol in reducing the contraction of horse bronchial muscle in this study seems to support earlier findings of a low efficacy both in vivo and in vitro (Derksen et al., 1987; Erichsen et al., 1994; Johnson, 1998; Torneke et al., 1998).

Ritodrine did not seem to be more effective than clenbuterol in reducing the contractions of horse bronchial muscle and, since data regarding its efficacy as a bronchodilator in vivo are lacking, being currently used only to prevent pre-term labour in humans (Kim et al., 2018), it is hard to foresee a possible future clinical utility of this drug in respiratory diseases of horses.

The good efficacy showed by fenoterol and salbutamol could instead promote further studies to assess if they may represent valid alternatives to clenbuterol to be used as bronchodilators in horses with asthma. The use of selective agonists with a higher selectivity for β_2-ARs versus β_1-ARs, such as fenoterol, could also grant a lower incidence of adverse effects due to the activation of β_1 receptors, such as tachycardia or cardiac arrhythmias. Inhaled fenoterol was indeed previously shown to improve clinical signs and respiratory parameters in horses with heaves, without evidence of side effects (Tesarowski et al., 1994). Additional in vivo studies are anyway required in order to expand the knowledge about the clinical efficacy and safety of fenoterol and salbutamol in horses with asthma.

Competing interest statement

All authors agree to the publication of this manuscript and have no conflicts of interest to declare.

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Figure legends

Fig. 1. Relaxing effects of increasing concentrations of clenbuterol, ritodrine, salbutamol, and fenoterol on the contractions of horse bronchial muscle induced by EFS. All data are expressed as mean±SEM from 8 experiments.

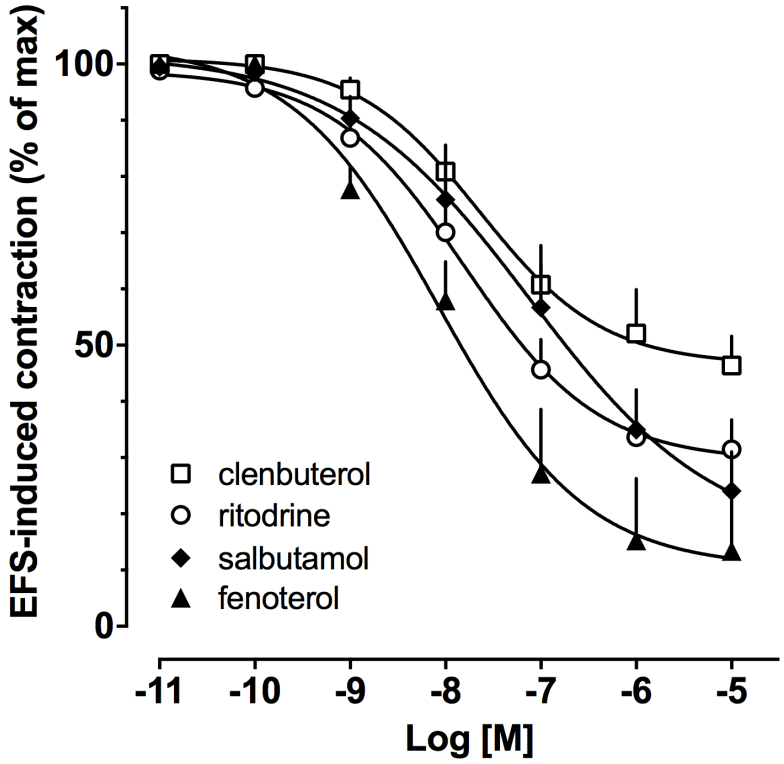
Fig. 2. Relaxing effects of increasing concentrations of clenbuterol, ritodrine, salbutamol, and fenoterol on the contraction of horse bronchial muscle induced by nonselective muscarinic agonist, carbachol 10^{-7} M. All data are expressed as mean±SEM from 8 experiments.

Fig. 3. Relaxing effects of increasing concentrations of clenbuterol, ritodrine, salbutamol, and fenoterol on the contraction of horse bronchial muscle induced by histamine 10^{-6} M. All data are expressed as mean±SEM from 8 experiments.

Fig. 4. Relaxing effects of increasing concentrations of clenbuterol, ritodrine, salbutamol, and fenoterol on the contraction of horse bronchial muscle induced by KCl $2x10^{-5}$ M. All data are expressed as mean±SEM from 8 experiments.

Fig. 5. Relaxing effects of increasing concentrations of salbutamol (A) and fenoterol (B) on carbachol-induced contraction of horse bronchial muscle without (Co) and in presence of selective β_2-AR antagonist ICI 118,551 10^{-8} M and 10^{-7} M. Both salbutamol and fenoterol were antagonised by ICI 118,551 in a competitive fashion.

435 **Fig. 1**



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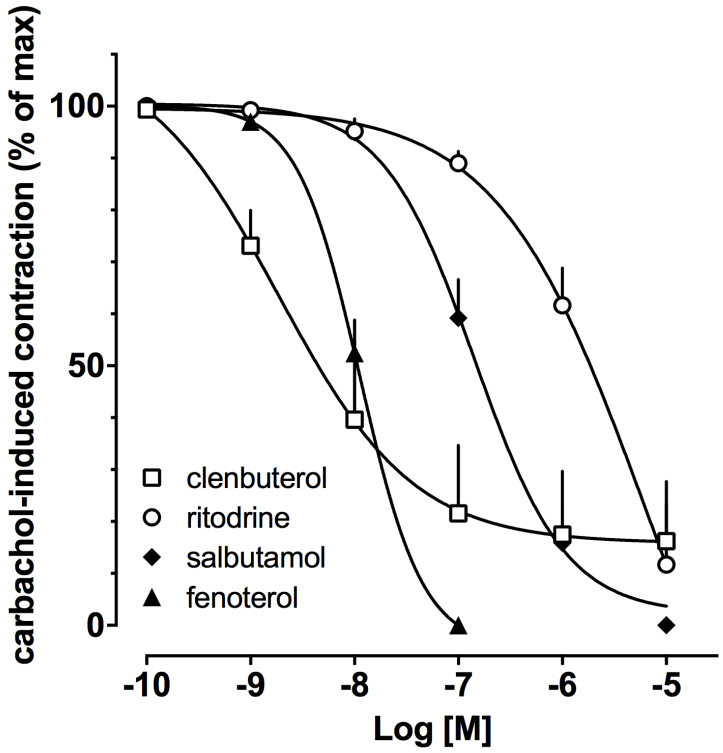
437 **Fig. 2**



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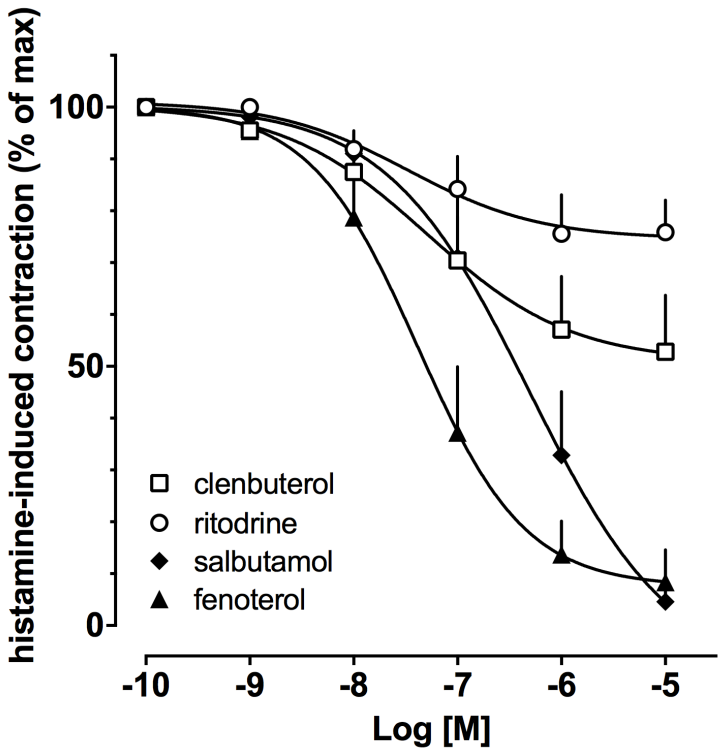
441 **Fig. 3**



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444 **Fig. 4**

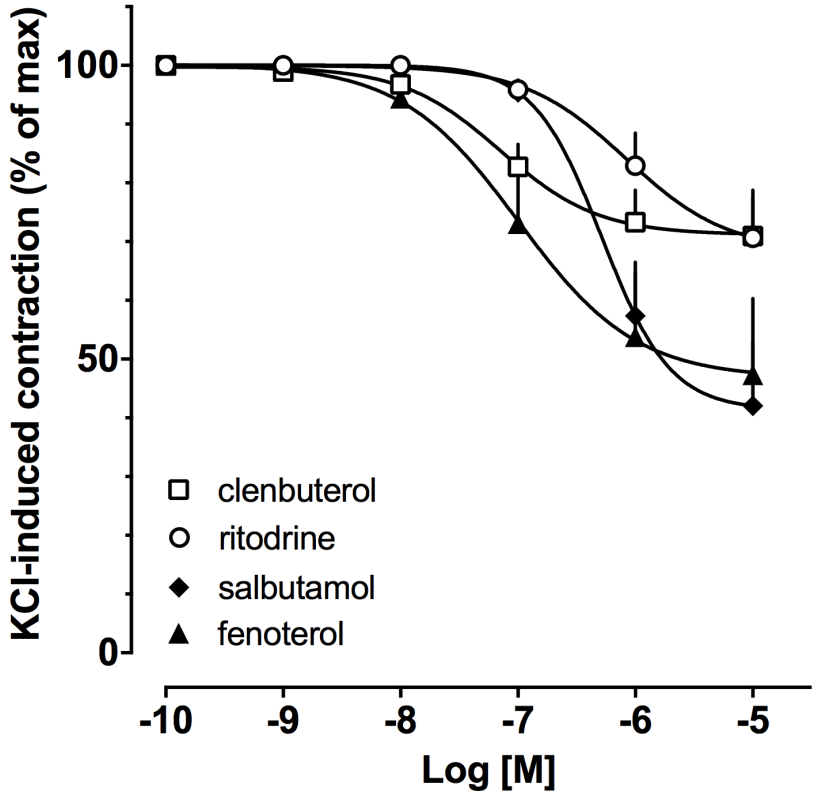

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Fig. 5

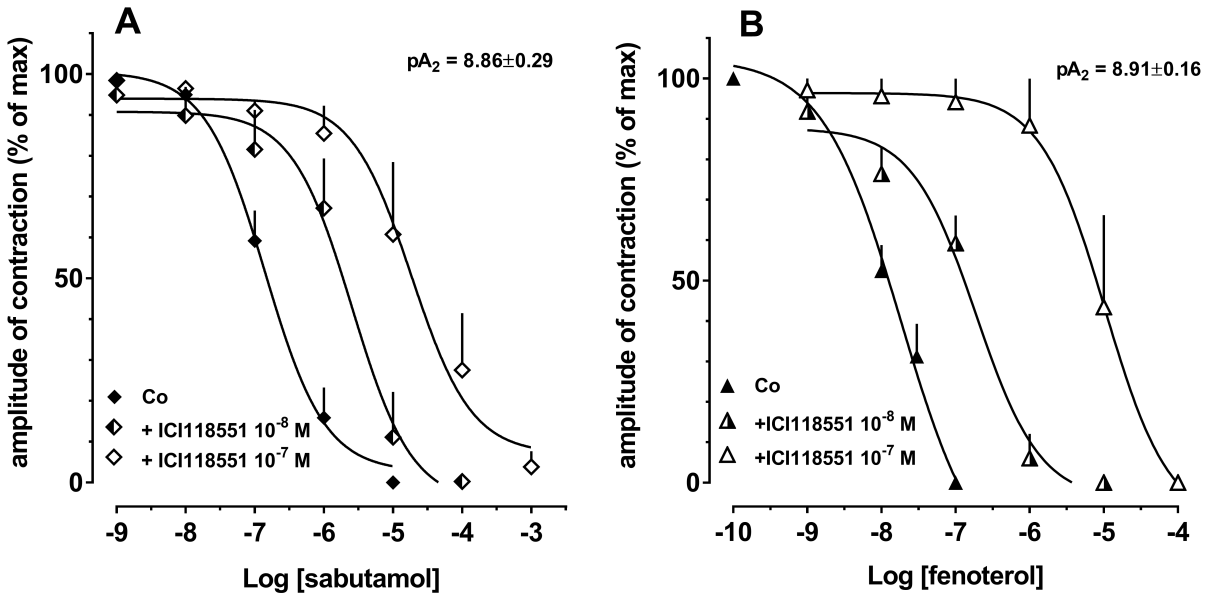

TABLE 1 Efficacy values (E_{max}) for selective β_2 agonists against the contractions of horse bronchial muscle induced by EFS, carbachol, histamine, and KCl. All values represent mean ± SEM of 8 experiments

	EFS	Carbachol	Histamine	KCl
Clenbuterol	-53.62 ± 5.21%	-83.80 ± 11.52%	-47.17 ± 10.92%	-29.02 ± 6.46%
Ritodrine	-68.55 ± 5.26%	-88.30 ± 11.70%	-24.14 ± 6.21%	-29.36 ± 8.92%
Salbutamol	-75.94 ± 6.95% [a]	-100%	-95.44 ± 3.51% [ab]	-55.25 ± 9.97%
Fenoterol	-86.60 ± 10.08% [a]	-100%	-91.63 ± 6.24% [ab]	-52.66 ± 12.95%

[a] $p < 0.01$ *vs* clenbuterol; [b] $p < 0.0001$ *vs* ritodrine.

TABLE 2 Potency values (pEC_{50}) for selective β_2 agonists against the contractions of horse bronchial muscle induced by EFS, carbachol, histamine, and KCl. All values represent mean ± SEM of 8 experiments

	EFS	Carbachol	Histamine	KCl
Clenbuterol	7.74 ± 0.20	8.39 ± 0.26 [ab]	7.39 ± 0.27	7.15 ± 0.06 [d]
Ritodrine	7.77 ± 0.17	5.49 ± 0.28	7.04 ± 0.28	6.07 ± 0.20
Salbutamol	7.30 ± 0.23	6.63 ± 0.14 [c]	6.45 ± 0.34	6.48 ± 0.14
Fenoterol	8.01 ± 0.13	7.68 ± 0.11 [ab]	7.34 ± 0.22	6.70 ± 0.18 [c]

[a] $p < 0.0001$ *vs* ritodrine; [b] $p < 0.001$ *vs* salbutamol; [c] $p < 0.05$ and [d] $p < 0.001$ *vs* ritodrine.