Functional and Biochemical Rationales for the 24-Hour-Long Duration of Action of Olodaterol

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ABSTRACT

β₂-Adrenoceptor (β₂-AR) agonists are powerful bronchodilators and play a pivotal role in the management of pulmonary obstructive diseases, such as asthma and chronic obstructive pulmonary disease. Although these agents first were used many years ago, progress in drug development has resulted in better tolerated, long-acting β₂-AR agonists (LABAs), such as formoterol and salmeterol. Although LABAs have been on the market for several years, relatively little is known on the rationale(s) behind their long duration of action. In this study, we focused on olodaterol (previously known as BI1744CL), a novel inhaled LABA, which provides a bronchodilating effect lasting 24 h and is currently in Phase III clinical trials. To understand the rationale behind its long duration of action, different aspects of olodaterol were analyzed (i.e., its lipophilicity and propensity to accumulate in the lipid bilayer as well as its tight binding to the β₂-AR). In line with its physicochemical properties, olodaterol associated moderately with lipid bilayers. Instead, kinetic as well as equilibrium binding studies indicated the presence of a stable [3H]olodaterol/β₂-AR complex with a dissociation half-life of 17.8 h due to ternary complex formation. The tight binding of olodaterol to the human β₂-AR and stabilization of the ternary complex were confirmed in functional experiments monitoring adenylyl cyclase activity after extensive washout. Taken together, binding, kinetic, and functional data support the existence of a stable complex with the β₂-AR that, with a dissociation half-life >17 h, might indeed be a rationale for the 24-h duration of action of olodaterol.

Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are conditions characterized by airway obstruction, which is variable and reversible in asthma but is progressive in COPD (Guerra, 2009). Both diseases are very common, and their incidence is increasing globally, placing a growing burden on patients and on health services in industrialized and developing countries (Pauwels and Rabe, 2004; Braman, 2006). β₂-Adrenoceptor (β₂-AR) agonists are among the most powerful known bronchodilators and play a pivotal role in the management of pulmonary obstructive diseases such as asthma and COPD. β₂-AR agonists act by binding to the β₂-AR, a member of the family of G protein-coupled receptors, which is present in high density in airway smooth muscle cells (aSMCs). Activation of the human β₂-AR in aSMCs induces increased levels of cAMP within cells, which lead to activation of protein kinase A and phosphorylation of several targets, ultimately resulting in relaxation (Johnson and Druey, 2002).

After the introduction of short-acting inhaled β₂-AR agonists such as salbutamol (Waldeck, 2002), progress in drug development has resulted in better tolerated, longer-acting β₂-AR-specific agents. In particular, two long-acting β₂-AR agonists (LABAs) that, when given by inhalation, produce bronchodilation for at least 12 h are available for clinical use, namely, formoterol (Anderson et al., 1994) and salmeterol (Johnson, 1995), and several are in development as once-daily treatments.

Recently, concerns were raised about LABA treatment as a monotherapy for asthma: for example, the Salmeterol Multicenter Asthma Research Trial found more asthma deaths and life-threatening events in the salmeterol-treated pa-
tent hypotheses have been presented. On the basis of the rationale(s) behind their long duration of action. Differ-
was dissolved in methanol, and Pd/C 105 was added to the solution; afterward, the reaction suspension was titrated in a special apparatus for tritium gas (iRC TRITEC Ltd., Teufen, Switzerland).

After evaporation and dissolution several times and purification by high-performance liquid chromatography (HPLC), the one-stage synthesis yielded 50 mCi (1850 MBq) [3H]olodaterol with a specific activity of 20 Ci/mmol (740 GBq/mmol). The specific radioactivity was calculated by measuring the activity of the product and by comparing the UV signal areas of the product solution with those of a solution with a known concentration. As determined by HPLC, the radiochemical purity was >98%. Chromatographic data for HPLC (for purification and analysis) was as follows: HPLC column, Waters (Milford, MA), XBridge C8 (5 μm), 4.6 × 150 mm; mobile phase A, water and 0.05% trifluoroacetic acid; mobile phase B, acetonitrile and 0.05% trifluoroacetic acid; flow rate, 1 ml/min; UV detection, 254 nm; temperature, 30°C.

Equilibrium binding experiments. In all of the radioligand experiments, the binding buffer consisted of 50 mM Tris-HCl, 2 mM MgCl2, and 1 mM EGTA, pH 7.3. After the indicated incubation period, bound and free [3H]olodaterol or [3H]CGP12,177 were separated by rapid vacuum filtration using a Brandel harvester (Gaithersburg, MD) on GF/B filters presoaked in 0.5% polyethyleneimine and rapidly washed three times with ice-cold binding buffer. Filter disks were added to 3 ml of scintillation fluid (Ultima Gold; PerkinElmer Life and Analytical Sciences) in pony vials, and radioactivity was quantified using liquid scintillation spectrometry on a Tri-Carb 2000TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences). In all of the experiments, total binding never reached 10% of that added, limiting complications associated with the depletion of the free radioligand concentration.

Saturation binding experiments were performed by incubating membranes expressing the human β2-AR (20 μg per sample, adjusted according to the Bmax of the cell line) with a range of concentrations of [3H]olodaterol (5 pM to 6 nM) or [3H]CGP12,177 (5 pM to 4 nM). Samples were incubated at room temperature overnight under gentle agitation before filtration. In some saturation experiments, the nonhydrolyzable GTP analog GppNHp was added (final concentration 10 μM) to induce receptor/G protein dissociation.

To ascertain which β-AR subtypes are expressed in aSMCs, heterologous competition experiments against [3H]CGP12,177 were performed with the β2-AR-selective antagonist CGP-20712A. Cells (150,000 per sample) were incubated in the presence of [3H]CGP12,177 (final concentration approximately 1 nM) and different concentrations of unlabeled antagonist at room temperature with gentle agitation for 2 h before filtration. Competition displacement binding data were fitted to the equation described by Hill (1909), and IC50 values obtained from the inhibition curves were converted to Kd values using the method of Cheng and Prusoff (1973).

Kinetic studies of the interaction [3H]olodaterol/human β2-AR. Parameters describing the association kinetics (Kass values) for [3H]olodaterol/human β2-AR were determined by adding [3H]olodaterol at time 0 (three different concentrations per experiment in the range of 0.05–1.5 nM) to membranes obtained from CHO human β2-AR cells (20 μg per sample) in a total volume of 1 ml. At different time points (i.e., after 1, 3, 5, 8, 12, 16, 20, 25, 30, 40, 60, 90, and 120 min), samples (triplicates per each radioligand concentration) were filtered, and radioactivity due to receptor association was quantified. Data were fit globally using the Prism equation “Association − Two or More Concentrations of Hot,” which derives a single best-fit estimate for Kass.

To determine dissociation kinetic parameters, membranes expressing the human β2-AR (20 μg per sample) first were allowed to equilibrate with multiple concentrations of [3H]olodaterol (usually in the range of 0.5–1.5 nM) at room temperature for at least 2 h (300 μl per sample). Afterward, samples were added to test tubes already containing 4 ml of binding buffer with 10−5 M nonradioactive olodaterol (time 0) to start dissociation. At the different time points, samples were filtered using a Brandel harvester, as described above. Care was taken to ensure that [3H]olodaterol was dissociated fully from the human β2-AR. Dissociation data were analyzed with Prism (GraphPad Software, San Diego, CA), allowing the program to compare the extra-sum-of-square F test the best fit between the equations “Dissociation: One-Phase Exponential Decay” and “Two-Phase Exponential Decay” (threshold p value ≤0.01). K1/2 values obtained were transformed into t1/2 values (dissociation half-life) using the following equation: t1/2 = ln 2/K1/2.

pKd Value and Log D Profile Determination. The pKd values of olodaterol were determined by a potentiometric and/or UV-metric titration approach using the GlpKa DPAS instrument from Sirius Analytical Ltd. (Riverside, UK). Due to the lower solubility of the free base at high pH values, the usage of an organic cosolvent (methanol) was indicated. A multiset titration with three different methanol contents was performed. The pKd values in pure aqueous systems were extrapolated from this multiset experiment.

The apparent partition coefficient was determined by performing potentiometric titrations similar to the experiments described above, except that n-octanol was used instead of methanol. Olodaterol was titrated with different mixtures of aqueous solution and n-octanol over the range of pH 2 to 12. From the shifts of the pKd values in the presence of n-octanol (apparent pKd values) in comparison to the pure aqueous pKd values, a pH-dependent partition coefficient profile was calculated.

cAMP Assay. To measure persistent receptor activation induced by the different agonists, changes in intracellular cAMP levels were determined with CHO human β2-AR and CHO human β2-AR cells using Lance technology (PerkinElmer Life and Analytical Sciences), according to a previously published protocol (Summerhill et al., 2008) with some modifications.

Cells were seeded overnight in 96-well view plates at 10,000 cells per well in growth media containing 1% fetal bovine serum. On the following day, a cAMP washout assay was performed in which all of the different steps were carried out at room temperature in medium consisting of Hank’s balanced salt solution with 0.1% bovine serum albumin and 5 mM HEPES (pH 7.4).

Cells were treated with either of the following protocols: 1) cells (referred to as “washed” cells) were stimulated with a range of concentrations of β-AR agonist in medium for 30 min, then washed for 1 h (with medium exchanged every 10 min). After this washing step, cells were incubated further with medium containing 0.5 mM isobutyl methylxanthine (a nonselective phosphodiesterase inhibitor) in the absence of any added β-AR agonist for 30 min. At the end of this incubation, the extracellular medium was discarded, and intracellular levels of cAMP were quantified. 2) Cells (referred to as control cells) were incubated with medium alone for 30 min, washed for 1 h (with medium exchanged every 15 min), and then stimulated with a range of concentrations of β-AR agonist in the presence of 0.5 mM isobutyl methylxanthine for 30 min. At the end of this incubation, intracellular levels of cAMP were quantified.

In both cases, cells only were treated with a single concentration
range of β-AR agonist: either before (referred to as washed cells) or after (control cells) the washing step. For each β-AR agonist, the EC_{50} was compared in washed versus control cells to generate a rightward shift concentration ratio, which was indicative of the persistence of receptor activation. Every plate contained washed and unwashed concentration-response curves to the same compound to control for plate-to-plate variation, and all of the cells were washed the same number of times.

**Data Analysis.** All of the experiments were analyzed by either linear or nonregression analysis with the equations mentioned under the different assay methodologies using Prism, version 5.02. Individual estimates (either K_{app}, K_{d}, K_{o}, pEC_{50} or pEC_{50} values) were obtained from each experiment and then averaged to provide mean data (±S.E.M.).

**Results**

**Generation and Characterization of [3H]Olodaterol.** To investigate the interactions of olodaterol with the human β_{2}-AR as well as with lipid membranes, a radioligand was generated as described under Materials and Methods and illustrated in Fig. 2.

To characterize the new radioligand, initial experiments were performed with membranes obtained from CHO cells selectively overexpressing the β_{2}-AR. In these experiments, the B_{max} obtained with [3H]olodaterol (2.22 ± 0.08 pmol/mg) was not statistically different from the B_{max} obtained with the β_{2}-AR antagonist [3H]CGP12,177 (2.13 ± 0.05 pmol/mg, n = 4 paired experiments), which is used classically as a nonspecific radioligand for β-AR.

Similarly, radioligand binding experiments were performed with human aSMCs and in purified membranes obtained from mouse lung homogenates (Fig. 3). Human aSMCs are reported to contain exclusively the β_{2}-AR (Green et al., 1995). This was confirmed by the displacement of [3H]CGP12,177 with the selective β_{1}-AR antagonist CGP-20712A: a monophasic displacement was found with a pK_{i} value of 6.14 ± 0.05 (n = 2), representing its dissociation constant for the human β_{2}-AR [reported pK_{i} at the human β_{2}-AR of 6.11 ± 0.05 (Baker, 2005)]. Similarly to results obtained with CHO human β_{2}-AR cells, the maximal numbers of receptor binding sites obtained in aSMCs with the two radioligands are in good agreement (10.304 versus 10.863 sites per cell with [3H]CGP12,177 and [3H]olodaterol, respectively, n = 2).

Because both β_{1}-AR [mostly in the vasculature (Lemoine, 1992)] and β_{2}-AR (in smooth muscle and epithelial cells) coexist in lung homogenates, B_{max} obtained with the unselective [3H]CGP12,177 is higher (Fig. 3A) compared to that obtained with [3H]olodaterol, which, when tested at low concentrations (here up to 3 nM), selectively labels the β_{2}-AR component [selectivity over the human β_{1}-AR is >200-fold (Bouyssou et al., 2010)]. In the presence of the β_{1}-AR-selective antagonist CGP-20712A (1 μM), the maximal numbers of receptor binding sites obtained with [3H]CGP12,177 and [3H]olodaterol are in good agreement (Fig. 3A).

Addition of the nonhydrolyzable GTP analog GppNHp did not affect B_{max} values obtained with either [3H]CGP12,177 or [3H]olodaterol in any of the described settings (see below and Fig. 7).

Taken together, these results indicate that [3H]olodaterol is able to label the whole β_{2}-AR population expressed at the cellular membrane independently from receptor association to G proteins, similarly to the antagonist [3H]CGP12,177.

**Physicochemical Properties of Olodaterol and Potential for Interaction with Lipid Membranes.** The pK values of olodaterol were determined by a potentiometric and UV-metric titration approach as described under Materials and Methods. A pK_{a} of 9.3 was obtained for the protonation of the secondary amine moiety, whereas a pK_{a} of 10.1 reflected the deprotonation of the phenolic function.

The apparent partition coefficient then was determined with different mixtures of aqueous solution and n-octanol over the range of pH 2 to 12. The obtained profile is depicted in Fig. 3B; the log D value at pH 7.4 is 1.2.

To evaluate the potential of olodaterol for interacting with lipids, experiments analyzing its association with membranes obtained from mock cells (i.e., CHO cells not expressing any β-AR) were performed. In line with its moderate lipophilicity, olodaterol showed little binding to mock membranes (20 μg, same amount as used in saturation binding experiments with human β_{2}-AR; Fig. 7), when tested at concentrations relevant for its interaction with the β_{2}-AR (i.e., low nanomolar range; its EC_{50} is 0.1 nM) (Fig. 4A) (Bouyssou et al., 2010). Likewise, unspecific binding of [3H]olodaterol (approximately 3 nM in the presence of a large excess of the unspecific β-AR antagonist CGP12,177A) to aSMCs as well as in mouse lung homogenates was approximately 10% of total binding (8.8 and 11.2%, respectively). To observe any accumulation in the lipid bilayer, the amount of membranes (150 μg per sample), radioligand (up to 180 nM), and volume of incubation (3 ml) were increased. With this protocol, which exposes membranes to large amounts of [3H]olodaterol (up to 600 pmol), olodaterol showed significant association with cellular membranes and linearity over increasing radioligand concentrations (Fig. 4B). However, also with these conditions the percentage of olodaterol bound to the lipid membranes is <1% of free olodaterol in the aqueous solution at any concentration tested, in line with its moderate log D value.

**Olodaterol Kinetics of Interaction with the β_{2}-AR Indicate Fast Association and Biphasic Dissociation.** As an alternative rationale to the microkinetic hypothesis, olodaterol interaction with the human β_{2}-AR as well as the stability of the ligand/receptor complex were investigated.

For kinetic association studies, membranes obtained from CHO cells expressing the human β_{2}-AR were incubated in the presence of three different concentrations of [3H]olodaterol within each experiment. Attention was paid to select [3H]olodaterol concentrations (in the range of 0.05–1.5 nM) at which binding to the human β_{2}-AR is significant (i.e., 20–80% receptor is bound; Fig. 5A) but negligible unspecific partitioning in the membranes takes place to analyze a simple model of ligand/receptor interaction.

The association kinetics followed a monoexponential relation and were fitted by nonlinear regression to the equation Association = Two or More Concentrations of Hot (see Materials and Methods). The parameter estimate of K_{on} (7.64 ± 0.45 × 10^{7} M^{-1} min^{-1}, n = 3) illustrates fast association of [3H] olodaterol, which correlates well with the reported fast onset of action of olodaterol in an in vivo model of acetylcholine-induced bronchoconstriction (Bouyssou et al., 2010).

Similarly, the kinetics of [3H]olodaterol/human β_{2}-AR dissociation were studied at radioligand concentrations at which negligible unspecific binding to the membranes takes place.
to ensure that our analysis exclusively reflects the ligand/receptor interactions. Analysis of the dissociation kinetics revealed a biphasic behavior ($K_{\text{off}}$; fast, $1.31 \pm 0.13 \text{ h}^{-1}$; $K_{\text{off}}$; slow, $0.039 \pm 0.008 \text{ h}^{-1}$; $n = 4$; Fig. 5B), with approximately 30 to 40% of the receptor pool showing very slow dissociation. Half-times of 32 min (fast dissociation $t_{1/2}$) and 17.8 h (slow dissociation $t_{1/2}$) were calculated according to the equation under Materials and Methods. Given the long time necessary to achieve complete dissociation of the [$^3$H]olodaterol/human $\beta_2$-AR complex (up to 50 h), control experiments were performed in parallel where dissociation was not started, and total binding was monitored at the different time points to rule out membrane degradation (data not shown).

As further proof for the existence of this stable long-lasting complex between the $\beta_2$-AR and olodaterol, membranes expressing the human $\beta_2$-AR were preincubated with nonlabeled olodaterol (3 nM), resulting in $90\%$ receptor occupation (saturation studies in Fig. 7). Small samples of pretreated membranes (50 $\mu$L) were diluted by adding 4 ml of binding buffer containing 3 nM [$^3$H]CGP12,177, thereby combining the dissociation of nonradioactive olodaterol by dilution (80-fold) with the association of an excess of competing radioligand. At different time points, membranes were filtered, and radioactivity was quantified (Fig. 6). According to the model built with the affinities of olodaterol and CGP12,177A for the human $\beta_2$-AR, at equilibrium $>98\%$ of receptor is labeled with [$^3$H]CGP12,177. Given the fast association of [$^3$H]CGP12,177, the "bottle neck" in reaching equilibrium within this binding assay is the dissociation of olodaterol from the human $\beta_2$-AR. As shown in Fig. 6,
it takes at least 24 h before [3H]CGP12,177 has labeled the whole /H9252 2-AR population. The profile of progressive recovery of the /H9252 2-AR binding sites reflects the slow dissociation component of olodaterol shown in the kinetic studies (Fig. 5B).

The /H9252 2-AR/G Protein Complex Is Responsible for the Slow Dissociation Component of [3H]Olodaterol. In contrast to antagonists, /H9252 2-AR agonists recognize two interconvertible states of the /H9252 2-AR, depending on the presence or absence of the G protein associated with the receptor (high- and low-affinity states, respectively). Keeping in mind that olodaterol is indeed a /H9252 2-AR agonist with an intrinsic activity close to full agonism [intrinsic activity 88% (Bouyssou et al., 2010)], we tested whether the biphasic dissociation observed depended on two different species of the [3H]olodaterol/human /H9252 2-AR complex, one in the presence (i.e., high-affinity, slow-dissociating complex) and one in the absence of the Gαs protein.

In these experiments, the nonhydrolyzable GTP analog GppNHp was used to induce the dissociation of the G protein from the human /H9252 2-AR and to convert all of /H9252 2-AR to the low-affinity, G protein-free binding state for agonists. Indeed, in the presence of GppNHp, the dissociation of the [3H]olodaterol/human /H9252 2-AR complex was fitted best by a monophasic equation with a Koff/2.04 ± 0.18 h⁻¹, n = 3 (dissociation t1/2 of 20.2 min), resembling the fast component seen previously. Taken together, these data indicate that the slow dissociation observed with [3H]olodaterol reflects its interaction with the /H9252 2-AR coupled to the Gαs protein (i.e., the signaling complex in its active state).

Saturation Experiments with [3H]Olodaterol. Kinetic studies indicate the presence of one association and two dissociation constants for the [3H]olodaterol/human /H9252 2-AR complex. Given that Kd = Koff/Kon, this should imply the
presence of two different equilibrium affinity constants. To confirm this, saturation experiments were performed in human β2-AR-expressing CHO membranes, comparing [3H]olodaterol and [3H]CGP12,177, which was used as a control (Fig. 7). As expected, analysis of [3H]olodaterol binding data indicated the model with two affinity constants as the best fitting model (p ≤ 0.001 versus one-site $K_d$). The $K_d$ values determined in the saturation binding experiments (0.04 ± 0.01 nM for the high-affinity site and 1.8 ± 0.3 nM for the low-affinity site, n = 3) are in reasonable agreement with the ones calculated from kinetic data according to the equation $K_d = K_{off}/K_{on}$ (0.01 and 0.4 nM for the high and low affinity, respectively).

When receptor/G protein association was prevented by the presence of the GTP analog GppNHp, a single binding site was observed with [3H]olodaterol ($K_d = 1.07 ± 0.4$ nM, n = 3), although $B_{max}$ was not affected (Fig. 7B). The loss of the high-affinity binding site is in line with the loss of the slow dissociation component observed in the kinetic experiments.

As expected, saturation data obtained with [3H]CGP12,177 were fit best by a model with a single $K_d$ ($K_d = 0.04 ± 0.01$ nM, n = 3; Fig. 7C), and the results did not vary in the presence of GTP (data not shown).

**cAMP Measurements for Stability of the Agonist/Receptor Complex.** Results obtained with kinetic as well as equilibrium binding studies indicated the presence of a stable, slow-dissociating complex between olodaterol and the Go$_s$-coupled β2-AR. Because β2-AR/Go$_s$ is the active receptor conformation, these results suggest that olodaterol is stabilizing a long-lasting signaling complex.

To prove this point and functionally measure the persistence of the olodaterol/β-AR signaling complex, a washout cAMP assay was devised. In brief, cells were incubated for 30 min with β2-AR agonists to allow binding to the β2-AR, then a procedure consisting of six consecutive washing steps of 10 min each was performed to remove any β2-AR agonist not bound to the cells. After this extensive and repetitive washing, adenylate cyclase activity in the cells was used as a measure of persistent occupancy of the β2-AR by agonists.

Persistence of agonist action then was expressed as a concentration ratio by comparing the response curves to agonists in washed versus control cells (i.e., cells that were stimulated with the β2-AR agonist without any washout afterward).

To validate this assay, several β-AR agonists were tested. As expected, hydrophilic compounds such as isoprenaline,
salbutamol, and fenoterol showed significant rightward shifts in their concentration-response curves after washing (Table 1 and exemplified by isoprenaline in Fig. 8A). This indicates that these compounds readily wash out of the β2-AR and thus have a very short duration of action. These results also prove that the extensive washout procedure is sufficient to eliminate cAMP generated in the cells during the first incubation with β-AR agonists, probably through active secretion in the extracellular medium and metabolism (phosphodiesterase activity is not blocked, because no inhibitor is present during the first incubation and washing steps), and validate the measurement of adenylate cyclase activity after washout as a proof of agonist persistence.

Compounds with a longer duration of action in clinical practice showed different profiles: with formoterol, there was a 105-fold shift to the right in comparison to the control curves (Fig. 8B).

The concentration-response relationships after washout for salmeterol (5.2-fold) and olodaterol (8-fold) were shifted less to the right in comparison to the control curves (Fig. 8; C and D), indicating persistent activation of the β2-AR.

To investigate whether the long-lasting effect observed with salmeterol and olodaterol is due to a stable complex with the human β2-AR resulting from slow ligand/receptor dissociation and therefore specific for this receptor subtype or a consequence of nonspecific interaction with lipid membranes and drug-depot formation, similar washout experiments were performed with CHO cells expressing the human β2-AR.

After washout at the human β2-AR, the concentration-response curve of isoprenaline was shifted significantly to the right compared with the control curves (Fig. 9C). Salmeterol and olodaterol this time showed a different behavior: whereas salmeterol washout induced a small shift (14-fold) (Fig. 9B) similar to that observed with human β2-AR, the olodaterol shift was 10 times more pronounced (87-fold) (Fig. 9C; Table 1). Therefore, the data indicate that the persistent effects of olodaterol at the β2-AR are specific to that receptor, whereas membrane loading seems to play a more dominant role for salmeterol.

**Discussion**

With chronic diseases such as COPD and asthma, patient adherence to medication plans is a major obstacle to successful management (Bender, 2002). One factor contributing to poor adherence is a complicated or multiple treatment regimen, and simplified dosing regimens are known to improve compliance with the human β2-AR. This strategy, which has been proven successful with the long-acting muscarinic antagonist tiotropium (Spiriva) (Tashkin et al., 2008), also is being pursued currently within a second class of bronchodilators, namely, the β2-AR agonists (Cazzola and Matera, 2008). Although there are a number of mechanisms by which duration of action may be extended, four in particular appear to be exemplified by β2-AR agonists: 1) avid biding to the receptor itself (i.e., “receptor kinetics”); 2) metabolic stability or low plasma clearance (i.e., “macrokinetics”); 3) plasma-lemma diffusion kinetics (i.e., “microkinetics”); and 4) local binding in the vicinity of the β-AR (i.e., exo site binding). The exo site theory, which was devised initially to explain the reassertion phenomenon observed with salmeterol (Ball et al., 1991; Johnson et al., 1993), postulates that the lipophilic

![Fig. 8. Persistence of action of β-AR agonists in CHO cells recombinantly expressing human β2-AR. CHO cells expressing human β2-AR were stimulated with isoprenaline (A), formoterol (B), salmeterol (C), and olodaterol (D) under control (filled circles) and washed (empty circles) conditions (see Materials and Methods for a detailed protocol description), then cAMP levels were quantified. Triplicate data points were used at each agonist concentration and expressed as percentage activity compared to 10 μM isoprenaline (as a reference full agonist) control wells on each plate. Data are shown as mean ± S.D. of a representative experiment out of three performed experiments, with each point performed in triplicate.](https://www.aspetjournals.org/atjournals/doi/10.1124/jpet.117.244837)

**Table 1**

<table>
<thead>
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<th>Agonist</th>
<th>Human β2-AR CHO Cells</th>
<th>Human β1-AR CHO Cells</th>
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<tr>
<td></td>
<td>pEC_{50} Control</td>
<td>pEC_{50} Washed</td>
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<tr>
<td>Isoprenaline</td>
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<td>9.39 ± 0.10</td>
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<tr>
<td>Olodaterol</td>
<td>10.00 ± 0.05</td>
<td>9.1 ± 0.04</td>
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N.T., not tested.
tail of salmeterol binds to a second site within the receptor (the so-called exo site), which functions to anchor the mole-
cule and provides persistent receptor activation. In contrast
to this hypothesis, Teschemacher and Lemoine (1999) dem-
Onstrated that in a cell membrane cAMP assay, the persist-
ence of action of salmeterol after washing was dependent on
the amount of salmeterol present in the solution (i.e., related
to depot formation), and Bergendal et al. (1996) demon-
strated that structural mimics of the side chain of salmeterol
failed to block the relaxation and extended duration of action
in guinea pig isolated trachea. These studies suggest that the
microkinetic theory (i.e., that duration is due to the lipid
solubility of the molecule and its ability to form a depot in the
membrane) might be a more appropriate model to interpret
salmeterol behavior. In line with these results, salmeterol
possesses a very high log D value (>2.5) and was shown to
significantly associate with liposomes resembling cellular
membranes in their lipid composition (Rhodes et al., 1992).

Differently from salmeterol, olodaterol shows a moderate
log D value (1.2), which reflects the chemical diversity of
these two molecules (Fig. 1). In line with its physicochemical
properties, olodaterol associates significantly with lipid bi-
layers only when high amounts are present in the equilib-
rating solution (Fig. 4). It is difficult to establish the relevance of
the microkinetic theory as rationale for the long duration of
action observed with olodaterol, because no data are cur-
cently available on local concentrations in the lungs after
inhalation. Olodaterol showed a significant dose-dependent
improvement in forced expiratory volume in one second at
doses ranging from 2 to 20 μg (Van Noord et al., 2009),
corresponding to 5 to 52 nmol, which are distributed on a
wide overall surface of the lungs (approximately 70 m²).
However, due to topical application, it is possible that in
some compartments the olodaterol concentration reaches lev-
els high enough for significant membrane association. As
such, the microkinetic theory cannot be fully dismissed at
this point in time. In any case, a second rationale, namely,
persistent interaction with the β2-AR, could be identified
when studying the kinetics of the olodaterol/human β2-AR
complex. Association was fast, in line with the rapid onset of
action measured in an acetylcholine-induced bronchocon-
striction model in guinea pigs (Bouyssou et al., 2010),
whereas dissociation showed a biphasic fitting.

Additional experiments performed in the presence of a
nonhydrolyzable analog of GTP indicated that the slow-dis-
sociating component is due to ternary complex formation
(i.e., a stabilized complex between the agonist, the receptor,
and the G protein). Because the ternary complex represents
the active, signaling moiety, a dissociation half-life of 17 h
(Fig. 5B) indeed might provide a rationale for olodaterol
duration of action in vivo. In our experiments performed in
CHO human β2-AR cells, which have a high receptor Bmax
and therefore a high receptor/G protein ratio, approximately
30 to 40% of the total human β2-AR pool was engaged in this
tight, slow-dissociating binding with olodaterol. However,
this is probably an underestimation of what would happen in
the airways, where the receptor/G protein ratio is likely to be
more even.

The tight agonist binding was confirmed in experiments
monitoring β2-AR reappearance after preincubation with olo-
daterol. In line with the dissociation kinetic experiments,
40% of the total human β2-AR pool showed tight binding to
olodaterol, and >24 h was necessary to reach the new equi-
librium (Fig. 6).

Similar results were obtained recently with 5-hydroxy-8-
[2-[2-(2-methylphenyl)-1,1-dimethyl-ethylamino]-1-hydroxy-
ethyl]-4H-benzol[1,4]oxazin-3-one (BI 167107), a close analog
of olodaterol (Rasmussen et al., 2011). Thanks to its high
affinity (84 pM) and dissociation kinetic profile (half-life lon-
ger than 40 h), BI 167107 was able to stabilize the active
state of the human β2-AR in the presence of a nanobody
mimicking Gα function, thus allowing crystallization. The
active-state crystal structure of the agonist-bound receptor
revealed rearrangements of the cytoplasmic ends of trans-
membrane segments 5, 6, and 7, which provide insights into
the process of agonist binding and activation (Rasmussen et
al., 2011).

Taken together, these biochemical data agree in showing a
long-lasting complex between agonist and G protein-coupled
receptor; however, they lack functional relevance, because
they can measure the presence of the complex but not its
signaling ability. To address this aspect, a functional cAMP
assay was devised to monitor the activity of the tightly bound
complex after extensive washout. Olodaterol showed signifi-
cant persistence of action at the human β2-AR (Fig. 8) that is
superior to that of twice-daily formoterol and similar to that
of salmeterol. Importantly, olodaterol achieved long-lasting
activation of human β2-AR with concentrations in the range
of 10⁻⁷ to 10⁻¹⁰ M and incubation volumes of 100 μl, result-
ing in low amounts of free olodaterol in solution even before
the extensive washout (0.01–10 pmol). Considering the low
propensity of olodaterol to interact with lipid membranes
unless exposed to high amounts (Fig. 4), it is highly unlikely that the persistent activation of human β2-AR is a consequence of depot formation for olodaterol, as outlined by the microkinetic theory. Instead, it is suggested that the specific tight binding to the human β2-AR and stabilization of the ternary complex are responsible for the persistent activation of the cAMP pathway. In support of this theory, olodaterol persistence at the human β2-AR is significantly higher (the EC50 shift is 10 times higher than the one measured at the human β2-AR) (Table 1), although the concentrations of olodaterol used (and as a consequence the free amount in solution) are higher due to its receptor subtype selectivity. Taken together, the data indicate that the persistent effects of olodaterol used (and as a consequence the free amount in solution) are higher due to its receptor subtype selectivity. Taken together, the data indicate that the persistent effects of olodaterol used (and as a consequence the free amount in solution) are higher due to its receptor subtype selectivity. Taken together, the data indicate that the persistent effects of olodaterol used (and as a consequence the free amount in solution) are higher due to its receptor subtype selectivity.

In conclusion, this study investigated in vitro different mechanistic rationales for the observed long duration of action of olodaterol in vivo. Our results indicate that olodaterol has a moderate propensity to accumulate in the lipid bilayer, and therefore the microkinetic theory cannot be dismissed fully. However, a second aspect, namely, the tight binding of olodaterol to the human β2-AR and formation of the ternary complex, was identified. To this end, binding, kinetic, and functional data support the existence of this complex, which, with a dissociation half-life of 18 h, indeed might be a rationale for the 24-h duration of action of olodaterol. Additional studies investigating the olodaterol/β2-AR interaction at the molecular level in, for example, the lung reperfusion model are deemed to establish the physiological relevance of these findings in the lung.

**Authorship Contributions**

- **Participated in research design:** Casarosa.
- **Conducted experiments:** Kollak, Kiechle, Ostermann, and Sieger.
- **Contributed new reagents or analytic tools:** Schnapp and Kiesling.
- **Performed data analysis:** Pieper.
- **Wrote or contributed to the writing of the manuscript:** Casarosa and Gantner.

**References**


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