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Different effects of salmeterol, formoterol and salbutamol on cholinergic responses in the ferret trachea

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1 In the present study, the inhibitory effects of the selective β_2-adrenoceptor agonists, salmeterol, formoterol and salbutamol, have been investigated on contractions of ferret trachea induced both by endogenous and exogenous acetylcholine. The aim of the study was to evaluate quantitative and/or qualitative differences in response which may indicate both pre-and post-junctional sites of action. The non-selective β-antagonist, sotalol, was used to estimate β-adrenoceptor involvement.

2 Isometric tension was measured in ferret isolated tracheal strips. The inhibitory effects of the drugs were studied on tonic contractions induced by pre-junctional activation with electrical field stimulation (EFS) (2 Hz, 700 mA) or post-junctional activation with exogenous acetylcholine (ACh) (0.5 μM, about EC_{80}), giving a similar degree of smooth muscle response.

3 Concentration-response experiments were performed with formoterol (0.3 nM–0.3 μM) and salmeterol and salbutamol (10 nM–10 μM). The experiments ended with the addition of sotalol (10 μM).

4 All three β-agonists inhibited the contractions in a concentration-dependent manner. Salbutamol, formoterol and salmeterol inhibited the EFS-induced contractions by 66(8)%, 105(5)% and 103(8)% (mean(s.e.mean)) respectively. ACh-induced contractions were inhibited by 37(6)%, 72(11)% and 33(8)%. Theophylline (10 nM–3 mM) inhibited the contractions to the same degree.

5 β-Adrenoceptor blockade by sotalol significantly antagonized the inhibitory effects of salbutamol and formoterol on both EFS- and ACh-induced contractions. The effect of salmeterol on ACh-induced contraction was also significantly antagonized, whereas the inhibition of EFS-induced contraction was virtually unaffected.

6 In conclusion, salbutamol, salmeterol and formoterol produced greater inhibitory effects in preparations contracted by EFS than in preparations contracted by exogenously-added ACh. In the case of formoterol and salbutamol, the effects on both levels are most probably due to β-adrenoceptor stimulation, whereas for salmeterol the dominant pre-junctional effect is probably not mediated via β-adrenoceptors. This non-β-mediated effect could represent an additional relaxant mechanism for salmeterol.

Keywords: β-Agonists; cholinergic responses; pre-junctional β-adrenoceptors; salmeterol; formoterol; salbutamol

Introduction

Two long-acting β-adrenoceptor agonists, salmeterol and formoterol, have recently been developed. The duration of the bronchodilator effect in human subjects is about 12 h compared with 4–6 h for earlier available β-agonists. The mechanism for the long duration is not fully understood and could be explained by a combination of properties. Lipophilicity is probably one important factor (Anderson *et al.*, 1994), but other properties, such as binding to an exosite close to the receptor have also been suggested (Ball *et al.*, 1991).

Lipophilicity is probably also important for the unspecific, non-β-mediated effects which has been shown for β-adrenoceptor ligands (Ijzerman *et al.*, 1987). In the case of salmeterol, several observations have been published showing non-β-mediated effects in both the airway smooth muscle and in other cells (Baker & Fuller, 1990; Jeppsson *et al.*, 1992; Lindén *et al.*, 1993; Coleman *et al.*, 1994; Nials *et al.*, 1994). For formoterol and salbutamol, there are no data concerning non-β-mediated effects.

The bronchodilator effect for β-agonists has been attributed to the stimulation of β-adrenoceptors on the smooth muscle. However, in several functional *in vitro* studies it has been suggested that β-agonists also exert a pre-junctional effect on cholinergic nerves, leading to a reduced output of acetylcholine (Vermeire & Vanhoutte 1979; Baker & Don 1987; Danser *et al.*, 1987; Ito, 1988; Rhoden *et al.*, 1988; Janssen & Daniel, 1990).

In the present study, we evaluated the relaxant effects of salmeterol and formoterol in the ferret isolated trachea. Possible non-β-mediated effects were estimated after treatment with the non-selective β-antagonist, sotalol. Contractions were induced either by exogenously added acetylcholine (ACh) or by electric field stimulation (EFS). ACh-induced contractions can only be inhibited by post-junctional mechanisms, whereas EFS-induced contractions can be inhibited by both post-junctional and pre-junctional mechanisms. To facilitate studies of the pre-junctional effects, we chose the ferret trachea, a preparation containing relatively few β_2-adrenoceptors mediating the post-junctional β-mediated relaxation (Skoogh *et al.*, 1989).

Thus, the aim of this study was to investigate the inhibitory effects of salmeterol, formoterol and salbutamol on cholinergic nerve-induced contractions (EFS) and exogenously-added ACh contractions in order to evaluate differences concerning the magnitude of the effects as well as to what extent these effects are β-adrenoceptor mediated.

Methods

The study was approved by the Animal Ethics Committee at the Medical Faculty at Göteburg University (DNr:260/88).

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Preparation

Eighteen male ferrets were killed instantly by electrocution and then exsanguinated. The trachea was rapidly removed and immediately immersed in oxygenated Krebs-Ringer (KR) solution with the following composition (in mM): NaCl 118, KCl 5.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.6. Ascorbic acid (0.3 mM) was added to the KR as an anti-oxidant. The trachea was dissected free and cut into 8 rings, each 7 mm wide. The rings were opened by cutting the cartilage opposite the smooth muscle and then connected to steel hooks. The epithelium covering the muscle was removed by gentle dissection in order to avoid the influences of epithelium-derived relaxant substances (Ullman *et al.*, 1988; 1990a,b).

The strips were mounted in 7 ml organ baths with KR maintained at 37°C and aerated with 94% O_2-6% CO_2 which produces a pH of 7.4. The baths were constantly flushed with KR, 1.5 ml min^{-1}, during the experiment. The isometric muscle tension was continuously recorded via Grass force transducers (FT03) connected to an NB-MIO-16 analogue/digital converting board and a MacIntosh II computer using the Labview signal-processing software (National Instruments, Austin, Texas, USA) (Ullman *et al.*, 1990a). This system permits the real-time monitoring of the experiment and the simultaneous and continuous saving of data for subsequent evaluation.

Experimental design

The tension was initially set at 8 g. After 60 min the tension was readjusted to 6 g (Skoogh *et al.*, 1982). A tonic contraction was induced either by exogenous acetylcholine (ACh) causing a post-junctional activation or by electric field stimulation (EFS) causing a pre-junctional activation of the cholinergic nerves. ACh was added in an EC_{80} concentration (0.5 μM) according to preliminary experiments and the parameters for EFS were chosen to match this degree of contraction. All the strips were treated with guanethidine (3 μM) (Löfdahl *et al.*, 1986; Brock & Cunnane 1988) to deplete the sympathetic nerves of neurotransmitter, and abolish sympathetic responses to EFS.

EFS was delivered by constant current generators, via rectangular platinum electrodes (5 × 30 mm) mounted parallel to the airway preparations (10 mm apart). Biphasic square waves with a pulse duration of 0.5 ms were applied at a frequency of 2 Hz. The current used was 700 mA. The cholinergic nature of the contractile response was demonstrated in separate experiments by addition of 1 μM atropine which abolished the response.

Thirty minutes after the administration of ACh and the start of EFS, the concentration-response experiments were performed. One animal (8 strips) was used in every experiment, and two treatments of the following four were used. Salmeterol, formoterol, salbutamol or vehicle were chosen randomly in each experiment. Salmeterol has a slow onset of action (Ball *et al.*, 1991; Jeppsson *et al.*, 1992; Ullman *et al.*, 1992) and, to guarantee maximum effect, a one-hour interval between successive administrations of agonist was chosen. This made it impossible to produce the entire cumulative concentration-response curve in the same experiment, and the experiment was therefore divided into two parts each using a separate animal. In the first group, we used 10 nM and 1 μM of salmeterol and salbutamol and 0.3 nM and 30 nM of formoterol. In the second group we used 0.1 μM and 10 μM salmeterol and salbutamol and 3 nM and 0.3 μM formoterol. All experiments ended with the addition of the non-selective β-adrenoceptor antagonist, sotalol (10 μM), to estimate β-adrenoceptor involvement (Figure 1).

In order to ascertain that the contractions induced by EFS and ACh were comparable, and thus equally inhibited by a drug with no pre-junctional effects, we performed concentration-response experiments with theophylline. The EFS and ACh contractions were induced in the manner described above and increasing concentrations of theophylline (10 nM–3 mM) were added cumulatively every 10 min or when the maximum effect was obtained. Isoprenaline (0.1 mM) was finally added to obtain maximum relaxation.

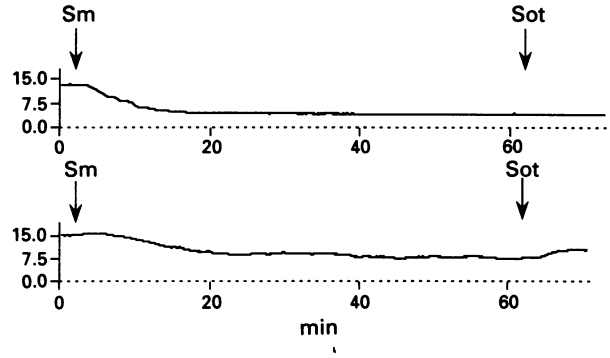

Figure 1 Original recording of the tension (in g) in the ferret tracheal strips. The upper panel shows the electrical-field-stimulation-induced contraction, and the lower panel shows the acetylcholine-induced contraction. Abbreviations: Sm; addition of 10 μM of salmeterol and Sot; addition of 10 μM of sotalol.

Drugs

Acetylcholine chloride (Sigma), atropine sulphate (Sigma), guanethidine sulphate (gift from Ciba-Geigy), (±)-sotalol hydrochloride (gift from Astra Hässle), (±)-isoprenaline hemisulphate (Sigma) and theophylline (Theofyllin inj. 20 mg ml^{-1}; gift from Astra Draco) were dissolved in distilled water. Salmeterol base (racemate) and salbutamol sulphate (gifts from Glaxo) and formoterol fumarate (racemate, pure R- and S-enantiomers; gift from Ciba-Geigy) were initially dissolved in 20 μl of glacial acetic acid and diluted in distilled water and frozen as stock solution.

Data analysis

The drug effects are presented as percentage inhibition of the initial contraction induced by either exogenous ACh or EFS. The concentration-response curves are constructed from the maximum effect seen during the incubation period. All the results are expressed as the mean (s.e.mean). Student's *t* distribution (two-tailed) for differences between data (paired or unpaired) was determined at 95% confidence intervals. The confidence intervals are presented in Table 1 and are referred to by capital letters in the text. *n* equals the number of airway preparations.

Results

There was no statistically-significant difference in the magnitude of the initial contraction in preparations contracted with exogenous ACh (15.9(0.8) g) compared with preparations contracted with EFS (15.1(0.8) g) (Table 1, **A**).

The inhibitory effects of theophylline were similar on contractions induced by EFS and exogenously-added ACh, as shown in the concentration-response curves in Figure 2.

Control strips

The EFS-contracted-preparations showed a slightly (17.9%) lower level of contraction at the end of the experiment than ACh-treated preparations (Table 2), and this small difference was significant (Table 1, **B**). When sotalol was added, there was a small increase in the contractile level, which was significant in EFS-contracted preparations (Table 1, **C**) but not in ACh-contracted preparations (Table 1, **D**). This small

Table 1 Statistical analysis of the results obtained in the study calculated as the difference in the level of inhibition after various treatments

	Comparison	*Mean and (95% confidence intervals) for differences between inhibitions (% inhibition of initial contraction)*	n
A	Degree of initial contraction ACh v. EFS	0.7 (−1.5 to 3.0) NS	61 and 60
B	Control strips ACh v. EFS (60 min after dose 2)	17.9 (11.0 to 24.9)	16
C	Control strips EFS: before v. after addition of sotalol, 10 μM	−8.6 (−13.5 to −3.6)	16
D	Control strips ACh: before v. after addition of sotalol, 10 μM	−3.0 (−6,3 to 0,3) NS	16
E	Salbutamol 10 μM ACh v. EFS	29.3 (7.4 to 51.2)	8
F	Salbutamol 10 μM ACh: before v. after addition of sotalol 10 μM	−35.3 (−21.7 to −49.0)	8
G	Salbutamol 10 μM EFS: before v. after addition of sotalol 10 μM	−43.8 (−34.2 to −53.4)	8
H	Formoterol 0.3 μM ACh v. EFS	33.1 (6.5 to 59.7)	7
I	Formoterol 0.3 μM ACh before v. after addition of sotalol 10 μM	−42.8 (−26.7 to −58.9)	7
J	Formoterol 0.3 μM EFS: before v. after addition of sotalol 10 μM	−58.5 (−44.8 to −72.3)	7
K	Salmeterol 10 μM ACh v. EFS	70.1 (44.8 to 95.4)	7 and 8
L	Salmeterol 10 μM ACh: before v. after addition of sotalol 10 μM	−19.9 (−29.7 to −10.1)	8
M	Salmeterol 10 μM EFS: before v. after addition of sotalol 10 μM	−11.7 (−23.6 to 0.2) NS	7

ACh; acetylcholine-induced contraction, EFS; electric-field-stimulation-induced contraction; NS; non-significant.

effect might be explained by a small amount of residual sympathetic nerve activation in spite of pre-treatment with guanethidine.

Salbutamol

Salbutamol (1−10 μM) caused a concentration-dependent inhibition of the contractions induced both by EFS and by ACh (Figure 3). The inhibition by 10 μM was significantly more pronounced in preparations contracted by EFS (66(8)% inhibition) than in preparations contracted by exogenous ACh (37(6)% inhibition) (Table 1, **E**).

Sotalol (10 μM) at the end of the experiment significantly reduced the salbutamol effect to 22(8)% inhibition in EFS-contracted preparations and totally blocked the effect in exogenous ACh-contracted preparations (1(1)% inhibition) (Figure 3) (Table 1, **F** and **G**).

Formoterol

Formoterol (3−300 nM) also caused a concentration-dependent inhibition of the contractions (Figure 3). At 300 nM, the inhibition in EFS-contracted preparations was total (105(5)%) and in ACh-contracted preparations the inhibition was 72(11)%, which was significantly less than in EFS-contracted preparations (Table 1, **H**).

Sotalol (10 μM) at the end of the experiment, significantly reduced the effect of formoterol to 47(6)% inhibition in EFS-induced contractions and to 29(8)% inhibition in ACh-induced contractions (Figure 3)(Table 1, **I** and **J**).

Salmeterol

Salmeterol (1−10 μM) caused a concentration-dependent inhibition of the EFS-induced contractions, whereas only the highest concentration (10 μM) inhibited ACh-induced contractions (Figure 3). At 10 μM, the inhibition was total (103(8)%) in EFS-contracted preparations, whereas it reached 33(8)% inhibition in ACh-treated preparations. This difference was significant (Table 1, **K**).

Sotalol (10 μM) at the end of the experiment had no significant effect on the salmeterol effect in preparations contracted by EFS (to 91(13)% inhibition), thereby indicating that this effect was not mediated by β-adrenoceptors (Table 1, **M**). In preparations contracted by ACh, however, the salmeterol effect (10 μM) was significantly reduced to 13(4)% inhibition (Table 1, **L**).

Discussion

In the present study we found that the inhibitory effects of salbutamol, formoterol and salmeterol on EFS-induced contractions were greater than those on ACh-induced contractions. Sotalol significantly counteracted the inhibition of all three drugs in ACh-contracted strips. However, in EFS-contracted strips, sotalol only antagonized the inhibition induced by salbutamol and formoterol, in contrast to salmeterol where no significant antagonism was found.

The discrepancy regarding the antagonist effect of sotalol in EFS-contracted preparations is probably an indication of

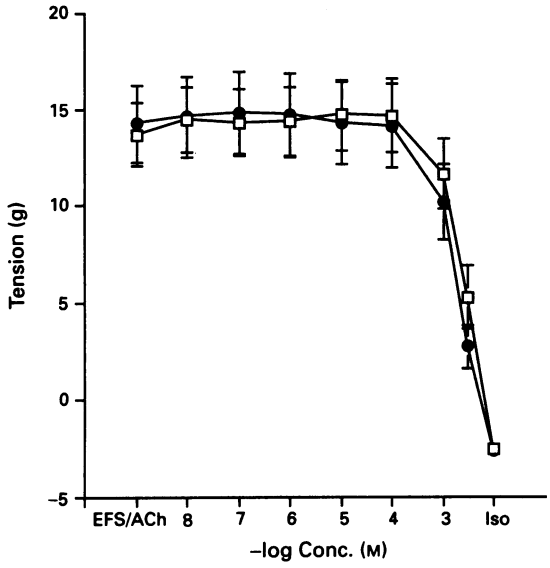

Figure 2 Inhibition of contraction induced by electric field stimulation, EFS (2 Hz, 700 mA) (●), or exogenously-added acetylcholine, ACh (0.5 μM) (□) by cumulatively added concentrations of theophylline. Abbreviations: Iso; 0.1 μM isoprenaline to obtain maximum relaxation.

Table 2 Degree of contraction in control strips without β-agonist treatment, 60 min after the time of β-agonist addition and 5 min after sotalol (10 μM) treatment

	Acetylcholine (% of initial contraction)	*Electric field stimulation* (% of initial contraction)
Dose 1	105.7 (2.1) $n = 16$	98.7 (2.0) $n = 14$
Dose 2	102.1 (1.8) $n = 16$	86.2 (2.9) $n = 16$
Sotalol (10 μM)	107.1 (1.8) $n = 16$	94.8 (2.6) $n = 16$

Values are means with s.e.mean in parentheses.

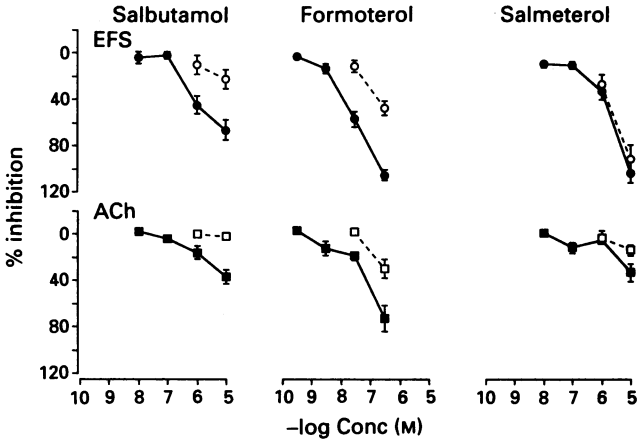

Figure 3 Inhibition of contraction induced by electrical field stimulation (2 Hz, 700 mA) or acetylcholine (0.5 μM), by increasing concentrations of salbutamol, formoterol and salmeterol (filled symbols). Values after sotalol (10 μM) with open symbols.

a non-β-mediated effect of salmeterol. Another possible explanation is that the sotalol concentration was too low to overcome the salmeterol effect. However, we think this is unlikely since theoretical calculations suggest a one log step shift of the salmeterol curve in the presence of 10 μM of sotalol (McKay, 1978). The shift in salmeterol-treated preparations is therefore expected to be similar to the shift seen in formoterol-treated preparations (Figure 3). As this is not the case, the dominant pre-junctional inhibitory effect of salmeterol is probably not mediated by β-adrenoceptors.

A non-β-mediated effect of salmeterol has been described before. In guinea-pig trachea, a sotalol-resistant relaxant effect of salmeterol was noted in both carbachol-induced contraction (Lindén *et al.*, 1993) and nerve-stimulated contraction (Jeppsson *et al.*, 1992; Coleman *et al.*, 1994). Furthermore, Baker & Fuller (1990) found an anti-inflammatory effect on human alveolar macrophages which was not reduced by propranolol.

The origin of the non-β-mediated effect is not known, but it is tentatively suggested that it could be a local anaesthetic effect on the cell membrane. A positive correlation between lipophilicity and local anaesthetic effects for β-adrenoceptor ligands has been demonstrated (Ijzerman *et al.* 1987), and salmeterol is highly lipophilic, as demonstrated in a study in which the octanol/water lipophilic distribution coefficient was measured (Jeppsson *et al.*, 1989a).

The non-β-mediated effect of salmeterol has not been studied in human subjects, normals or asthmatics. Although the concentrations of salmeterol used in these experiments are quite high, the result might still be relevant for man since the local concentration in the lungs after inhalation is also high, although the exact concentration is difficult to determine. Theoretical calculations have suggested concentrations of 0.1–1 mM in the airway lumen (Kerrebijn, 1990). Furthermore, one *in vitro* study involving a tracheal tube preparation showed that the concentration-difference between lumen and tracheal tissue is about tenfold after the intraluminal administration of a lipophilic substance. The concentration in the surrounding bath was close to nil for the lipophilic substance whereas 20% of a hydrophilic substance was found there (Jeppsson *et al.*, 1989b). Thus, *in vitro* studies at least suggest high concentrations in the tissue and an ability of lipophilic substance to remain in the tissue. In our study, inhibitory non-β-mediated effects on the cholinergic nerves were seen with 1 μM of salmeterol. We therefore suggest that this possible mechanism of action for salmeterol should be evaluated in human studies.

In the present study we found that the EFS-induced contractions were more effectively inhibited than the ACh-induced contractions by all the agonists. There was no significant difference in the magnitude of the initial contraction and theophylline inhibited the contractions in a similar way. We have found no reports in the literature about pre-junctional effects of theophylline in addition to its post-junctional effects and our own results do not indicate pre-junctional effects. This argues that the difference between the effects of the β-agonists on responses to EFS and ACh really reflect an additional β-effect on the pre-junctional level.

In our study, we used guanethidine to prevent sympathetic nerve effects. In spite of this, addition of sotalol resulted in a small increase in the response to EFS, indicating that a small remaining sympathetic nerve activation was present. This effect is, however, too small to explain the findings with the β-agonists showing a more pronounced inhibition of the EFS-contraction compared to the ACh-contraction. Besides, if the sympathetic effect were responsible for the difference, the same difference should have been found in the theophylline experiments which was not the case.

The more pronounced inhibition in EFS-contracted preparations indicates a pre-junctional mechanism in addition to the post-junctional β_2-adrenoceptor effect on the smooth muscle. In formoterol- and salbutamol-treated preparations this appears to be a β-mediated mechanism, as it was antagonized by sotalol. In a functional study, it is however, impossible to determine the exact mechanism. It could be directly mediated by β-adrenoceptors on the cholinergic nerves leading to a reduced output of ACh, or alternatively indirectly via release of other endogenous

mediators. The present study indicates, however that β_2-adrenoceptors are involved, since specific β_2-agonists were used, although this does not exclude the existence of a β_1-mediated mechanism as well.

The evidence in favour of a β-mediated inhibitory effect on cholinergic nerves is mostly indirect. Several authors have shown in functional studies in canine isolated airways and human isolated airways that β-agonists inhibit cholinergic nerve-induced contractions in concentrations that have little effect on comparable contractions induced by exogenous acetylcholine (Vermeire & Vanhoutte 1979; Danser *et al.*, 1987; Rhoden *et al.*, 1988; Ito, 1988; Janssen & Daniel, 1990). In addition to functional studies, excitatory junctional potentials have been measured, and these results also indicate pre-junctional receptors (Ito, 1988; Janssen & Daniel, 1990). The most direct method would be to evaluate the ACh release from cholinergic nerves. Martin & Collier (1986) found in muscle preparations from guinea-pig ileum that there was a significant inhibition of the ACh output after administration of noradrenaline. In canine isolated airways, however, they failed to find a significant inhibition of the release of ACh, although there was a tendency towards a decline in the trachea. The discrepancy could be due to a lack of sensitivity in the method, since the amount of ACh released was 20–40 times lower in the airways than in the ileum (Martin & Collier, 1986).

In conclusion, we have found a more pronounced inhibitory effect by salbutamol, salmeterol and formoterol in preparations contracted by EFS than in preparations contracted by exogenously added ACh. For all three β-agonists, the post-junctional effect is due to β-adrenoceptor stimulation. The pre-junctional effect of salbutamol and formoterol is also at least largely due to β-adrenoceptor stimulation, whereas the dominant pre-junctional effect of salmeterol is probably not mediated via β-adrenoceptors. The non-β-mediated effect could represent an additional relaxant mechanism for salmeterol.

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