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RESEARCH PAPER

Slow receptor dissociation is not a key factor in the duration of action of inhaled long-acting β_2-adrenoceptor agonists

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BACKGROUND AND PURPOSE

β_2-Adrenoceptor agonists are important bronchodilators used for the treatment of chronic obstructive pulmonary disease and asthma. Clinical data on β_2-adrenoceptor agonists show a range of onset and duration of action. We have investigated whether the receptor binding kinetics of β_2-adrenoceptor agonists can explain their observed onset of action and duration of effect in the clinic.

EXPERIMENTAL APPROACH

[^{3}H]-DHA was used to label β_2-adrenoceptors expressed in CHO-cell membranes (K_d of 0.084 nM). Competition kinetic experiments were performed in the presence of unlabelled β_2 agonists at 37°C in HBSS containing GTP. To determine the kinetic parameters, three concentrations (10, 3 and 1 × K_i) of the unlabelled compound were employed against a fixed concentration of [^{3}H]-DHA (0.6 nM).

KEY RESULTS

The clinically used β_2-adrenoceptor agonists exhibited a range of association and dissociation rates. The kinetic K_d and the competition K_i values of the eight β_2-adrenoceptor agonists examined were strongly correlated, suggesting that the method had produced accurate k_{off} and k_{on} rates. The kinetic on-rate was highly correlated with equilibrium binding affinity.

CONCLUSIONS AND IMPLICATIONS

Although the β_2-adrenoceptor agonists displayed a range of kinetic rate parameters, simulations at relevant drug concentrations suggest that receptor kinetics do not play an important role in determining onset of action in the clinic. In addition, it is unlikely that receptor kinetics exert an important influence on the duration of action of these agonists, as indacaterol (once daily dosing) had a shorter residency time at the receptor than salmeterol (twice daily dosing).

Abbreviations

[^{3}H]-DHA, 1-[4,6-propyl-^{3}H] dihydroalprenolol; NSB, non-specific binding; [^{125}I]-CYP, [^{125}I]-iodo-(-)-cyanopindolol; COPD, chronic obstructive pulmonary disease; LABA, long-acting β_2-adrenoceptor agonist

Introduction

Long-acting β_2-adrenoceptor agonists (LABAs) play an important role in the treatment of asthma and chronic obstructive pulmonary disease (COPD), providing improved symptom control. One aspect of the biology of LABAs which remains unresolved is the factors which determine their duration of action. Duration of action often depends on many pharmacokinetic factors, including absorption, distribution and clearance (Smith *et al.*, 1996). However, the direct kinetics of drug receptor interaction can also play a significant role in drug duration (Copeland *et al.*, 2006).

Of the clinically approved bronchodilators, only indacaterol (150 μg) achieves a 24 h duration of action in COPD patients (Beier *et al.*, 2006; Beeh and Beier, 2010). In contrast, formoterol (12 μg) and salmeterol (50 μg) require twice daily dosing (Wegener *et al.*, 1992; van Noord *et al.*, 1996; Palmqvist *et al.*, 1997; Sutherland *et al.*, 2009), while salbutamol (200 μg) must be given up to four times a day in order to achieve a clinically useful effect (Tashkin and Fabbri, 2010).

It is widely accepted that these agonists have the capacity to 'reassert' airway smooth muscle relaxation *in vitro* despite repeat washing of isolated tissue (Anderson *et al.*, 1994). The retention and reassertion of salmeterol in tissue have been attributed to binding of its aliphatic tail to a so-called 'exosite' or 'exoceptor', a site distinct from the β_2 adrenoceptor, allowing the active saligenin head structure to freely angle on and off the receptor (Johnson, 1992). However, it is now becoming clear that the persistent *in vitro* relaxant activity and reassertion effect are properties common to several lipophilic β_2 agonists that do not possess a long aliphatic side chain (Summerhill *et al.*, 2008), questioning the validity of the exosite model. Perhaps a more compelling model is the plasmalemma diffusion microkinetic theory, in which the plasmalemma lipid bilayer of airway smooth muscle acts as a depot for β_2 adrenoceptor agonists with moderate to high lipophilicity (Anderson *et al.*, 1994).

Another potential factor that might influence the duration of action of β_2-adrenoceptor agonists is the kinetics of agonist–receptor dissociation. It has been demonstrated that slow dissociation kinetics plays an important role in the duration of drug action of inhaled muscarinic antagonists (Disse *et al.*, 1999). In the case of β-adrenoceptors, several slowly dissociating antagonists from this receptor have been described (Lucas *et al.*, 1979; De Blasi *et al.*, 1988; Pauwels *et al.*, 1988; Keith *et al.*, 1989; Doggrell, 1990; Deyrup *et al.*, 1999). In keeping with their slow dissociation, several of these antagonists, including bornaprolol (FM 24) and ICI 147,798, have been shown to have a relatively long duration of action *in vivo* that is independent of their plasma levels (Le Fur *et al.*, 1980; Keith *et al.*, 1989).

The long duration of action of the β_2-adrenoceptor agonist carmoterol (CHF-4226, TA-2005) has been attributed to its 'slow dissociation' from the receptor (Voss *et al.*, 1992). However, this claim is based on *in vitro* 'wash out' experiments which do not directly measure the dissociation kinetics of the molecule as it may also be influenced by membrane interactions and drug 'rebinding' (Vauquelin and Charlton, 2010).

In addition to a long duration of action, a fast onset of action is a desirable property of inhaled β_2-adrenoceptor agonists, providing rapid relief of symptoms. Fast-acting β_2-adrenoceptor agonists such as indacaterol, formoterol and salbutamol can produce bronchodilation within 1–5 min (Brookman *et al.*, 2007; Van Noord *et al.*, 1998), whereas the slower-acting β_2-adrenoceptor agonist salmeterol can take between 6 and 30 min to produce a significant bronchodilatory effect (Palmqvist *et al.*, 1997; Brookman *et al.*, 2007). The kinetics of drug–receptor interaction could be one factor important in determining the onset and subsequent relief of symptoms, as receptor kinetics will determine the initial rate of receptor occupancy. A recent review by Tashkin and Fabbri, (2010) details the onset and duration of action of therapies currently used to treat COPD.

The aim of this work was to investigate the kinetic properties of several clinically relevant β_2-adrenoceptor agonists with widely varying onset and durations of action to determine if any relationships exist. The association and dissociation rates of compounds are traditionally assessed directly by monitoring the specific binding of a labelled form (often radiolabelled) of the ligand of interest. Motulsky and Mahan (1984) have previously described a method to quantify the kinetic parameters of unlabelled compounds. The practical application of this method was demonstrated by Dowling and Charlton (2006), and more recently we have used this technique to explore the kinetics of muscarinic M_3 receptor agonists (Sykes *et al.*, 2009). In brief, a kinetically characterized radioligand is added simultaneously with an unlabelled ligand to the receptor preparation of interest. The experimentally derived rate of specific radioligand binding can then be modelled to provide the association and dissociation rates of the unlabelled compound.

Kinetic competition models rely on the radiolabel having a rapid enough off-rate such that the competing ligand is able to reach equilibrium with the receptor in the time frame of the experiment. We have characterized two commercially available radiolabels, the commonly used β_2-adrenoceptor radiolabel [^{125}I]-CYP and the less widely used [^{3}H]-DHA. Following these initial exploratory binding studies, we selected [^{3}H]-DHA as the most suitable ligand for determining the kinetic properties of our unlabelled β_2-adrenoceptor agonists.

Methods

Cell culture

CHO cells stably transfected with the human β_2-adrenoceptor were grown adherently in Ham's F-12 Nutrient Mix GlutaMAX-1, containing 10% fetal calf serum, and 0.5 mg·mL^{-1} Geneticin (G-418). Cells were maitained at 37°C in 5% CO_2/humidified air. Cells were routinely subcultured at a ratio between 1:10 and 1:20 twice weekly using tryspin-EDTA to lift cells.

Cell membrane preparation

CHO cells expressing the human β_2-adrenoceptor were grown to 80–90% confluency in 500 cm^2 cell culture plates at 37°C in 5% CO_2. All subsequent steps were conducted at 4°C to avoid receptor degradation. The cell culture media were removed, and ice-cold buffer [1 × 10 ml; 10 mM HEPES, 0.9% (w/v) NaCl, and 0.2% (w/v) EDTA, pH 7.4] was added to the cells, which were then scraped from the plates into a 50-ml Corning tube (Corning Inc., Corning, NY, USA) and subsequently centrifuged at 250 *g* for 5 min to allow a pellet to form. The supernatant fraction was aspirated, and 10 ml per 500-cm^2 tray of wash buffer (10 mM HEPES and 10 mM EDTA, pH 7.4) was added to the pellet. This was homogenized using an electrical homogenizer Ultra-Turrax (Ika-Werk GmbH & Co. KG, Staufen, Germany) (position 6, 4 × 5-s bursts) and subsequently centrifuged at 48,000 *g* at 4°C (Beckman Avanti J-251 Ultracentrifuge; Beckman Coulter, Fullerton, CA, USA) for 30 min. The supernatant was dis-

carded, and the pellet was rehomogenized and centrifuged as described above in wash buffer. The final pellet was suspended in ice-cold 10 mM HEPES and 0.1 mM EDTA, pH 7.4, at a concentration of 5 to 10 mg/ml. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) based on the method of Bradford (1976) using BSA as a standard, and aliquots were maintained at –80°C until required.

Common procedures applicable to all radioligand binding experiments

All radioligand experiments were conducted in 96-deep well plates, in assay binding buffer, HBSS pH 7.4 containing 0.1% BSA, 0.01% ascorbic acid and 100 μM GTP. In all cases, non-specific binding (NSB) was determined in the presence of 1 μM propranolol. After the indicated incubation period, bound and free radiolabels were separated by rapid vacuum filtration using a FilterMate™ Cell Harvester (PerkinElmer Life and Analytical Sciences, Beaconsfield, UK) onto 96 well GF/B filter plates previously coated with 0.5% (w/v) polyethylenimine and rapidly washed three times with ice-cold 75 mM HEPES, pH 7.4. After drying (>4 h), 40 μL of Microscint™ 20 (PerkinElmer Life and Analytical Sciences) was added to each well and radioactivity was quantified using single photon counting on a TopCount™ microplate scintillation counter (PerkinElmer Life and Analytical Sciences). Aliquots of radiolabel were also quantified accurately to determine how much radioactivity was added to each well using liquid scintillation spectrometry on LS 6500 scintillation counter (Beckman Coulter, High Wycombe, UK). In all experiments, total binding never exceeded more than 10% of that added, limiting complications associated with depletion of the free radioligand concentration (Carter *et al*., 2007).

Saturation binding studies

CHO cell membranes containing the β_2-adrenoceptor were incubated in 96-deep well plates at 37°C in assay binding buffer with a range of concentrations of [^{125}I]-CYP (~200–0.05 pM) and [^{3}H]-DHA (~3–0.001 nM) at 3 and 15 μg per well respectively, for 180 min with gentle agitation to ensure equilibrium was reached. Saturation binding was performed in a final assay volume of to 1.5 mL to avoid significant ligand depletion.

Determination of the association rate (k_{on}) and dissociation rate (k_{off}) of [^{125}I]-CYP and [^{3}H]-DHA

To accurately determine k_{on} and k_{off} values, the observed rate of association (k_{ob}) was calculated at least three different concentrations of either [^{125}I]-CYP or [^{3}H]-DHA. The appropriate concentration of radioligand was incubated with β_2-adrenoceptor CHO cell membranes (3 and 15 μg· per well) in assay binding buffer with gentle agitation (final assay volume 1000 μL). Exact concentrations were calculated in each experiment by liquid scintillation counting. Free radioligand was separated by rapid filtration at multiple time points to construct association kinetic curves as described previously by Sykes *et al*. (2009). The resulting data were globally fitted to the association kinetic model to derive a single best fit estimate for k_{on} and k_{off} as described under Data analysis.

Determination of agonist affinity constants (K_i)

To obtain affinity estimates of unlabelled agonists, [^{3}H]-DHA competition experiments were performed at equilibrium. [^{3}H]-DHA was used at a concentration of approximately 0.6 nM (~25 000 c.p.m. final assay volume of 0.5 mL), such that the total binding never exceeded more than 10% of that added. Radioligand was incubated in the presence of the indicated concentration of unlabelled agonist and CHO cell membranes (15 μg· per well) at 37°C, with gentle agitation for 180 min.

Competition binding kinetics

The kinetic parameters of unlabelled agonists were assessed using a competition kinetic binding assay as described by Sykes *et al*. (2009). This approach involves the simultaneous addition of both radioligand and competitor to receptor preparation, so that at t = 0 all receptors are unoccupied. Approximately 0.6 nM [^{3}H]-DHA (a concentration which avoids ligand depletion in this assay volume) was added simultaneously with the unlabelled compound (at t = 0) to CHO cell membranes containing the human β_2-adrenoceptor (15 μg· per well) in 500 μL assay buffer. The degree of [^{3}H]-DHA bound to the receptor was assessed at several time points by filtration harvesting and liquid scintillation counting, as described previously. NSB was determined as the amount of radioactivity bound to the filters and membrane in the presence of propranolol (1 μM) and was subtracted from each time point, meaning that t = 0 was always equal to 0. Each time point was conducted on the same 96-deep well plate incubated at 37°C with constant agitation. Reactions were considered stopped once the membranes reached the filter, and the first wash was applied within 1 s. Three different concentrations of unlabelled competitor were tested to ensure that the rate parameters calculated were independent of ligand concentration. All compounds were tested at one-, three- and 10-fold their respective K_i and data were globally fitted using Equation 3 to simultaneously calculate k_{on} and k_{off}.

Data analysis and statistical procedures

As the amount of radioactivity varied slightly for each experiment (<5%), data are shown graphically as the mean ± range for individual representative experiments, whereas all values reported in the text and tables are mean ± SEM for the indicated number of experiments unless otherwise stated. All experiments were analysed by either Deming regression or non-linear regression using Prism 4.0 (GraphPad Software, San Diego, CA, USA).

Competition binding. Competition displacement binding data were fitted to sigmoidal (variable slope) curves using a four-parameter logistic equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{(\log EC_{50} - X)\cdot HillSlope}) \quad (1)$$

IC_{50} values obtained from the inhibition curves were converted to K_i values using the method of Cheng and Prusoff (1973). Equation 1 was utilized for data presented in Figure 3.

Association binding. [^{125}I]-CYP and [^{3}H]-DHA association data were globally fitted to the following equation, where L is the

concentration of radioligand in nM using GraphPad Prism 4.0 to determine a best fit estimate for k_{on} and k_{off}. Equation 2 was utilized for data presented in Figure 2A,B.

$$k_{ob} = [L] \cdot k_{on} + k_{off} \quad (2)$$

Competition kinetic binding. Association and dissociation rates for unlabelled agonists were calculated using the equations described by Motulsky and Mahan (1984) using a global fitting model:

$$\begin{aligned} K_A &= k_1[L] + k_2 \\ K_B &= k_3[I] + k_4 \\ S &= \sqrt{\left((K_A - K_B)^{2+4 \cdot k_1 \cdot k_3 \cdot L \cdot I \cdot 10^{-18}}\right)} \\ K_F &= 0.5 \cdot (K_A + K_B + S) \\ K_S &= 0.5 \cdot (K_A + K_B - S) \\ DIFF &= K_F - K_S \\ Q &= \frac{B_{max} \cdot K_1 \cdot L \cdot 10^{-9}}{DIFF} \\ Y &= Q \cdot \left(\frac{k_4 \cdot DIFF}{K_F \cdot K_S} + \frac{k_4 - K_F}{K_F} \cdot \exp^{(-K_F \cdot X)} - \right. \\ &\quad \left. \frac{k_4 - K_s}{K_s} \cdot \exp^{(-K_S \cdot X)} \right) \end{aligned} \quad (3)$$

where X is time (min), Y is specific binding (c.p.m.), k_1 is k_{on} [^{3}H]-DHA, k_2 is k_{off} [^{3}H]-DHA, L is the concentration of [^{3}H]-DHA used (nM) and I is the concentration of unlabelled agonist (nM). Fixing the above parameters allowed the following to be simultaneously calculated: B_{max} is total binding (c.p.m.), k_3 is association rate of unlabeled ligand (M^{-1} min^{-1}) or k_{on}, and k_4 is the dissociation rate of unlabelled ligand (min^{-1}) or k_{off}. Equation 3 was utilized for data presented in Figure 4A–H.

Simulations. The observed association of ligand to receptor (k_{ob}) (see Figure 7A–C) was simulated in Prism 4.0 using Equation 2. Fixed kinetic parameters (k_{1-2}) for the ligands determined in the competition kinetic studies were used to simulate the binding of ligand to receptor over time, at the concentration of ligand required to occupy 50% of available receptors (K_d), or at an EC_{25} concentration equivalent to 25% of the maximal cAMP response to isoprenaline (values taken from Battram *et al.*, 2006). In addition, simulations were performed at relative clinical doses. In the clinic, salmeterol, indacaterol, salbutamol and formoterol are dosed at 50, 150, 200 and 12 μg respectively. A concentration of salmeterol at 10-fold its K_d (7.5 nM) was chosen for modelling purposes and concentrations of indacaterol, formoterol and salbutamol were calculated based on their clinical doses relative to this concentration of salmeterol. For example, doses of salbutamol and indacaterol are four and threefold higher than that of salmeterol, so concentrations of 22.5 nM and 30 nM were used for modelling purposes. In contrast, formoterol is given at a 4.17-fold lower dose than salmeterol, so a lower relative concentration of 1.8 nM was used. Dissociation rates for the β_2 agonists were modelled in Prism 4.0 using Equation 4 (Figure 7D).

$$Y = \text{Span} \cdot \exp^{(-k_{off} \cdot X)} + \text{Plateau} \quad (4)$$

Materials

1-[4,6-propyl-^{3}H]dihydroalprenolol ([^{3}H]-DHA specific activity 91 Ci·mmol^{-1}) was obtained from Amersham Biosciences UK Ltd. (GE Healthcare, Chalfont St Giles, UK) and [^{125}I]-iodo-(-)-cyanopindolol ([^{125}I]-CYP specific activity 2200 Ci·mmol^{-1}) was obtained from PerkinElmer. 96-deep well plates and 500 cm^2 cell culture plates were purchased from Fisher Scientific (Loughborough, UK). 96-well GF/B filter plates were purchased from Millipore (Watford, UK). Sodium bicarbonate, ascorbic acid, EDTA, sodium chloride, GTP, propranolol (-)isoprenaline hydrochloride, formoterol fumarate, and (-)adrenaline were obtained from Sigma Chemical Co Ltd. (Poole, UK). Salmeterol and salbutamol hemisulfate were obtained from Tocris Cookson Inc. (Bristol, UK). A related sulfonamide analogue of salmeterol 3-[4-[[6-[[(2*R*)-2-hydroxy-2-[4-hydroxy-3 (hydroxymethyl)phenyl]ethyl]amino]hexyl] oxy]butyl]benzenesulfonamide (Compound 1; Procopiou *et al.*, 2009; Rosethorne *et al.*, 2010) and indacaterol were synthesized by Global Discovery Chemistry (Novartis, Horsham, UK). All cell culture reagents including HBSS and HEPES were purchased from Gibco (Invitrogen, Paisley, UK).

Results

Characterization of [^{125}I]-CYP and [^{3}H]-DHA saturation binding

Specific [^{125}I]-CYP and [^{3}H]-DHA binding to human β_2-adrenoceptors expressed in CHO membranes was saturable and best described by the interaction of each radioligand with a single population of high-affinity binding sites. The expression level of the human β_2-adrenoceptor recombinantly expressed in CHO cells was assessed, using [^{125}I]-CYP saturation binding, as 499 ± 129 fmol·mg^{-1} protein (Figure 1A). A similar value of 529 ± 16 fmol·mg^{-1} protein was obtained when saturation binding was carried out with [^{3}H]-DHA (Figure 1B). From these studies, the equilibrium dissociation constant (K_d) of [^{125}I]-CYP and [^{3}H]-DHA was determined to be 4.10 ± 0.93 pM and 83.5 ± 11.1 pM respectively.

Characterization of [^{125}I]-CYP and [^{3}H]-DHA kinetic parameters

In order to establish a suitably robust system for determining the kinetic parameters of unlabelled β_2-adrenoceptor agonists, a comparison was made of two commercially available antagonist radiolabels [^{3}H]-DHA and [^{125}I]-CYP in HBSS at 37°C. The observed association rate of a ligand is in part dependent upon the concentration of radiolabel used, so we constructed a family of association curves using a range

Table 1

Kinetic binding parameters and affinity values of [^{3}H]-DHA and [^{125}I]-CYP for human β_2-adrenoceptor receptors

Radiolabel	Kinetic K_d (pM)	k_{on} (M^{-1} min^{-1})	k_{off} (min^{-1})	Dissociation half-life $t_{1/2}$ (min)	Saturation K_d (pM)
[^{3}H]-DHA	33.9 ± 12.3	2.86 ± 0.32 × 10^9	0.083 ± 0.020	8.4	83.5 ± 11.1
[^{125}I]-CYP	1.6 ± 0.2	3.68 ± 0.32 × 10^9	0.0056 ± 0.0001	123.7	4.1 ± 0.9

Data are mean ± SEM for ≥3 experiments performed in duplicate.

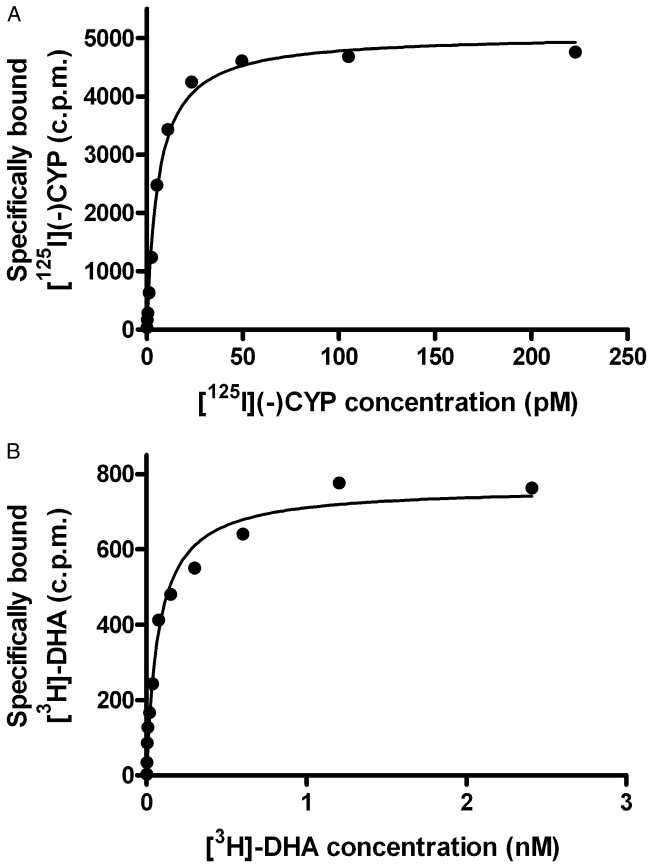

Figure 1

Saturation analysis of the binding of (A) [^{125}I]-CYP and (B) [^{3}H]-DHA to CHO membranes expressing the human β_2-adrenoceptor. CHO-β_2 cell membranes (3 and 15 μg· per well respectively) were incubated for 180 min with gentle agitation with increasing concentrations of radiolabel. NSB was defined by 1 μM propranolol. Specific binding is presented as the mean from a representative of three experiments performed in duplicate.

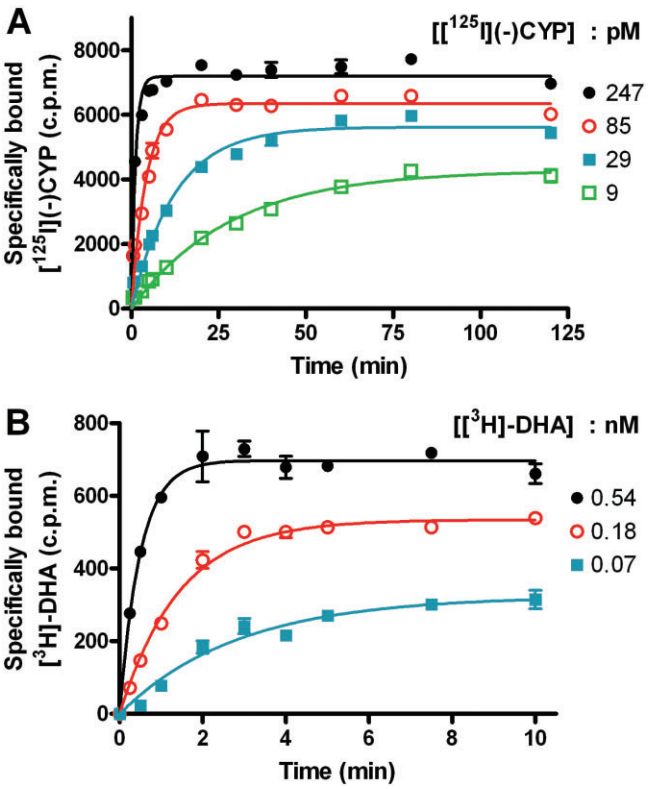

Figure 2

Kinetics of the interaction of [^{125}I]-CYP and [^{3}H]-DHA with CHO membranes expressing the human β_2-adrenoceptor. The k_{on} and k_{off} values for (A) [^{125}I]-CYP and (B) [^{3}H]-DHA were determined by incubating CHO-β_2 cell membranes (3 and 15 μg· per well, respectively) with the indicated concentrations of [^{125}I]-CYP and [^{3}H]-DHA for various time periods. Association data were fitted to a global fitting model using GraphPad Prism 4.0 to simultaneously calculate k_{on} and k_{off}. Data are presented as the mean ± range from a representative of ≥3 experiments performed in duplicate.

of [^{125}I]-CYP (~250–10 pM) and [^{3}H]-DHA concentrations (~0.6–0.1 nM) concentrations.

Each association curve was monitored to equilibrium, the point at which no further binding was observed (Figure 2A,B). Binding followed a simple law of mass action model, k_{ob} increasing in a linear manner with radioligand concentration (data not shown). Consequently, [^{125}I]-CYP and [^{3}H]-DHA association data were globally fitted to derive a single best fit estimate for k_{on} and k_{off} of each radioligand. Kinetic k_{on} and k_{off} values determined for [^{125}I]-CYP and [^{3}H]-DHA are shown in Table 1. Association rates for both ligands were similar; however, there was a 10-fold difference in their dissociation rates, with [^{125}I]-CYP being considerably slower than [^{3}H]-DHA to dissociate (0.0056 vs. 0.083 min^{-1}). This difference in dissociation rate is largely responsible for the difference in the speed at which these two ligands reach equilibrium with the receptor. The kinetically derived

Table 2

Affinity values and kinetically derived parameters for unlabelled ligands

Compound	k_{on} (M^{-1} min^{-1})	k_{off} (min^{-1})	$t_{1/2}$ (min)	pK_d (k_{off}/k_{on})	pK_i
Isoprenaline	$2.47 \pm 1.39 \times 10^7$	3.06 ± 1.53	0.23	6.89 ± 0.08	6.73 ± 0.15
Salmeterol	$4.31 \pm 1.34 \times 10^9$	0.76 ± 0.06	0.91	9.70 ± 0.20	9.19 ± 0.07
Salbutamol	$2.05 \pm 1.03 \times 10^7$	4.06 ± 1.19	0.17	6.65 ± 0.14	6.54 ± 0.07
Formoterol	$2.15 \pm 0.45 \times 10^8$	3.29 ± 0.79	0.21	7.83 ± 0.09	7.28 ± 0.10
Indacaterol	$8.74 \pm 2.12 \times 10^7$	3.48 ± 0.42	0.20	7.37 ± 0.11	7.04 ± 0.05
Adrenaline	$3.15 \pm 0.61 \times 10^6$	5.12 ± 1.39	0.14	5.85 ± 0.10	5.82 ± 0.05
Compound 1	$3.25 \pm 1.7 \times 10^8$	0.41 ± 0.04	1.69	8.79 ± 0.22	8.80 ± 0.08
Carmoterol	$8.66 \pm 0.46 \times 10^7$	0.46 ± 0.09	1.51	8.32 ± 0.11	8.16 ± 0.11

Data are mean ± SEM for ≥3 experiments performed in duplicate.

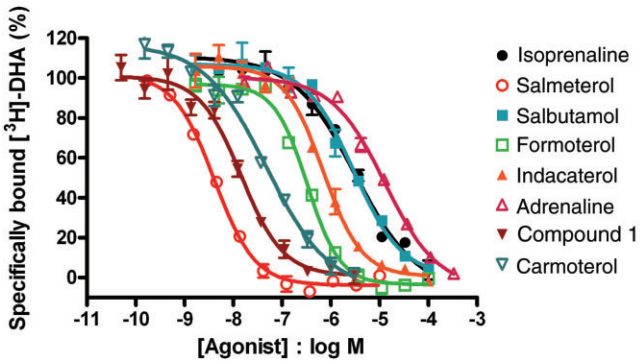

Figure 3

Competition between [^{3}H]-DHA and increasing concentrations of isoprenaline, salmeterol, salbutamol, formoterol, indacaterol, adrenaline, Compound 1 and carmoterol for human β_2-adrenoceptors expressed in the CHO cells in the presence of GTP. Membranes (15 µg· per well) from CHO-β_2 cells were incubated in HBSS containing 0.1 mM GTP at 37°C (as described in Methods) with 0.6 nM [^{3}H]-DHA and the indicated concentrations of competitor for 180 min. Data are presented as the mean ± range from a representative of ≥3 experiments performed in duplicate.

K_d value for [^{3}H]-DHA was in good agreement with the K_d estimated from the saturation analysis (33.9 ± 12.3 vs. 83.5 ± 11.1 pM), as was that for [^{125}I]-CYP (1.6 ± 0.2 vs. 4.10 ± 0.93 pM). The small differences between the two approaches are most likely because the incubation time for the saturation studies (3 h, limited by membrane stability) was insufficient to achieve full equilibrium between receptor and radioligand.

Kinetic observations are accurate only if the competition kinetic curves are allowed to approach equilibrium. The slower the off-rate of the radioligand from its receptor, the longer the time taken to reach equilibrium not only for kinetic determinations but also for equilibrium competition experiments. A faster dissociating radioligand ensures that the total incubation period of the assays is reduced; which is an important practical consideration. In preliminary experiments, inadequate competition was observed between CYP and the competing agonists, thus competition kinetic parameters were determined using DHA which has a relatively faster dissociation rate.

[^{3}H]-DHA equilibrium competition binding

The β_2-adrenoceptor binding profile of the agonists was determined in buffer containing GTP (0.1 mM) to ensure that agonist binding only occurred to the uncoupled form of the β_2 receptor. Each of the β_2 agonist ligands produced concentration-dependent inhibition of the specific binding of [^{3}H]-DHA to sites on CHO-β_2-adrenoceptor membranes. Examples of competition data are shown in Figure 3. Equilibrium competition binding data were fitted to a four-parameter logistic equation to obtain pIC_{50} and Hill slope parameter estimates. Equilibrium dissociation constants (pK_i) were subsequently determined from pIC_{50} values using the Cheng and Prusoff equation (Cheng and Prusoff 1973). The binding affinity of the β_2-adrenoceptor agonists for the β_2-adrenoceptor is shown in Table 2.

Competition kinetic binding

The association and dissociation rates of [^{3}H]-DHA were determined in each experimental run and these values were used to calculate the k_{on} (k_3) and k_{off} (k_4) of the unlabelled compound using Equation 3, as detailed in Methods. Representative curves for the β_2-adrenoceptor agonists tested are shown in Figure 4A–H. To ensure that each ligand displayed classical competitive and reversible binding, each agonist was assayed at three different concentrations, one-, three- and 10-fold K_i.

The pattern of [^{3}H]-DHA binding over time was dependent upon the off-rate of the competing agonist. [^{3}H]-DHA association in the presence of more slowly equilibrating competitors was bi-phasic. Progression curves for [^{3}H]-DHA alone and in the presence of three different concentrations of competitor were globally fitted to Equation 3, enabling the calculation of both k_{on} (k_3) and k_{off} (k_4) for each of the agonists, as reported in Table 2. As the k_{off} values determined were similar across the cohort, we tested whether the data were sufficient to discriminate between the agonists. The quality of fit was worse when the k_{off} was fixed to any value outside that predicted by

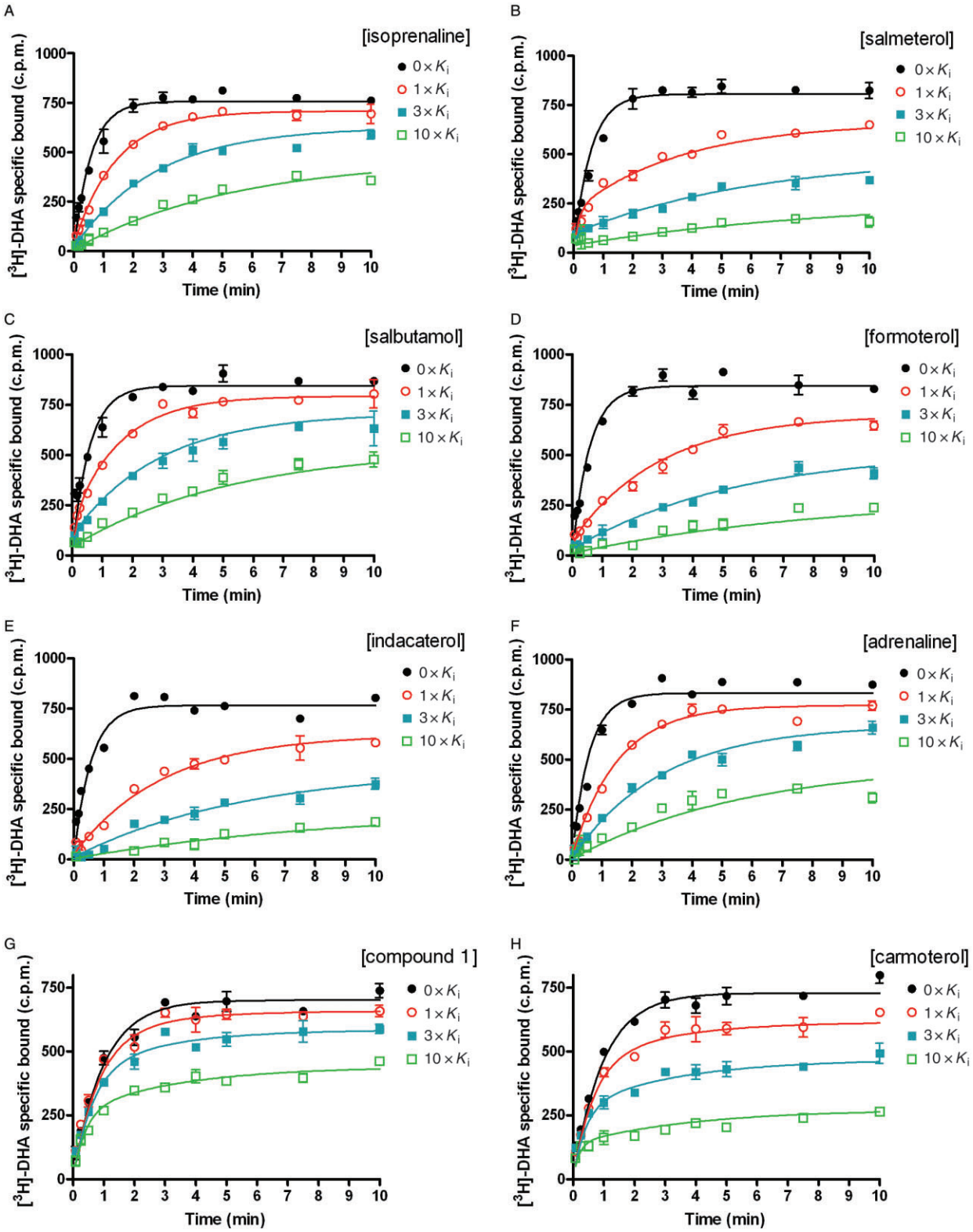

Figure 4

[^{3}H]-DHA competition kinetic curves in the presence of isoprenaline (A), salmeterol (B), salbutamol (C), formoterol (D), indacaterol (E), adrenaline (F), Compound 1 (G) and carmoterol (H). CHO-β_2 membranes were incubated with ~0.6 nM [^{3}H]-DHA and either 0-, 1-, 3 or 10-fold K_i. Plates were incubated at 37°C for the indicated time points and NSB levels were determined in the presence of 1 μM propranolol. Data were fitted to the equations described in the Methods to calculate k_{on} and k_{off} values for the unlabelled agonists; these are summarized in Table 2. Data are presented as mean ± range from a representative of ≥3 experiments performed in duplicate.

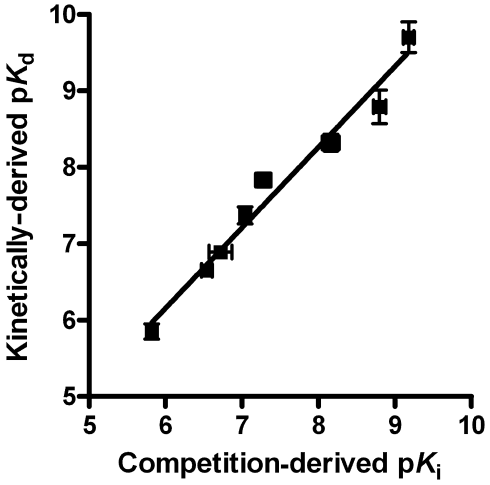

Figure 5

Correlation between pK_i and kinetically derived pK_d for the eight test agonists. pK_i values were taken from [^{3}H]-DHA competition binding experiments at equilibrium. The values comprising the kinetically derived K_d (k_{off}/k_{on}) were taken from the experiments shown in Figure 4. Data are presented as mean ± SEM from three or more experiments.

simultaneous fitting. To validate the rate constants, the kinetically derived K_d values (k_{off}/k_{on}) were compared with the affinity constant (K_i) obtained from equilibrium competition binding experiments (Figure 5). There was a very good correlation ($r^2 = 0.97$, $P < 0.0001$) between these two values.

A relationship between k_{off} and competition-derived pK_i has been suggested previously for β_2-adrenoceptor antagonists (Affolter *et al.*, 1985; Contreras *et al.*, 1986). A correlation plot of log k_{off} versus kinetically derived pK_d for the eight agonists tested in this study also revealed a significant correlation (Figure 6A, $r^2 = 0.64$, $P < 0.05$). Interestingly, there was a more highly significant correlation between log k_{on} and pK_d values (Figure 6B, $r^2 = 0.92$, $P < 0.0002$) than that achieved for k_{off}. These data imply that k_{on} plays a more important role in defining the equilibrium dissociation constant of β_2-adrenoceptor agonists.

Using kinetic parameters to model the rate of agonist occupancy and dissociation from the receptor

The rate of receptor occupancy is one factor which could potentially play a significant role in the rate of onset of the actions of β_2-adrenoceptor agonists. When k_{ob} was simulated at K_d concentration, salmeterol had a slower rate of receptor occupancy than the other clinically used ligands tested (Figure 7A). Comparing agonists at the same level of receptor occupancy does not, however, account for any differences in potency that are a consequence of different intrinsic efficacies (Charlton, 2009). To address this, the rate of agonist association was simulated using agonist concentrations based on potency from *in vitro* experiments (equivalent to 25% of the maximal cAMP response to isoprenaline, data taken from Battram *et al.*, 2006). Under these conditions, there were no clear differences in the rate of association of the four clinically used β_2-adrenoceptor ligands, suggesting that binding rate is not a key determinant of clinical onset of action (Figure 7B).

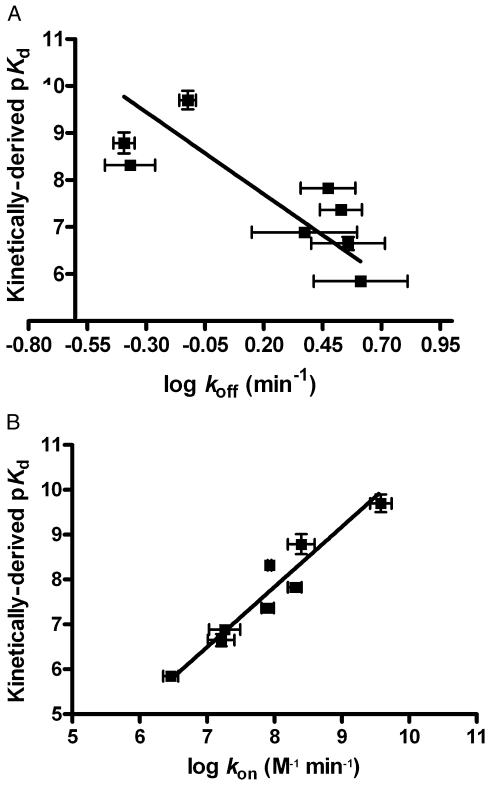

Figure 6

Correlation of pK_i with log k_{off} (A) and log k_{on} (B) determined for the eight test agonists. pK_i values were taken from [^{3}H]-DHA competition binding experiments. All data used in these plots are detailed in Table 2. Data are presented as mean ± SEM from three or more experiments.

Although comparing *in vitro* potency data provides more relevant simulations than comparing agonist occupancy, it is still far removed from the relative doses of each compound used in the clinic. Ideally, clinical pharmacokinetic (PK) data are used to develop a pharmacokinetic /pharmacodynamic (PK/PD) model but, for inhaled compounds, plasma levels do not reflect the pharmacodynamically relevant concentrations, but rather the spillover of drug from the effect compartment. In the absence of direct PK measurements in the lung, we have considered the clinical doses of the compounds administered by inhalation and simulated receptor association rates at relative concentrations of the compounds assuming complete dissolution following dosing.

In the clinic, salmeterol, indacaterol, salbutamol and formoterol are given at doses of 50, 150, 200 and 12 µg respectively. Figure 7C models the rate of receptor occupancy of these agonists at concentrations based on the relative clinical doses of the four drugs. A concentration of salmeterol at 10-fold its K_d (7.5 nM) was chosen for modelling purposes, and concentrations of indacaterol, formoterol and salbutamol were calculated based on their clinical doses relative to this concentration of salmeterol. For example, salbutamol and indacaterol are dosed four- and threefold higher than

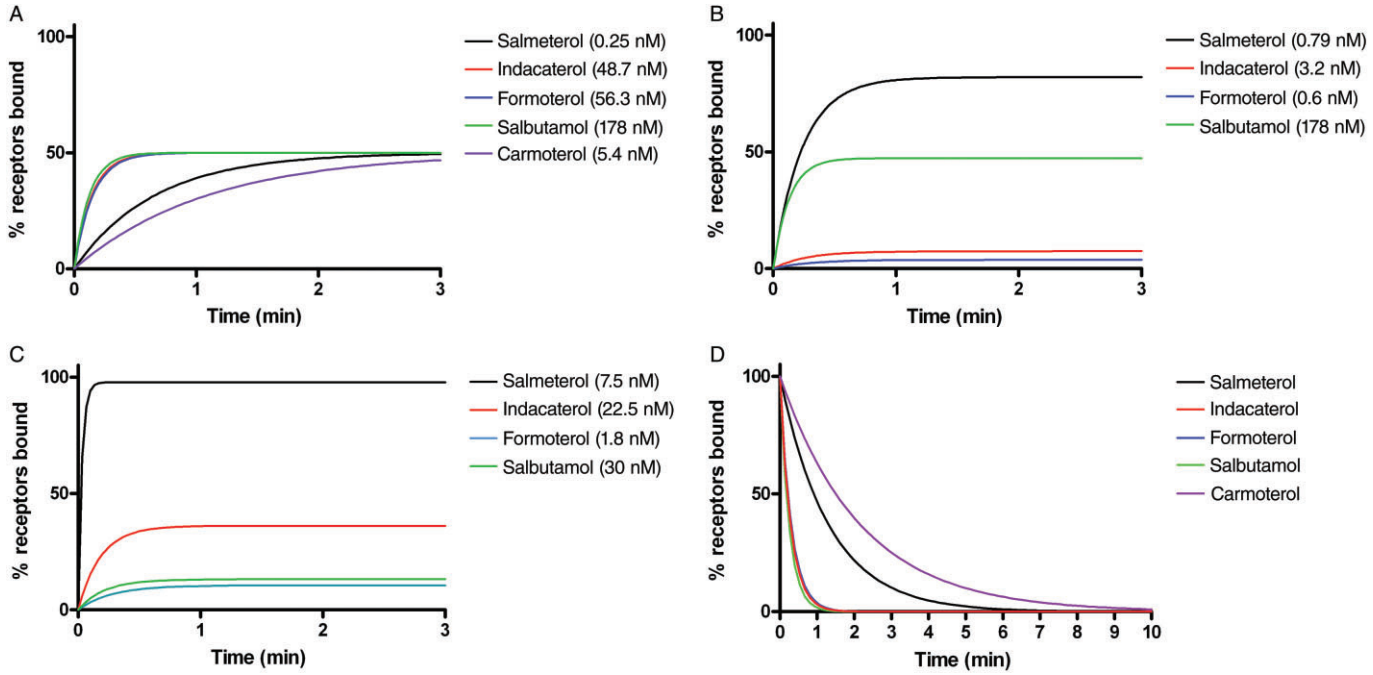

Figure 7

Modelling the association and dissociation of clinically relevant LABAs. Simulated binding of clinically relevant LABAs salmeterol, indacaterol, formoterol, salbutamol and carmoterol to human β_2-adrenoceptors, at (A) K_d concentrations (see Table 2 for details), (B) EC_{25} concentrations salmeterol (0.79 nM), indacaterol (3.2 nM), formoterol (0.6 nM) and salbutamol (178 nM), (EC_{25} values from Battram *et al.*, 2006) and (C) concentrations derived from relative clinical doses. For simulation purposes salmeterol at a concentration 10× its K_d (7.5 nM) was chosen and concentrations of indacaterol (22.5 nM), formoterol (1.8 nM) and salbutamol (30 nM) were calculated based on their clinical doses (see Results section) relative to this concentration of salmeterol. (D) Stimulated dissociation rates of clinically relevant β_2 ligands from β_2-adrenoceptors based on off-rates determined in competition kinetic binding experiments. All parameters derived from these plots are detailed in Table 3.

salmeterol, so concentrations of 22.5 nM and 30 nM were used for modelling purposes. In contrast, formoterol is dosed 4.17-fold lower than salmeterol so a lower relative concentration of 1.8 nM was used. Under these conditions, salmeterol occupies the receptors more rapidly than the other agonists, reinforcing the conclusion that receptor kinetics does not have an important influence on onset of action. As observed in the previous simulation, salmeterol occupies a far larger proportion of receptors than the other agonists when their clinical doses are compared. Indacaterol has the longest duration of action in the clinic (>24 h) of all the ligands tested in this study, with salbutamol having the shortest duration of action at 4–6 h (Brookman *et al.*, 2007). When the relationship between receptor occupancy and dissociation for all four ligands was simulated in Figure 7D, all were fully dissociated within 10 min, suggesting that dissociation rate has little or no role to play in the duration of action of clinically used long-acting β_2 ligands. Dissociation $t_{1/2}$ values and k_{ob} values from these simulations are detailed in Table 3.

Discussion

The aim of the present study was to measure the kinetic properties of a series of β_2-adrenoceptor agonists to determine if the kinetic rate constants influenced the observed onset of action and duration of effect in the clinic. Direct kinetic analysis of the binding of ligands to β_2-adrenoceptors has been limited primarily to the study of the interactions of antagonists with the receptor. Only a few kinetic studies with radiolabelled agonist have been performed due to the limited number of radiolabelled agonists available. In addition, the studies with agonists have largely been performed in the absence of GTP, thus molecular interpretation is likely to be complicated by interactions between the receptor and its associated G protein. Kinetic parameters for isoprenaline have been determined previously in a competition kinetic format but at non-physiological temperature (–10°C) designed to slow ligand dissociation (Contreras *et al.*, 1986). To our knowledge, the current study is the first time that kinetic rate constants have been derived for β_2-adrenoceptor agonists in a binding assay performed at physiological temperature and sodium concentrations in the presence of guanine nucleotide. These studies were designed to more closely mimic *in vivo* conditions, allowing better extrapolation to the clinical situation. Indirect isoprenaline k_{off} measurements have been made previously in HBSS using the rate of cAMP decline following the addition of a high concentration of propranolol, as a measure of k_{off} (Deyrup *et al.*, 1999). The value of 5.5 min^{-1} was in agreement with the reported k_{off} (4 min^{-1}) for isoprenaline based on recovery of a chemo attractant-mediated cellular response inhibited by β_2-adrenoceptor activation in neutrophils (Mueller *et al.*,

Table 3

Simulated $t_{1/2}$ and k_{ob} values for clinically relevant β_2-adrenoceptor agonists determined at binding K_d concentration, CHO-β_2 cAMP assay derived EC_{25} concentration and concentrations derived from relative clinical doses (see Figure 7 for graphical representation)

Compound	Dissociation $t_{1/2}$ (min)	Binding assay – K_d concentration k_{ob} (min^{-1})	% receptor occupied	cAMP assay – EC_{25} concentration k_{ob} (min^{-1})	% receptor occupied	Concentration relative to clinical dose k_{ob} (min^{-1})	% receptor occupied
Salmeterol	0.91	1.52	50	4.2	81.9	33.1	97.7
Indacaterol	0.20	6.96	50	3.8	7.4	5.5	36.1
Formoterol	0.21	6.58	50	3.4	3.8	3.7	10.5
Salbutamol	0.17	8.12	50	7.7	47.3	4.7	13.1
Carmoterol	1.51	0.92	50	ND	ND	ND	ND

ND, not determined.

1988). More recently, the interaction of ligands with purified β_2-adrenoceptors has been measured by plasmon waveguide resonance spectroscopy. Using this method, isoprenaline and adrenaline produced off-rates of 4.68 min^{-1} and 7.20 min^{-1}, respectively, following displacement by alprenolol (Devanathan *et al.*, 2004). These values, as well as the values obtained for isoprenaline in functional studies (Mueller *et al.*, 1988; Deyrup *et al.*, 1999), agree closely with the value of 3.06 min^{-1} for isoprenaline and 5.12 min^{-1} for adrenaline obtained in our competition kinetic studies.

The values for k_{off} for the binding of agonists correlated with the equilibrium dissociation constant (r^2 = 0.64, P < 0.05), which is in agreement with previous studies suggesting that affinity and kinetic off-rate are linked (Affolter *et al.*, 1985; Contreras *et al.*, 1986 and Deyrup *et al.*, 1999). However, the fastest and the slowest dissociating ligands tested in this study differed in k_{off} only by a factor of 10-fold.

The association rate constant, k_{on}, is thought to depend largely on diffusion (Weiland and Molinoff, 1981). Interestingly, k_{on} values for the binding of β_2-adrenoceptor agonists have been shown to be consistently lower when compared with β_2-adrenoceptor antagonists when determined at –10°C (Contreras *et al.*, 1986). This finding is thought to be the result of isomerization of the receptor on binding of the agonist. Our data obtained at physiological temperature would seem to support this theory as [^{3}H]-DHA and [^{125}I]-CYP have very fast on-rates, compared with the β_2-adrenoceptor agonists with high intrinsic activity such as adrenaline. There was, however, a much larger difference in k_{on} values between the β_2 agonists. These ranged from 4.31 × 10^9 M^{-1} min^{-1} for the partial agonist salmeterol to 3.15 × 10^6 M^{-1} min^{-1} for the full agonist adrenaline. It is tempting to speculate that the rapid on-rate for the partial agonist salmeterol compared with the full agonist adrenaline (>1000-fold difference) is due to a reduction in the magnitude of the isomerization step it undergoes when activating the receptor. Interestingly, k_{on} correlated better with the K_d than k_{off} values, suggesting that the on-rate is more important for determining the equilibrium affinity of these agonists than the off-rate. We have previously observed this phenomenon in a study of muscarinic M_3 agonist kinetics (Sykes *et al.*, 2009).

Our simulations would suggest that the kinetic rate parameters determined for the clinically used β_2-adrenoceptor ligands are unlikely to play a significant role in determining the duration of action of these molecules. For example, salbutamol is a short-acting β_2 agonist which requires dosing several times a day. However, we have shown that the kinetic off-rate of salbutamol is similar to indacaterol (4.06 vs. 3.48 min^{-1}), which is a long-acting β_2-adrenoceptor ligand requiring only once daily administration for a 24 h duration of action in asthma (Kanniess *et al.*, 2006; Beeh *et al.*, 2007; Chuchalin *et al.*, 2007) and COPD patients (Beier *et al.*, 2006, Beeh and Beier, 2010). Salmeterol has a slower off-rate than indacaterol and yet requires twice daily administration (Sutherland *et al.*, 2009). Thus, duration of action and kinetic off-rate do not appear to be linked for the β_2-adrenoceptor agonists tested in this study. This is in contrast to a recent study by Casarosa and colleagues (2011) who reported a dissociation half-life for [^{3}H]-olodaterol from the G-protein coupled form of the β_2-adrenoceptor of 17.8 h, concluding this contributes to its 24 h duration of action. However, these studies were conducted at non-physiological temperature, in the absence of sodium ions and GTP. This absence of guanine nucleotide in particular means there is likely to be two populations of receptors, a low agonist affinity state that is uncoupled from G-proteins and a high-affinity agonist state that is stabilized by the guanine nucleotide-free Gα subunit in a ternary complex. This ternary complex is stable in a well-washed membrane preparation, but because GTP is present in the cytoplasm at high concentrations, this ternary complex is very short lived in the whole cell (Lemoine, 1992). The stable agonist high-affinity state can therefore be considered as an artefact of the experimental design, meaning it is unlikely that the very slow off-rate of olodaterol observed in this study will be relevant in the clinic. Indeed, the twice daily β_2-adrenoceptor [^{3}H]-formoterol has been reported to have a very slow dissociation from this same receptor species, so it is unlikely this property is unique to olodaterol (Lemoine, 1992). It has also been suggested that the long duration of carmoterol observed *in vitro* in 'wash out' experiments can be attributed to its slow dissociation from the β_2 receptor (Voss *et al.*, 1992); however, based on its measured kinetic k_{off} (0.46 min^{-1}), we would predict almost complete dissociation of carmoterol from β_2 receptors within 5 min. Therefore, the long duration of action of carmoterol is more likely to be attributable to membrane interactions and

so-called drug 'rebinding' which are inherent features of 'washout' experiments (Vauquelin and Charlton, 2010).

Recently, a mathematical model describing the washout of LABAs from the β_2-adrenoceptor was reported, but due to the lack of published kinetic binding data, it relied on receptor dissociation values estimated indirectly from functional studies looking at the reversibility of agonist effects (Szczuka *et al.*, 2009), creating debate over the accuracy of the conclusions (Coleman, 2009). The incorporation of our new kinetic parameters into such models would be interesting, although in this particular case the estimated k_{off} value for salmeterol of 0.23 min^{-1} used by Szczuka *et al.* in their mathematical simulations was only threefold different to the value determined in our competition binding assay (0.76 min^{-1}).

The clinically used β_2-adrenoceptor agonists vary in terms of their onset of action, with salmeterol taking longer to achieve full bronchodilator efficacy than salbutamol, formoterol and indacaterol (Palmqvist *et al.*, 1997; Brookman *et al.*, 2007). To test whether the kinetics of receptor binding was in part responsible for this difference, we simulated receptor association rates using the kinetic parameters measured in this study. Initial simulations at a K_d concentration of each agonist appeared to support this notion, with salmeterol taking longer to reach equilibrium than the other ligands. However, at concentrations that give equivalent functional responses, the onset of action was similar, while at concentrations based on their relative clinical doses, salmeterol occupied the receptors more rapidly than the other agonists. This reinforces the conclusion that receptor kinetics does not have an important influence on onset of action. An interesting observation in these simulations is that salmeterol occupies a far larger proportion of receptors than the other agonists when their clinical doses are compared. This suggests that salmeterol is potentially overdosed relative to the other agonists, perhaps reflecting its poorer efficacy and requirement to bind more receptors to achieve its pharmacological response. In contrast, despite having a relatively low efficacy, salbutamol occupies almost the same proportion of receptors as formoterol (high-efficacy ligand). This suggests that salbutamol is relatively under-dosed in the clinic, which may contribute to the need for four doses in a day to produce a sustained effect on FEV.

In summary, the competition binding studies described here produced accurate kinetic parameters for the binding of a cohort of β_2-adrenoceptor agonists to the human β_2-adrenoceptor. Although the β_2-adrenoceptor agonists exhibited a range of dissociation rates from the receptor, it is doubtful that kinetic rate parameters play a significant role in determining either onset of duration of action. It is more likely that other factors such as lipophilicity (Beattie *et al.*, 2010) and agonist efficacy (Rosethorne *et al.*, 2010) determine the overall onset of action in the clinic, and that partitioning of drug into lipophilic compartments (Anderson *et al.*, 1994; Teschemacher and Lemoine, 1999) after inhalation is the key determinant of their long duration of action.

Conflict of interest

None.

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