Why are long-acting beta-adrenoceptor agonists long-acting?

G.P. Anderson*, A. Lindén**, K.F. Rabe†

ABSTRACT: The extended duration of bronchodilation due to formoterol and salmeterol greatly exceeds that of short acting beta2-adrenoceptor agonists, such as salbutamol or terbutaline. This extended duration and their capacity to "reassert" airway smooth muscle relaxation in vitro despite repeated washing has prompted considerable debate on the underlying mechanism(s). The comparative pharmacology, and molecular modelling of these drugs and of the beta2-adrenoceptor and its ligand binding core have cast doubt on the exosite/exoceptor model previously proposed to explain the behaviour of salmeterol.

We present evidence supporting a unifying hypothesis that the duration of action both of formoterol and salmeterol is determined principally by their physicochemical interactions with membrane lipid bilayers (plasmalemma diffusion microkinetic model), rather than putative distinct exosite/exoceptor binding sites in or near the beta2-adrenoceptor. This model provides a clearer understanding of the pharmacological profile of these drugs (rate of onset, duration, "reassertion", interaction with hydrophilic and hydrophobic beta2-adrenoceptor antagonists), and explains why in human airway smooth muscle in vitro a true relaxation-concentration response may not exist for salmeterol.

Eur Respir J., 1994, 7, 569–578.

Two new long-acting inhaled selective beta2-adrenoceptor agonists, formoterol and salmeterol, have recently become available for the treatment of reversible airflow obstruction in asthma. Extensive clinical trial data have been obtained with these drugs, which indicate that they both cause significant bronchodilation for at least 12 h after a single administration [1–5]. Both compounds are well-tolerated and highly potent; the principal difference in their clinical pharmacology being the faster onset of action of formoterol compared to salmeterol [6–8]. The extended duration of action of formoterol and salmeterol, which greatly exceeds that of more familiar beta2-adrenoceptor agonists, such as salbutamol and terbutaline, has prompted considerable debate on the underlying mechanism(s). This review describes the evidence supporting a unifying hypothesis that the duration of action of both formoterol and salmeterol is determined principally by their physicochemical interactions with membrane lipid bilayers, rather than putative exoceptor binding sites in or near the beta2-adrenoceptor.

Pharmacological and physicochemical properties of formoterol and salmeterol

The basic pharmacological properties of formoterol and salmeterol are somewhat similar. Both drugs are highly potent relaxants of human bronchial smooth muscle in vitro (negative log10 molar concentration for half maximal relaxation±SEM: formoterol 9.6±0.1; salmeterol 7.6±0.2; potency ratio 107 at basal tone) [9]. In addition, they both display a high degree of selectivity for the beta2-adrenoceptor subtype [10–13]. The important differences observed in vitro are that formoterol has a higher intrinsic efficacy [9, 12, 14] and a faster onset of relaxation than salmeterol [15]. The dissociation constant, -log10 KD, is a measure of affinity of drugs for receptors. Formoterol has a very high binding affinity for the beta2-adrenoceptor, with a -log10 KD=8.12 for displacement of ICI 118,551, a selective beta2-adrenoceptor antagonist, versus 6.44 for salbutamol [12]. The apparent binding affinity of salmeterol is also relatively high (-log10 KD salmeterol 7.82) and greater than that of salbutamol (-log10 KD 5.63) for displacement of 125iodine-labelled pindolol from guinea-pig lung tissues [16].

The structures of formoterol, a formanilide substituted phenoethanolamine, and salmeterol, a saligenin derivative of phenylethanolamine, are shown in figure 1. Computer-assisted molecular modelling (CAMM) studies predict that formoterol is approximately 13–15 Å long in aqueous solution in its lowest energy conformations. Despite the apparently lengthy aliphatic side-chain attached to its saligenin head group, salmeterol is predicted to be less than twice the length of formoterol (25 Å) (CAMM...
studies were kindly performed by N. Cohen (Ciba-Geigy, Basel, Switzerland), using the "MacroModel" Modelling System analysis software supported on a VAX mainframe computer [17]. The side-chain does, however, profoundly affect the lipophilicity of salmeterol, which is at least an order of magnitude more lipid soluble than the moderately lipophilic formoterol molecule: the octanol-to-water partition coefficients are: formoterol=2.6 and salmeterol=63 at pH 7.4 [15]. As argued below, it is the physicochemical nature of the interactions of formoterol or salmeterol with the membrane lipid bilayer that may determine the duration of action and other behaviours of these molecules in vitro and in vivo. It should be stressed at this point that lipophilicity (octanol:water partition) and membrane lipid bilayer affinity (Kp(mem)) are by no means identical, and the octanol:water partition coefficient is only a crude predictor of the true extent of drug interactions with biological membranes [18–20].

**Structure of the beta_2-adrenoceptor and the nature of its ligand binding site**

Definitive 3-D X-ray crystallography studies of beta_2-adrenoceptor or of agonist-receptor complexes have not yet been made. However, a number of indirect methods based on the primary amino acid sequence, such as molecular modelling and hydropathy analysis, and direct epitope mapping of the beta_2-adrenoceptor using monoclonal antibodies, support the concept that the beta_2-adrenoceptor is composed of seven transmembrane spanning sequences arranged in alpha-helices. These helices cluster together, forming a binding clef or active site accessible to ligands approaching through the extracellular aqueous biophase [21–24].

The structure of the beta_2-adrenoceptor is thought to be an analogue of bacteriorhodopsin, the only protein of this structural class for which definitive 3-D structural information has been obtained [25, 26], and the mammalian G-protein-coupled visual pigment rhodopsin [27, 28]. The dimensions of bacteriorhodopsin are known to be 25×35 Å in the plane of the lipid membrane bilayer and 45 Å in depth spanning both sides of the bilayer [25]. Lefwell [29] has calculated the dimensions of the beta_2-adrenoceptor to be 18×33×45 Å. The peptide backbone of the individual alpha-helices is approximately 5 Å in diameter, and the helices are thought to be separated by approximately 10 Å. A schematic structural model of the beta_2-adrenoceptor, highlighting the agonist and antagonist binding regions, is shown in figure 2.

A body of evidence supports the concept that beta_2-adrenoceptor agonists bind to a hydrophobic pocket or active site located at least 11 Å within the core of beta_2-adrenoceptor, i.e. approximately 30–40% into the depth of the receptor [22, 23]. This location corresponds to the predicted location of several key amino acid residues, aspartate 113, serine 204, serine 207 and phenylalanine 290, known from molecular biology studies on genetically engineered recombinant point-mutated receptors to be crucial for ligand binding [21–24].

The binding of adrenaline to key amino acid residues in the active site of the beta_2-adrenoceptor has recently been predicted using computer modelling techniques [24]. Based on the known structure-activity relationships, binding affinity states, selectivity and efficacy, it is possible that formoterol and salmeterol interact with the beta_2-adrenoceptor in a manner in which at least some of the bonds formed between the agonists and their receptor are similar to those formed by adrenaline. If this were the case, salmeterol and formoterol would be expected to interact with (using the nomenclature of Hibert et al. [24] which numbers amino acids from the predicted start of each alpha-helix rather than from the N terminus), serine 413 (transmembrane domain 4), a highly conserved residue common to all beta-adrenoceptors. Specificity for the beta_2-adrenoceptor subtype might be determined by interactions with serines 504 and 507 (i.e. serines 204 and 207, located on transmembrane domain 5).

**Reassertion, duration and the "exosite" hypothesis**

An extraordinary property of salmeterol is its capacity to "reassert" relaxation of airway smooth muscle [10, 16, 17].
This retention of salmeterol in the tissue and "reassertion" is very persistent, since both relaxation and "reassertion" effects have been documented even after 10 complete wash-out cycles or several hours of continuous superfusion of the tissue with drug-free medium [10, 16, 30–34] (fig. 3). This property, which has been held to be unique to salmeterol, has previously been explained in terms of a putative "exosite" or "exoceptor", distinct from the beta2-adrenoceptor, which was proposed to bind the pharmacologically inactive long aliphatic tail with high affinity, allowing the active saligenin head structure to angle on and off the receptor in a manner that would allow a beta-adrenoceptor antagonist, such as sotalol, access to the active site of the beta2-adrenoceptor (fig. 4) [13, 16, 31]. However, it should be noted that persistent in vitro relaxant activity and the "reassertion" effect, are properties common to several lipophilic beta2-adrenoceptor agonists. These effects have previously been reported for clenbuterol; salmefamol, a saligenin derivative of formoterol; and, the experimental compound D2489, the resorcinol analogue of salmeterol [15, 35, 36]. In recent experiments, in which preparations of guinea-pig trachea have been precontracted with carbachol to induce submaximal contraction and then relaxed with sub- or supra-maximal concentrations of salbutamol or formoterol, salbutamol showed virtually no capacity to reassert relaxation after repeated washing of the tissues. In contrast, formoterol produced consistent reassertion of relaxation of 78±7% (mean±SEM) (fig. 3). Furthermore, in human airway smooth muscle, formoterol protects against acetylcholine-induced contraction, despite repeated washing of the tissue, over a period of more than 6 h [9].

The capacity of several lipophilic beta2-adrenoceptor agonists lacking a long side-chain to behave as if they were interacting with an "exosite" raises fundamental questions about the mechanism of long duration of action for formoterol, and about the validity of the "exosite".
model. Furthermore, an "exoceptor" is not consistent with a recent computer modelling study of the interaction of salmeterol with the beta2-adrenoceptor, which predicted that the tail of salmeterol intercalates between the alpha-helices inside the beta2-adrenoceptor itself [37]. X-ray diffraction and nuclear magnetic resonance (NMR) studies on ligand-receptor complexes will be required to resolve the exosite theory definitively, but it should also be noted that the apparent binding affinity of salmeterol for the beta2-adrenoceptor is actually lower than that of formoterol, and formoterol can be slowly washed from airway smooth muscle in vitro (fig. 3) [35], whereas the biological activity of salmeterol persists for periods in excess of 10 h [13, 16, 31, 38]. An alternative explanation of duration of action accommodating all experimental observations is clearly required.

Plasmalemma diffusion microkinetic theory of the duration of action of beta2-adrenoceptor agonists

To understand the duration of action of beta2-adrenoceptor agonists, we propose that it is necessary to consider the plasmalemma diffusion microkinetics of these drugs, i.e. what happens to beta2-adrenoceptor agonists in the cell membrane lipid bilayer (plasmalemma) and in the aqueous biophase closest to the active site of the beta2-adrenoceptor. When a beta2-adrenoceptor agonist is inhaled, surprisingly high topical concentrations are achieved in the periciliary fluid of the bronchi. KERREBIJN [39], basing his estimates on earlier work, has suggested that a single inhalation of terbutaline will lead to topical concentration of up to 100 µmol·l⁻¹ in the major bronchi [39, 40]. Correcting for differences in the inhaled dose, it could be anticipated that formoterol and salmeterol would achieve instantaneous topical concentrations at least as high as 1 µmol·l⁻¹ in the main bronchi. This represents a substantial bulk concentration, which moves efficiently across the epithelium and into the lamina propria as the drug diffuses towards airway smooth muscle. This bulk effect after inhalation may be essential for long duration, since formoterol is not long-acting when an equieffective bronchodilator dose is administered orally to patients [41]. It is at this point, as molecules of agonist approach the airway smooth muscle cell membrane, that we propose that the physicochemical properties of formoterol and salmeterol become the principal determinants of their duration of action.
Fig. 5. – Diffusion microkinetic model. The top panel is an overview of the model which explains duration and reassertion in relation to interactions of salbutamol (left), formoterol (middle) and salmeterol (right) with lipid membranes adjacent to the beta_2-adrenoceptor. The small arrows at the left of each panel show the drug-lipid equilibrium position. The large shaded arrows show the major movement of drug. During drug association with the receptor (middle panel) the interaction of salbutamol with membrane lipid ($K_{\text{min}}$) in very energetically unfavourable (dark shaded barrier) due to its high hydrophilicity and salbutamol associates with the receptor directly ($K_{\beta 2\text{on}}$) from the aqueous biophase [18]. Therefore, salbutamol exhibits rapid onset, but the drug diffuses from tissues rapidly causing short duration of effect. The association of formoterol with both receptor and lipid is relatively thermodynamically favourable, allowing fast onset [11, 42]. Formoterol is retained in the lipid to be released over an extended period, continually activating the $\beta$-adrenoceptor. Salmeterol associates predominantly with lipid, and its interaction with the $\beta$-adrenoceptor is proposed to be energetically unfavourable causing slow onset of action [18]. In the lower panel, the dissociation of salbutamol and formoterol from the $\beta$-adrenoceptor is not impeded ($K_{\beta 2\text{off}}$), although the affinity of formoterol for the $\beta$-adrenoceptor is higher than that of salbutamol [11]. Formoterol is retained with moderate affinity by lipid ($K_{\text{mout}}$) [42]. Salmeterol may slowly form a stable complex with the $\beta$-adrenoceptor, but is also avidly retained by lipid, accounting for slow onset but long duration [18].
Figure 5 shows a schematic representation of the plasmalemma diffusion microkinetic theory of duration of action of beta₂-adrenoceptor agonists. The essential feature of the model is that the plasmalemma lipid bilayer of airway smooth muscle acts as a depot for beta₂-adrenoceptor agonists with moderate to high lipophilicity. Beta₂-adrenoceptor agonists, once having partitioned into the bilayer, remain available to interact with the beta₂-adrenoceptor active site. This model appears at first to be inconsistent with traditional views of a ligand approaching the receptor only via the aqueous biophase and of the plasmalemma as an inert substratum for the receptor. In fact, the balance of evidence currently indicates that the membrane itself is a major determinant of the nature of agonism at the beta₂-adrenoceptor for lipophilic compounds. Aliphatic amines appear to have a natural affinity for partitioning into lung tissue [43, 44], but the molecular basis of the relationship between membrane affinity and duration has not previously been explained.

As indicated in figure 5, in the case of hydrophilic drugs, such as salbutamol (very low solubility in lipid), the partition equilibrium is strongly in favour of the extracellular aqueous compartment. Salbutamol does not partition into the bilayer [20], and is readily removed from the biophase by diffusion into the microcirculation in vivo or by wash-out with fresh buffer in vitro. As such, the onset of effect is rapid, since the molecule can rapidly diffuse to the active site of the receptor, but no persistent relaxation of airway smooth muscle occurs (fig. 3).

In the case of formoterol, which is moderately lipophilic, the partition equilibrium allows the molecule to enter the plasmalemma and to be retained. Concurrently, sufficient drug is available in the aqueous biophase to allow immediate interaction with the active site of the receptor, accounting for the rapid onset of bronchodilation observed clinically. Subsequently, small amounts of formoterol leak out from the plasmalemma and become available for activation of the beta₂-adrenoceptor, explaining the experimental observation of persistent relaxation of airway smooth muscle after wash-out. It should be noted that as little as 30% of maximal contraction is sufficient to profoundly increase airway resistance in vivo [45]. By analogy, very little muscle length increase is likely to be required to relieve airflow limitation in vivo, suggesting that even small amounts of a potent drug released over time can cause effective bronchodilation (the threshold concentration for relaxation of human airway smooth muscle by formoterol is less than 0.10 nM [9], i.e. approximately four orders of magnitude lower than the possible instantaneous topical concentration achieved in major bronchi). This model also explains the reassertion effect after beta-blockade, since a hydrophilic beta-antagonist, such as sotalol, would compete with formoterol diffusing from the membrane for the beta₂-adrenoceptor active site according to the law of mass action, but sotalol would wash-out from the tissue readily, whereas formoterol would tend to be retained for a longer period. The partitioning interaction of formoterol into the outer lipid bilayer has recently been demonstrated [42].

For highly lipophilic drugs, such as salmeterol, the partition equilibrium is very much in favour of the plasmalemma lipid bilayer. This partitioning behaviour has been unequivocally demonstrated by the studies of RHÖES and co-workers [19]. Using cholesterol and dioleoylphosphatidylycholine as artificial membrane lipid components reconstituted as uni- or multi-lamella liposomes, H-labelled salmeterol was found to partition into the lipid extremely rapidly (<1 min) and completely (Kp_{mem} 22,500, compared to salbutamol, Kp_{mem} 4) but to diffuse from the lipid only slowly (half-life (T1/2) approximately 25 min at 25°). Salmeterol enters only the outer layer of uni-lamella liposomes and does not cross into underlying lipid layers of multi-lamella liposomes to any appreciable extent, implying that salmeterol maintains a highly specific orientation in the outermost leaf of the cell membrane [20]. Neutron diffraction studies, to determine the absolute orientation of salmeterol in the outer lipid bilayer, indicate that salmeterol assumes an orientation analogous to the phospholipids themselves, with its saligenin head in the plane of the phosphate groups and its aliphatic tail extending perpendicularly to the membrane aligned with neighbouring phospholipid groups [46]. Since salmeterol was found to be present in a ratio of one molecule in the aqueous phase to every 22,500 molecules in the lipid membrane at equilibrium, it would therefore be expected that little active salmeterol substance would be present in the aqueous biophase shortly after inhalation. It is actually likely that the majority of salmeterol which activates beta₂-adrenoceptor, diffuses laterally between the phospholipid layers, and its aliphatic tail precludes grounded interaction being thermodynamically unfavourable when the agonist approaches the receptor via the lipid membrane (fig. 5). The lack of effect of removing the epithelium on in vitro relaxation time precludes retarded penetration through the epithelium alone as a determinant of slow onset [51].

Very recent computer models of the interaction between salmeterol and the beta₂-adrenoceptor point to three possible drug receptor interactions. Each possibility assumes that the saligenin head binds to the active core but the lipophilic tail has different orientation in each case: 1) the tail points outward from the receptor into the extracellular space, 2) the tail points down into the receptor towards Asp 79; and 3) the tail remains in the lipid bilayer, while the head enters the receptor [29]. There would be two possible modes of interaction between salmeterol and the beta₂-adrenoceptor predicted by the diffusion microkinetic model. In the first case, it is physically possible that the pharmacologically inactive
Fig. 6. – Relaxation of human airway smooth muscle by β-adrenoceptor agonists. a) Shows original recordings from continually superfused human bronchi *in vitro* exposed to superfusions with β-adrenoceptor agonists at the indicated concentrations. The traces demonstrate that for isoprenaline, fenoterol and formoterol onset of relaxation is rapid and concentration dependent. At the conclusion of drug superfusion, isoprenaline-induced relaxation is rapidly lost, higher concentrations of fenoterol, and particularly of formoterol, produce sustained relaxations and in all cases the rate of loss of relaxation is lower for higher drug concentrations. In the case of formoterol, little loss of relaxation is observed at concentrations between 10^{-8} to 10^{-6} M (*i.e.* 10 nmol·l^{-1} to 1 µmol·l^{-1}. b) Results obtained with salmeterol. In contrast, the onset of salmeterol-induced relaxation is always slow but increases somewhat with increased concentration. At the conclusion of drug superfusion the relaxation is not lost but continues to increase at a very slow rate until a maximal response, which is independent of initial concentration over the tested concentration range, is achieved up to 11 h later. c) Illustrates that for isoprenaline and formoterol the length of time that drug is superfused over the tissues determines both the rate of onset and duration of effect. In the case of salmeterol, the rate of onset is related to duration of administration but the maximal effect is independent of contact time. All recordings were made from human bronchioles of 2–5 mm internal diameter, under spontaneous tone as described previously [55, 56].
The diffusion microkinetic theory applies equally to the behaviour of beta-adrenoceptor antagonists. Propranolol, for example has been clearly demonstrated to partition in membrane bilayers and adopt a specific location, approximately 10 Å into the membrane just under the phospholipid head groups, as detected by neutron diffraction. This property has also been observed for timolol which, unlike propranolol, is not formally charged at physiological pH [18, 57, 58]. The diffusion microkinetic model would predict that antagonists interacting with membranes in airway smooth muscle tissue pre-relaxed by a beta2-adrenoceptor agonist would assert their antagonism at a rate broadly related to their lipophilicities. Such a rank order of behaviour has already been demonstrated for the time needed to antagonize salmeterol-induced relaxation of guinea-pig airway smooth muscle: the rank order is propranolol > ICI 118,551 > timolol > sotalol > atenolol [33, 34]. This order is comparable to the rank order of lipophilicity of these drugs [18, 57–60].

The diffusion microkinetic theory, presented here, has the advantages that it explains the major experimental observations for beta2-adrenoceptor agonists of different molecular structures, including the rate of onset and reassertion of smooth muscle relaxation. In addition, the theory also explains the clinically observed anomaly that the plasma concentrations of inhaled beta2-adrenoceptor agonists do not necessarily correlate with time course of clinical bronchodilation [59]. Furthermore, the hypothesis is consistent with the major known properties of the long-acting beta2-adrenoceptor agonists. The diffusion microkinetic model provides a reasonable and testable hypothesis based on experimental data to explain the duration and "reassertion" behaviour of formoterol and salmeterol, and should help to unravel the puzzling behaviour of these new and intriguing molecules.

**Acknowledgements**

Johnson et al. [15] were first to discuss the relationship between the octanol-water partition coefficient of beta2-adrenoceptor agonists and the duration of action in *vitro*. The first proposal that interaction with lipid membranes may relate to "reassertion" was made by Lodish [35]. Herbst and Rhodes demonstrated membrane partition of propranolol in 1988. Their subsequent work [20] in collaboration with Newton (Head of Chemistry, Glaxo, UK), has proved that rapid membrane interactions occur with salmeterol. The authors are most grateful for information provided by Rhodes and Newton regarding their studies on salmeterol. The authors are also grateful to Aventis et al. [9] (Paris, France, for access to work "in press" on responses of human bronchi to salmeterol and formoterol, to N. Cohen (Basel, Switzerland) for molecular modelling studies of salmeterol and formoterol and, to I. Drewett for comments on the manuscript. A preliminary account of the diffusion microkinetic hypothesis was represented in lecture form by G.P.A. in 1991, and subsequently published in outline [47].
References


38. Nials AT, Butchers PR, Coleman RA, Johnson M, Vardey CJ. Salmeterol and formoterol: are they both long-acting β2-adrenoceptor agonists. Br J Pharmacol 1990; 99(Suppl.): 120P.


42. Jaekel K, John E, Anderson GP. Comparative biophysical analysis of interactions between formoterol, salbutamol or salmeterol and lipid membranes (Abstract). European Respiratory Society Proceedings, 1993; (In press).


53. Frielle T, Daniel KW, Caron MG, Lefkowitz RJ. Structural basis of β-adrenergic sub-type specificity studied with chimeric β1/β2-adrenergic receptors. Proc Natl Acad Sci (USA) 1988; 85: 9494–9498.


