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Extent of salmeterol-mediated reassertion of relaxation in guinea-pig trachea pretreated with aliphatic side chain structural analogues

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1 Salmeterol is a potent, selective and long acting β_2-adrenoceptor agonist. *In vitro*, salmeterol exerts 'reassertion' relaxation of airways smooth muscle. Reassertion relaxation refers to the capacity of salmeterol to cause repeated functional antagonism of induced contraction when airway smooth muscle is intermittently exposed to, then washed free from, β-adrenoceptor antagonists such as sotalol. The mechanism(s) underlying reassertion relaxation are unknown but may relate to high affinity binding of the long aliphatic side chain of salmeterol to an accessory site, distinct from the agonist recognition site, in or near the β_2-adrenoceptor (exosite binding hypothesis).

2 In order to test the exosite hypothesis, three pure analogues of salmeterol, each exactly preserving the molecular structure of the aliphatic side chain but with zero or low efficacy at the β_2-adrenoceptor were synthesized. The effect of pre-incubating guinea-pig tracheal smooth muscle with these analogues on salmeterol-induced reassertion relaxation was determined.

3 Computer Assisted Molecular Modelling of these molecules revealed that each of them exactly preserved the low energy linear conformation of the aliphatic side chain of salmeterol. Measurement of lipophilicity (octanol : water partition coefficient; log P) and direct partition into synthetic membranes (membrane partition coefficient; K_{pmem}) showed that all compounds had high affinity for lipids and membranes. In particular the biophysical properties of CGP 59162 (log P 1.89, K_{pmem} 16500) were very similar to salmeterol (log P 1.73, K_{pmem} 16800).

4 Two of the analogues, CGP 54103 and D 2543 (1 μM), which are structural mimics of the side chain of salmeterol, differing slightly in their length, did not prevent either the initial relaxation induced by salmeterol (0.1 μM) or the reassertion relaxation; however, it was not possible to determine whether either of these molecules occupied the β_2-adrenoceptor.

5 The third analogue, CGP 59162, which has the substituents on the active saligenin head group of salmeterol in transposed positions, itself exerted a weak β_2-adrenoceptor-mediated relaxation antagonized by ICI 118551 (β_2-selective antagonist) but not CGP 20712 (β_1-selective antagonist) and, at higher concentrations CGP 59162 caused reassertion relaxation suggesting that it may occupy and activate the β_2-adrenoceptor in a manner analogous to salmeterol.

6 CGP 59162, at concentrations up to ten fold molar excess, did not prevent or reduce salmeterol-induced reassertion relaxation.

7 In conclusion these data are not consistent with the existence of a distinct 'exosite' recognising the aliphatic side chain of salmeterol mediating reassertion.

Keywords: β-Adrenoceptor agonists; salmeterol; salmeterol analogues; airway smooth muscle relaxation; duration of action; reassertion; exosite

Introduction

Salmeterol is a highly selective β_2-adrenoceptor agonist for the maintenance treatment of reversible airways obstruction in diseases such as asthma. Clinically, salmeterol is distinguished from traditional inhaled β_2-adrenoceptor agonists such as salbutamol or terbutaline, by its very long duration of action extending over at least 12 h and a relatively slow onset of action (Ullman & Svedmyr, 1988; Pearlman *et al.*, 1992; Lötvall *et al.*, 1994). This slow onset of action and extended duration of relaxation is also observed in isolated airway smooth muscle *in vitro* (Jeppsson *et al.*, 1989; Ball *et al.*, 1991; Ullman *et al.*, 1992).

When studied on isolated airway smooth muscle, salmeterol but not shorter acting β_2-adrenoceptor agonists, shows the unique property of 'reassertion' of relaxation. Reassertion relaxation is the reproducible phenomenon observed in contracted airways smooth muscle treated *in vitro* with long acting β_2-adrenoceptor agonists where the induced relaxation can be reversed pharmacologically by a β_2-adrenoceptor antagonist yet relaxation is again observed ('reassertion' occurs) when the antagonist is washed from the tissue by exchange of the bathing medium (Ball *et al.*, 1991; Lindén *et al.*, 1991; Voss, 1994). To date no mechanism which can account for the long duration of action for salmeterol and its ability to exert reassertion has been unequivocally proven although at least two plausible hypotheses, the 'exosite' hypothesis and the 'diffusion micro kinetic model' have been advanced (Ball *et al.*, 1991; Anderson *et al.*, 1994).

Chemically, salmeterol is a saligenin like salbutamol, yet it differs from salbutamol and other short acting β_2-adrenoceptor agonists in that it posseses an extended aliphatic side chain (Figure 1). In the 'exosite' hypothesis it has been suggested that

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this aliphatic side chain binds with high affinity to an accessory site ('exosite') in or near the β_2-adrenoceptor but distinct from the active site (agonist recognition site). This would permit the active saligenin head of salmeterol to exert agonism at the agonist recognition site of the β_2-adrenoceptor but also anchor salmeterol in the vicinity of the active site if the saligenin head were to be displaced by a competing antagonist. Binding of the aliphatic side chain to a specific 'exosite' would therefore explain both the extended duration of action and the reassertion phenomenon.

In the present study we have synthesized chemically pure structural analogues of the aliphatic side chain of salmeterol which exactly preserve the same low energy structural conformation as salmeterol in aqueous solution. We have used these molecules as pharmacological probes to test the possible functional importance of a putative 'exosite', using an *in vitro* model of reassertion relaxation of guinea-pig isolated tracheal smooth muscle. Two of these probe molecules represent direct structural mimics of the side chain itself. The third molecule is a transposed saligenin where the substituents of the saligenin aromatic head group were exchanged, producing a molecule almost identical to salmeterol in terms of its physicochemical properties and molecular shape but with profoundly attenuated efficacy at the β_2-adrenoceptor. We hypothesized that if an 'exosite' mediating high affinity binding of the aliphatic side chain exists, these salmeterol analogues should be capable of interacting with this 'exosite' and thereby attenuate reassertion.

A preliminary account of these results has been presented in abstract form (Bergendal *et al.*, 1995b).

Methods

The study was approved by the Animal Ethics Committee of the Medical Faculty of University of Göteborg (Dno 158/91).

Synthesis and chemical characterization of salmeterol analogues

Salmeterol analogues (Figure 1) were synthesized from commercially available starting materials. For the synthesis of CGP compounds, all intermediates and the final products were characterized by spectroscopical methods to ensure their chemical structure and purity. D2543 (6-[4-phenyl-butoxyl]-hexylamine), the shorter of the aliphatic side chain analogues has a terminal amino group, whereas CGP 54103 (2-[6-(4-phenyl-butoxy)-hexylamino]-ethanol) contains an additional hydroxy-ethyl substituent at the amino group. For the preparation of CGP 59162 (2-hydroxymethyl-5-[1-hydroxy-2-[6-(4-phenyl-butoxy)-hexylamino]-ethyl]-phenol) as a racemate, a synthesis route was designed so that the compound has the hydroxy- and hydroxymethyl-substituents in exchanged positions when compared with salmeterol. The synthetic pathway was designed to ensure that no authentic salmeterol could be formed during the preparation of CGP 59162. The structures of CGP compounds were confirmed by high field proton nuclear magnetic resonance (^{1}H.-n.m.r.), IR and elemental analysis. This analysis of CGP 59162 confirmed that the compound did not contain any salmeterol as a contaminant. All compounds had a purity greater than 99.5% where the detected contaminants were traces of the solvents used in the synthesis.

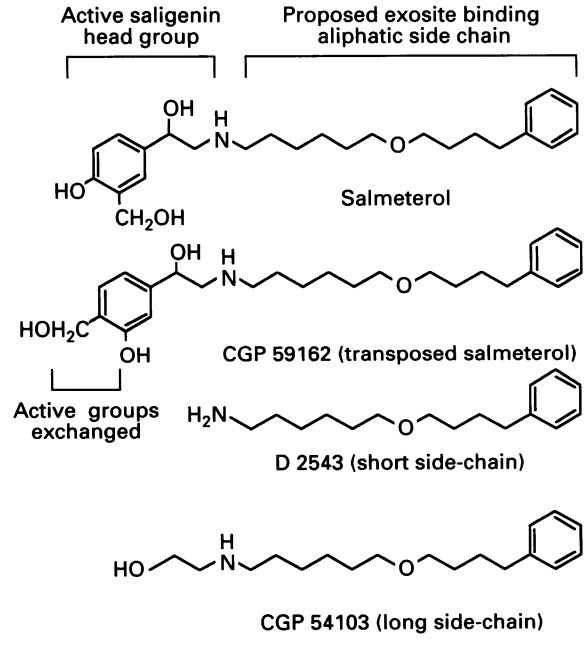

Figure 1 Molecular structure of salmeterol and its analogues indicating important structural features.

Studies of biophysical properties and computer assisted molecular modelling of salmeterol and its analogues

The lipophilicity of the compounds was determined as the classical $\log_{10}$ octanol: water partition coefficient (log P) with octanol and 0.067 M aqueous phosphate buffer as the solvent phases and the concentrations were measured by u.v. absorbance spectroscopy. In the case of the side chain analogues, log P values were calculated by validated physicochemical algorithm as detergent-like properties of the molecules prevented accurate direct measurements (Leo, 1993).

K_{pmem} values for CGP 59162 and salmeterol were determined by measuring the concentrations of drug partitioning into phospholipids adsorbed to semipermeable membrane substrates. Partition measurement cells of 4.5 ml volume were constructed using the lipid phase (POPS and OOPS; 9:1 liposomes) adsorbed to two cellulose membranes (Visking dialysis membranes, 0.025 mm wall thickness, average pore diameter 48Å, Union Carbide). These cells were submerged in buffer solution (0.01 M PBS-buffer pH 7.0) containing 1 μM test drug and continually stirred until equilibrium was achieved. Drug partitioning into the lipid phase was extracted into an equivalent volume of isopropanol. Residual drug in the aqueous buffer was extracted into n-octanol, concentrated by evaporation and measured. Drug concentrations were determined by u.v. absorbance spectrophotometry.

To assess the lowest energy three dimensional structure of salmeterol and its analogues, the structural co-ordinates in aqueous solution were calculated by computer-assisted molecular modelling (CAMM) using Macro model Software on a VAX mainframe computer (Mohamadi *et al.*, 1990). Individually calculated solution structures were superimposed onto the low-energy conformation of salmeterol with the same software and printed as images in two dimensions.

Organ bath studies

Preparations Thirty-four female Dunkin-Hartley guinea-pigs (280–430 g) were killed by inhalation of carbon dioxide for 3 min in a closed box. The trachea was rapidly removed and put in an oxygenated dissection bath filled with Krebs-Ringer (KR) solution at room temperature. The solution had the following composition (mmol l^{-1}): 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. Indomethacin (10 μM) was also present in the Krebs-Ringer solution to abolish spontaneous tone. The medial trachea was dissected free and mounted as four isolated strips, each consisting of approximately four cartilage rings, in 9 ml organ baths filled with oxygenated (94% O_2: 6% CO_2) Krebs-Ringer solution maintained at 37°C.

The isometric muscle tension was recorded continuously

with Grass force transducers (FT03) connected to a NB-MIO-16 analogue/digital converting board and a Macintosh Quadra 800 computer using the LabVIEW II signal-processing software (National Instruments, Austin, Texas, U.S.A.). This system permits real-time monitoring of the experiment and the simultaneous and continuous saving of data for subsequent evaluation.

Experimental design Preparations continuously flushed with KR (0.4 ml min^{-1}) were pre-stretched to 2.5 g (24.5 mN) and adjusted after 5 min to 1.4 g (13.7 mN). Following 25 min of equilibration, the preparations were readjusted to 1.4 g and then contracted by 0.1 μM carbachol (EC_{50} ($n=4$) in preliminary experiments) added to the KR. The flushing was stopped after 10 min and the chosen salmeterol analogue was added to half of the baths and incubated for 15 min. Salmeterol was subsequently administrated to half of the baths and incubated for 40 min. This procedure gave four different treatment groups in each experiment i.e. control, salmeterol analogue, salmeterol and salmeterol analogue *plus* salmeterol. Thereafter all preparations were treated in an identical manner. The hydrophilic, nonselective β-adrenoceptor antagonist, sotalol (10 μM) was added and 15 min later a wash-out procedure with fresh KR solution, was started. The bathing fluid was exchanged five times and then 30 min of continuous flushing was started with KR solution containing 0.1 μM carbachol. During this period the relaxant effect of salmeterol returned or was 'reasserted'. In order to antagonize this β_2-adrenoceptor-mediated relaxation, 10 μM sotalol was added. Thereafter a new wash-out procedure was performed and a new reassertion period followed. Finally 10 μM sotalol was added (Figure 2).

The structural mimics of the side chain (CGP 54103 and D2543) were used in a ten fold higher concentration than salmeterol i.e. 1 μM of the side-chains and 0.1 μM salmeterol. In the experiments with CGP 59162 (transposed salmeterol) three different concentration combinations (0.01 μM +0.01 μM, 0.1 μM+0.03 μM and 1 μM+0.1 μM) of CGP 59162 + salmeterol, respectively, were used.

In addition we performed experiments with the same protocol for four different concentrations of CGP 59162 (0.1, 0.3, 1, 3 μM) to evaluate any concentration-response relationship for the reassertion effect.

In separate experiments the weak relaxant effect of 1 μM CGP 59162 was studied in the presence of 1 μM ICI 118 551 (highly selective β_2-antagonist) or 1 μM CGP 20712 (highly selective β_1-antagonist) added in the KR at the start of the experiment (45 min prior to addition of CGP 59162).

Data analysis

Data are presented as mean (with s.e.mean) of the absolute values of the isometric tension recorded, or as mean of the percentage of the stable tension after contraction by carbachol (initial contraction-basal tension). Statistical evaluations were performed with non-parametric tests for paired data i.e. Friedman followed by Wilcoxon signed rank test. The concentration-dependent reassertion of salmeterol (Figure 6) was tested by the Kruskal-Wallis test followed by the Mann Whitney test. $P<0.05$ was regarded as significant; n equals the number of animals used.

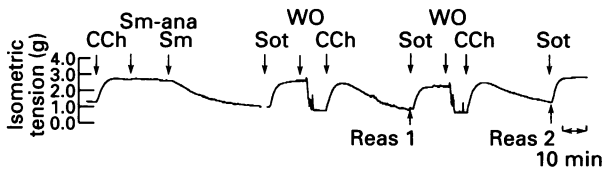

Figure 2 Original tracing from a typical experiment. Abbreviations: CCh = carbachol 0.1 μM, Sm-ana = salmeterol analogue (CGP 59162 0.1 μM); Sm = salmeterol (0.03 μM); Sot = sotalol 10 μM; WO = wash-out procedures; Reas = reassertion.

Drugs

Carbachol (Sigma), sotalol hydrochloride (Sotacor 10 mg ml^{-1}, Bristol-Myers Squibb) ICI 118 551 HCl (D,L-erythro-1-(7-methylindan-4-yl-oxy)-3-isopropylamino-butan-2-ol-hydrochloride; RBI-U.S.A. MA), CGP 20712 ((1-[2-(3-carbamoyl-4-hydroxy-phenoxy) ethylamino]-3(4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy) 1-propanol-methane sulphonate; gift from Ciba-Geigy) and indomethacin (Confortid 50 mg, Dumex) were dissolved and diluted in distilled water. Salmeterol base (racemate, gift from Glaxo), CGP 59162 (racemate) and CGP 54103 (gifts from Ciba-Geigy) were initially dissolved in glacial acetic acid (approx. 1 μl mg^{-1}) and then diluted in distilled water. D2543 (gift from Astra Draco) was dissolved in dimethylsulphoxide, (DMSO, 25 μl mg^{-1}) and then diluted in distilled water. All stock solutions, except for carbachol, were frozen. L-α-Phosphatidylcholine-β-oleoyl-γ-palmitoyl (POPS) and L-α-phosphatidyl-L-serine dioleoyl (OOPS) were obtained from Sigma.

Results

Computer-assisted molecular modelling and biophysical properties of salmeterol and its analogues

CAMM studies revealed that each of the salmeterol analogues exactly preserved (less than one Angstrom deviation) the linear conformation and three dimensional space filling properties of salmeterol (Figure 3).

Transposition of the functional substituents on the aromatic head group was found not to alter the linear conformation of the aliphatic side chain on CGP 59162 nor to distort the planar position of the aromatic head group relative to salmeterol. The relative position of the ether oxygen in the aliphatic side chain was extremely well preserved in relation to salmeterol. Measurement or calculation of the log P values (octanol: water partition coefficient), a measure of lipophilicity, revealed that all analogues were highly lipophilic and in the case of CGP 59162 the log P value was similar to that of salmeterol (Table 1). The partitioning into synthetic membranes (K_{pmem}) was determined for CGP 59162 and salmeterol and showed very similar high affinity partition of both of the compounds into lipid membranes (Table 1).

Organ bath studies

Effect of short side-chain (D2543) on reassertion D2543 at 1 μM did not significantly alter the reassertion effect of salmeterol (0.1 μM), nor was the initial relaxant effect of salmeterol affected. The effects of D2543 alone did not differ significantly from the control group treated with the vehicle (Figure 4).

Effect of long side-chain (CGP 54103) on reassertion CGP 54103, 1 μM, did not significantly alter the reassertion effect or the initial relaxant effect of salmeterol (0.1 μM) and CGP 54103 alone did not differ significantly from controls (Figure 4).

Effects of transposed salmeterol (CGP 59162) The transposed salmeterol (CGP 59162) did not, at any concentration, significantly alter the reassertion effect of salmeterol (Figure 5).

CGP 59162 exerted a concentration-dependant relaxant effect and at high concentrations this effect reasserted (Figure 6). The initial relaxant effect was β_2-mediated as the highly selective β_2-antagonist, ICI 118 551, totally blocked the initial relaxant effect of 1 μM CGP 59162 whereas the highly β_1-selective antagonist, CGP 20712 did not affect it at all (Figure

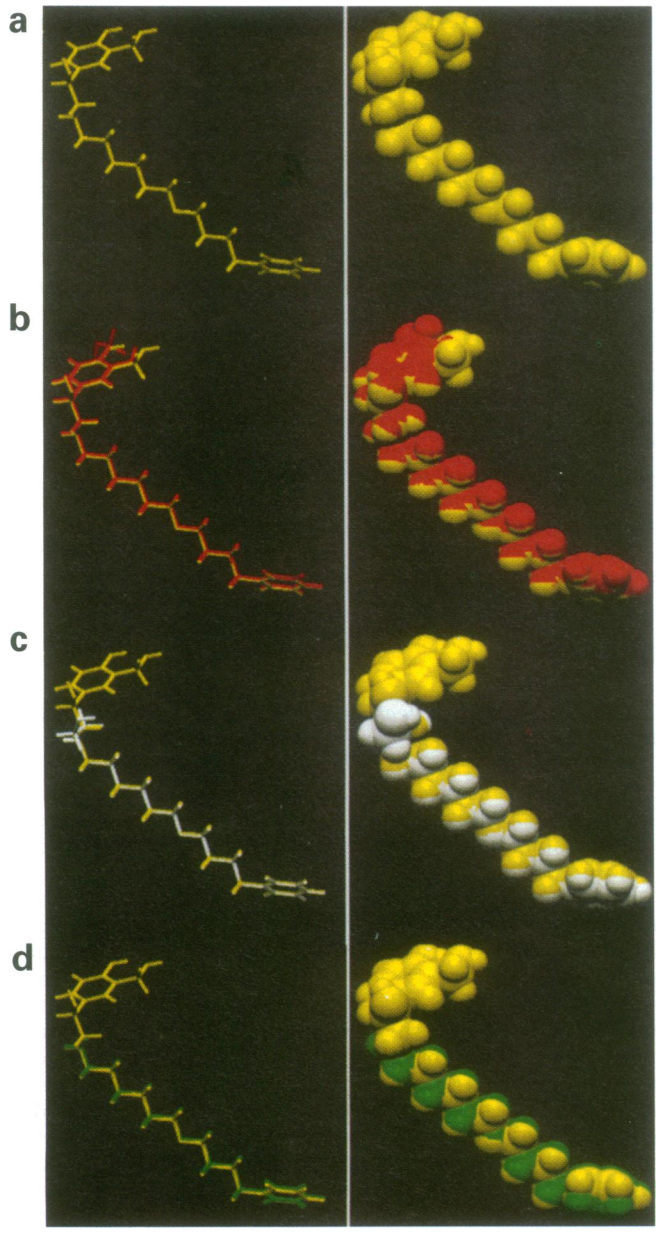

Figure 3 Computer-assisted molecular modelling (CAMM) images of salmeterol and aliphatic side chain analogues. Two images of salmeterol (yellow), CGP 59162 (red), CGP 54103 (white) and D2543 (green), corresponding to stick figure chemical structures and space-filling molecular structures are shown. Each image represents the lowest energy state conformation of the molecule in aqueous solution. In three panels salmeterol is shown overlayed by an aliphatic side chain analogue. Half filled spheres represent exact superimposition of structures.

Table 1 Octanol: water partition coefficient (log P) and synthetic membranes: water partition coefficient (K_{pmem}) for salmeterol and its analogues

Compound	*log P*	K_{pmem}
Salmeterol	1.73[a]	16800 ± 400
CGP 59162	1.89[a]	16500 ± 400
CGP 54103	0.75[b]	ND
D 2543	0.35[b]	ND

[a]Measured values; [b]calculated values; ND = not determined.

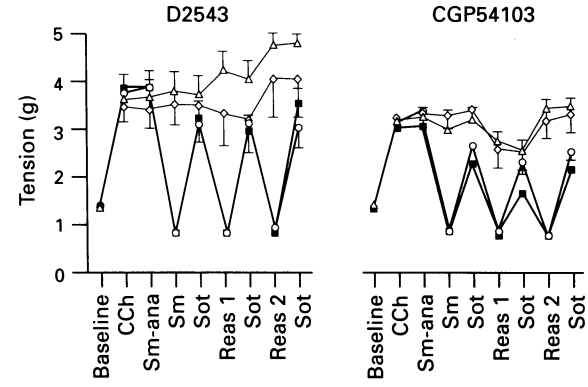

Figure 4 Influence of D2543, short ($n = 6$) and CGP 54103, long side chain ($n = 7$) (1 μM) on the reassertion relaxation of salmeterol (0.1 μM). Abbreviations: CCh = carbachol 0.1 μM; Sm-ana = salmeterol analogue; Sm = salmeterol (0.1 μM); Sot = sotalol 10 μM; Reas = reassertion. (△) Controls; (◇) side-chain; (○) salmeterol; (■) side chain + salmeterol.

7). However, the potency and efficacy of transposed salmeterol were considerably lower than those for salmeterol (Figure 6).

Salmeterol showed concentration-related reassertion (Figure 6). At 0.01 μM, the decrease in relaxation at reassertion 2 compared to the initial relaxation was significantly more pronounced than the decrease in relaxation for 0.1 μM salmeterol at reassertion 2 compared to initial relaxation ($P = 0.005$).

The initial level of contraction induced by carbachol was not statistically different among the groups except in one group of experiments where the control group (2.9 [0.2]g) had a slightly lower tension compared to the group treated by 0.01 μM CGP 59162 (3.5[0.3]g, $P = 0.02$) (Figure 5a).

In one of three experiments (Figure 5b) the mean relaxation induced by salmeterol (0.03 μM) was slightly but statistically significantly smaller in tissues treated with 0.1 μM CGP 59162 than with salmeterol alone. However, this is unlikely to represent a direct effect of CGP 59162 on salmeterol responses since the magnitude of salmeterol relaxation was not altered in the two other experiments with an identical protocol (Figure 5a, c).

Discussion

In the present study we have examined the interaction between salmeterol and three salmeterol analogues using a pharmacological model of 'reassertion' relaxation. We observed that all three structural analogues of the proposed exosite binding side chain did not affect the reassertion relaxation exerted by salmeterol.

A number of *in vitro* studies have revealed striking properties of salmeterol in airway smooth muscle isolated preparations which lend support to the side chain/exosite binding hypothesis. Salmeterol has been shown to exert an extremely long acting relaxation (> 12 h) of continually superfused guinea-pig trachea (Ball *et al.*, 1991). In contrast to the normally rapid onset of action exerted by saligenins, such as salbutamol which shares identical functional substituents on its aromatic group with salmeterol, the salmeterol relaxation has been shown to be of slow onset (Jeppsson *et al.*, 1989; Ball *et al.*, 1991; Ullman *et al.*, 1992). However, the slow onset of relaxation exerted by salmeterol appears neither to be due to retention of the drug in the β_2-adrenoceptor rich epithelial cell layer which overlays the airway smooth muscle, nor due to the epithelium acting as a functional barrier but rather to an interaction at the level of smooth muscle (Jeppsson *et al.*, 1989; Hamid *et al.*, 1991; Ullman *et al.*, 1992). Reassertion relaxation has been demonstrated in pre-

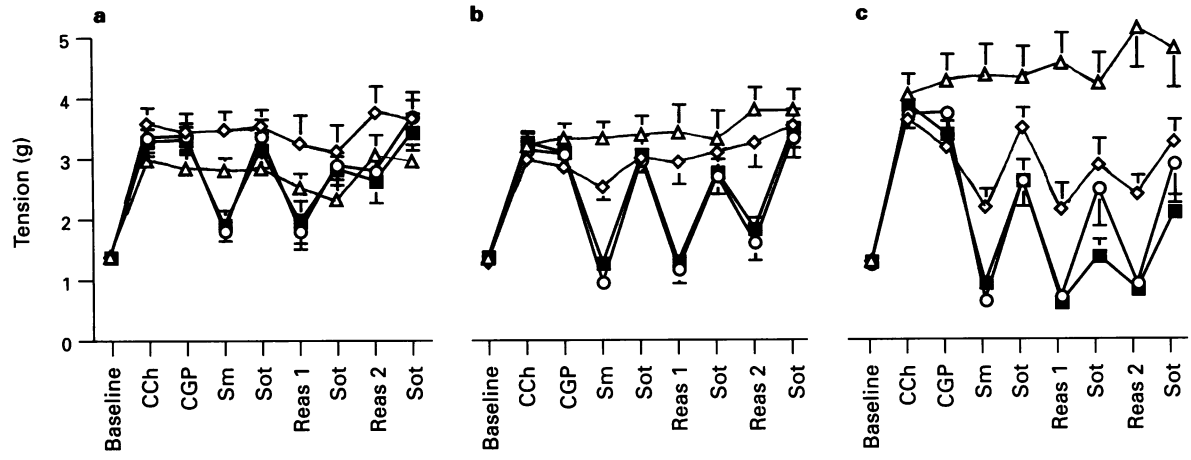

Figure 5 Influence of CGP 59162 (CGP) on the reassertion effect of salmeterol. (a) CGP and salmeterol 0.01 μM ($n=7$); (b) CGP 0.1 μM, salmeterol 0.03 μM ($n=8$); (c) CGP 1 μM, salmeterol 0.1 μM ($n=6$). Abbreviations: CCh = carbachol 0.1 μM, Sm = salmeterol, Sot = sotalol 10 μM, Reas = reassertion. (△) Controls; (◇) CGP 59162; (○) salmeterol; (■) CGP 59162 + salmeterol.

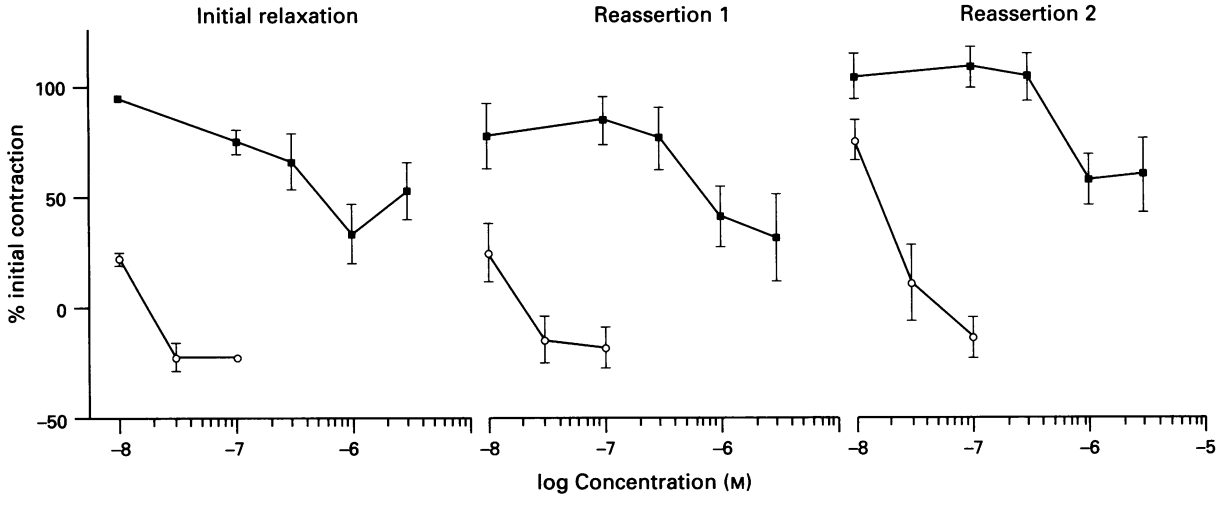

Figure 6 Non-cumulative concentration-response curves for salmeterol (○) and CGP 59162 (■) ($n=7-12$) regarding initial relaxation and reassertion. Data are taken from reassertion experiments.

parations of superfused guinea-pig trachea contracted by periodic field stimulation as well as in carbachol-contracted trachea (Ball *et al.*, 1991; Lindén *et al.*, 1991; Voss, 1994). Moreover, although direct radioligand binding studies of salmeterol to β_2-adrenoceptors have not been reported, indirect radioligand displacement suggests that salmeterol may form a very stable, perhaps irreversible and active, complex with the β_2-adrenoceptor (Nials *et al.*, 1993).

The outlined 'exosite' hypothesis is consistent with the characteristic behaviour of salmeterol: slow onset, long duration and reassertion. The exosite model suggests that reassertion occurs when a β_2-adrenoceptor antagonist physically displaces the active part of salmeterol from the agonist recognition domain of the β_2-adrenoceptor, but salmeterol remains anchored to the adrenoceptor. Reassertion relaxation is therefore a convenient model to investigate the nature of the interaction between salmeterol and the β_2-adrenoceptor.

The physical location of the proposed exosite has not been identified nor has the affinity of the exosite binding been directly measured. However, the affinity for the saligenin head group of salmeterol for the agonist recognition site has been measured, both in binding studies (Brittain, 1990) and in functional studies (Dougall *et al.*, 1991). The estimates obtained (pK_D of 7.82 in binding studies and pK_B of 7.3 in functional studies) indicate that salmeterol has relatively high affinity for the agonist recognition site. Since salmeterol cannot be washed from the tissue the binding of the aliphatic side chain to the proposed exosite must be inferred to be greater (and perhaps very much greater) than the above measured values for the binding of the saligenin head group.

To understand better the pharmacology of salmeterol we synthesized structural analogues of the salmeterol aliphatic side chain. We reasoned that if an exosite exists it might recognise analogues of the salmeterol side chain with equally high affinity. We hypothesized that such an interaction would be likely to affect the capacity of salmeterol to exert reassertion relaxation if airway smooth muscle was pre-incubated with the side chain analogues prior to salmeterol. Three compounds were prepared. Two of these compounds (D2543 and CGP 54103) were prepared as analogues of the aliphatic side chain portion of salmeterol. A third compound, CGP 59162, with identical molecular formula to salmeterol, differing only in the relative position of the substituents on the saligenin head group, was also prepared.

CGP 54103 and D2543 when pre-incubated at concentrations up to 10 fold that of salmeterol did not affect the reassertion behaviour of salmeterol. As reasoned each of these molecules might represent a molecule capable of interacting with the proposed exosite and blocking the effect of salmeterol. Computer-assisted molecular modelling studies (CAMM) were performed to explore whether the analogues were capable of adopting a conformation in aqueous solution that was at least

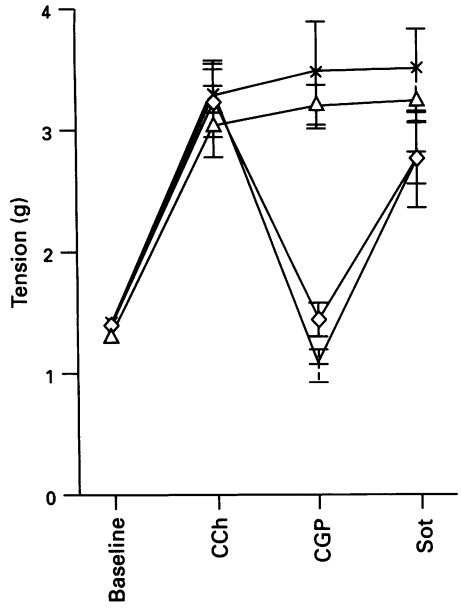

Figure 7 Influence of 1 μM CGP 20712 (β_1-blocker) or 1 μM ICI 118 551 (β_2-blocker) on the relaxant effect of CGP 59162 (1 μM) ($n=3-4$). Abbreviations: CCh = carbachol 0.1 μM, CGP = CGP 59162, Sot = sotalol 10 μM. (△) Controls; (◇) CGP 59162; (+) CGP 20712 + CGP 59162; (×) ICI 118 551 + CGP 59162.

similar to the side chain of salmeterol. The results of the computer projection suggest that each of the two side chain molecules could adopt a low energy conformation, which was similar to the side chain of salmeterol, with calculated deviations of less than one Ångstrom from authentic salmeterol. However, computer modelling provides absolutely no evidence to test whether either CGP 54103 or D2543 was actually capable of binding to an exosite in or close to the β_2-adrenoceptor.

It is in this context that the results obtained with CGP 59162, where the substituents on the saligenin head group of salmeterol were exchanged, offer more insight into potential mechanisms underlying the reassertion behaviour of salmeterol. We reasoned that, unlike CGP 54103 or D2543, CGP 59162 was more likely to retain the biophysical properties of salmeterol maximizing the possibility of interaction with the proposed exosite. Our biophysical studies which measured both the lipophilicity (logP) and lipid membrane affinity (K_{pmem}) directly support this assumption. Furthermore, CAMM studies suggested an almost identical conformation with salmeterol, with the expected lack of superimposition at the functional groups on the saligenin head group.

CGP 59162 was prepared based on original structure activity studies performed by Collins *et al.* (1970). In this study a saligenin with the head group identical to CGP 59162, was described as having no efficacy at the β_2-adrenoceptor measured as relaxation of guinea-pig airway smooth muscle. However, in the present study we found that CGP 59162 directly relaxed airway smooth muscle, albeit, with very low efficacy. This relaxation was characterized and found to be prevented by the highly β_2-adrenoceptor-selective antagonist, ICI 118 551, but not by the highly selective β_1-adrenoceptor antagonist, CGP 20712, suggesting that CGP 59162 directly occupied and activated the β_2-adrenoceptor. Since even a slight contamination of CGP 59162 with authentic salmeterol would give the appearance that the CGP 59162 occupied the β_2-adrenoceptor and exerted reassertion relaxation, it was important to exclude contamination. High field n.m.r. analysis confirmed that the synthetic pathway selected to produce CGP 59162 excluded the formation of salmeterol as a side product, even in minute quantities. CGP 59162 also showed reassertion of relaxation with a maximal effect very much less than salmeterol but with an identical pattern suggesting that it may occupy the proposed exosite.

Despite the similarities of CGP 59162 and salmeterol in their biophysical properties, their very similar conformation in aqueous solution as assessed by CAMM and the clear evidence that both compounds activate the β_2-adrenoceptor, CGP 59162 had no effect whatsoever on the reassertion relaxation exerted by salmeterol, even when pre-incubated in 10 fold molar excess.

Taken together the experimental data obtained with CGP 59162 question the existence of a distinct exosite recognising the aliphatic side chain of salmeterol. Interpretation of these data depends, however, on the nature of the binding interaction between salmeterol and the β_2-adrenoceptor. The balance of evidence currently at hand suggests that salmeterol forms a very stable, perhaps irreversible complex with the β_2-adrenoceptor (Nials *et al.*, 1993). The potency and the apparent binding affinity for salmeterol is higher compared to salbutamol (Brittain, 1990; Dougall *et al.*, 1991; Nials *et al.*, 1993) and this may be due to the interaction of the aliphatic side chain with an exosite not directly involved in recognising the saligenin head group at the receptor agonist binding domain. Such interactions have been predicted by computer modelling studies (Lewell, 1992).

In the case where salmeterol forms a very stable complex with the β_2-adrenoceptor, the interaction of the aliphatic side chain to the exosite must produce a very high affinity binding. It is therefore reasonable to assume that CGP 59162, which clearly activates the β_2-adrenoceptor and manifests salmeterol-like reassertion would be a useful tool to pre-block this exosite binding. It would then seem reasonable to speculate that if the exosite were blocked by prior treatment with CGP 59162, the dissociation of CGP 59162 from this site would be at least as slow as that of salmeterol and that excess salmeterol would be unable to displace CGP 59162 from the exosite binding domain. Since CGP 59162 has a very low efficacy at the β_2-adrenoceptor, the functional consequences of pre-absorbing exosite binding with this molecule would be that the reassertion relaxation of salmeterol would be expected to be, at the very least, partially attenuated. This was not observed.

If however the aliphatic side chains of salmeterol and CGP 59162 display true competitive binding at the proposed exosite according to the laws of mass action it must be considered whether the 10 fold molar excess of CGP 59162 would be sufficient to pre-block subsequent salmeterol binding to this site. If the exosite model is valid and CGP 59162 and salmeterol did compete for the same exosite in our experiments it would however be expected that either the salmeterol or CGP 59162 responses might be attenuated during repeated washing of the tissue since the unbound fraction of either drug should be free to wash from the tissue. In this case differences in the magnitude of relaxation of smooth muscle at paired time points would be expected to be consistently significantly lower for salmeterol *plus* CGP 59162 treated tissues compared to those treated with salmeterol alone. This, too, was not observed.

It was not possible to test higher concentrations of CGP 59162 because of the development of non-β_2-adrenoceptor-mediated relaxation of the tissue which confounded interpretation of the results. Non-β_2-adrenoceptor-mediated relaxation of airway smooth muscle has previously been observed in tissue incubated with high concentrations of salmeterol (Lindèn *et al.*, 1993; Bergendal *et al.*, 1995a). Salmeterol, and its close structural analogue CGP 59162 as we demonstrated in this study, partition avidly into lipid membranes (high K_{pmem} values) and the non-specific relaxation may be a consequence of perturbance of the airway smooth muscle cell membrane.

The objective of the present study was to study the effect of possible exosite binding molecules in the reassertion relaxation model induced by salmeterol. Although our results, particu-

larly those obtained with CGP 59162, do not favour the existence of a distinct exosite recognising the aliphatic side chain of salmeterol, our studies do not provide direct evidence of the actual mechanism underlying salmeterol-induced reassertion. An interesting and perhaps informative side observation was made during the course of the study. It has previously been observed, and suggested as evidence in support of the exosite binding model, that salmeterol-induced relaxation has a duration of action unrelated to the concentration of drug used to relax guinea-pig tracheal smooth muscle. If salmeterol bound irreversibly to an exosite such a persistent relaxation, independent of the magnitude of the initial response, is easily understandable. However, in our experiments we observed a clear concentration-dependence in the duration of salmeterol-induced relaxation. One possible explanation for this concentration-dependence of duration of relaxation, which has also been observed in human clinical trials with salmeterol (Sandström *et al.*, 1989), is that the drug partitions in a dose-proportional manner into the lipid compartment of the tissue, perhaps in a manner proposed in the lipid membrane partition-dependent 'diffusion microkinetic' hypothesis (Anderson *et al.*, 1994).

In conclusion we have found that three structural analogues which exactly preserved the physical structure of the proposed exosite binding side chain of salmeterol had no effect on salmeterol-induced reassertion relaxation. One of the test molecules, CGP 59162, was demonstrated in functional studies to occupy the β_2-adrenoceptor and, itself, exert weak reassertion relaxation. Our data are not consistent with the theory that the reassertion behaviour of salmeterol and, by inference, long duration are due to the binding of its aliphatic side chain to a specific exosite.

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