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

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ABSTRACT

$\beta_2$-Adrenoceptor (\(\beta_2\)-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 $\beta_2$-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 olopatadine (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 olopatadine were analyzed (i.e., its lipophilicity and propensity to accumulate in the lipid bilayer as well as its tight binding to the $\beta_2$-AR). In line with its physicochemical properties, olopatadine is associated moderately with lipid bilayers. Instead, kinetic as well as equilibrium binding studies indicated the presence of a stable [3H]olodaterol/$\beta_2$-AR complex with a dissociation half-life of 17.8 h due to ternary complex formation. The tight binding of olopatadine to the human $\beta_2$-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 $\beta_2$-AR that, with a dissociation half-life >17 h, might indeed be a rationale for the 24-h duration of action of olopatadine.

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). $\beta_2$-Adrenoceptor ($\beta_2$-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. $\beta_2$-AR agonists act by binding to the $\beta_2$-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 $\beta_2$-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 Druy, 2002).

After the introduction of short-acting inhaled $\beta_2$-AR agonists such as salbutamol (Walde, 2002), progress in drug development has resulted in better tolerated, longer-acting $\beta_2$-AR-specific agents. In particular, two long-acting $\beta_2$-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-
tients (Perera, 2003). As a result, the U.S. Food and Drug Administration added a black-box warning to LABAs, stating that their use is contraindicated in the absence of an asthma controller medication, such as an inhaled corticosteroid. However, despite all of the concerns raised by the Salmeterol Multicenter Asthma Research Trial, inhaled β₂-AR agonists remain the most effective bronchodilators available for the immediate relief of asthma symptoms and, as such, an important component of asthma management.

Formoterol and salmeterol, although sharing a long duration of action that allows twice-daily administration in patients, differ in their onset of action and intrinsic activity. These pharmacological differences are mirrored in the chemical diversity of the two molecules (Fig. 1). Although long-acting bronchodilation by the formoterol was found by chance in clinical studies (Löfdahl and Svedmyr, 1989), salmeterol was the result of a specific research program to design long-acting drugs through increasing the lipophilicity of the β₂-AR agonist salbutamol (Johnson, 1995). Although LABAs have been on the market for several years, relatively little is known on the rationale(s) behind their long duration of action. Different hypotheses have been presented. On the basis of the resistance of [³H]formoterol to displacement by high concentrations of β₂-AR agonists or antagonists, it was postulated that the stability of the formoterol/β₂-AR complexes may contribute to its long-lasting therapeutic action (Lemoine, 1992). However, the prolonged duration of action of salmeterol, accompanied by the characteristic resistance to washout (a phenomenon termed “reassertion”) (Ball et al., 1991), led to the hypothesis that salmeterol binds to two sites of the β₂-AR: the classic active site interacting with the saligenin moiety of the compound and a second site within the β₂-AR, called the “exo site,” to which the hydrophobic tail of the molecule is supposed to bind quasi-reversibly. As a consequence of this hypothesis, the interaction of the hydrophobic tail with the exo site is thought to keep salmeterol in the vicinity of the receptor and to restore its action by flipping in and out of the active site after the withdrawal of the antagonists (Johnson et al., 1993). There is some evidence to support a defined region for the exo site in the human β₂-AR (Green et al., 1996), but it is not conclusive. Other studies (Teschemacher and Lemoine, 1999) have suggested instead the “diffusion microkinetic model” as rationale for salmeterol’s long duration of action on the basis of the high lipophilicity of the compound (Rhodes et al., 1992). The essential feature of the microkinetic model is that, after the inhalation of salmeterol, a bulk concentration of the drug enters the plasmalemma lipid bilayer of aSMCs and acts as an agonist depot even after the withdrawal of the drug. In this model, drug access to the active site of the β₂-AR occurs via lateral diffusion between the α helices into the receptor rather than via a direct approach from the extracellular aqueous biophase, thus accounting for the slow onset and long duration of action.

In this study, we focused on olodaterol (previously known as BI1744CL), a novel, chirally pure inhaled LABA (Bouyssou et al., 2010), currently in Phase III clinical trials for the treatment of COPD in patients. Olodaterol was identified as part of a program aimed at the discovery of selective β₂-AR agonists with the potential for once-daily administration, as shown in clinical studies in both asthma and COPD patients (O’Byrne et al., 2009; Van Noord et al., 2009).

In this study, we analyzed the ability of olodaterol to interact with lipophilic membranes as well as with the β₂-AR to identify potential rationales for its long duration of action shown in clinical trials.

**Materials and Methods**

**Chemicals and Reagents.** MgCl₂, isoprenaline hydrochloride, salbutamol hemisulfate, 2-[(3-carbamoyl-4-hydroxy)phenoxymethyl]phenoxymethylamino)-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]2-propanol dihydrochloride (CGP-20712A), 4-[(3-tetraarybutylamino-2-hydroxy-2-propoxy)benzimidazole-2-one hydrochloride (CGP12,177A), (±)-cyclopropyl-(S,S)-1-(1,1-dimethyl-3-(1H-inden-1-yl)oxy)-2-(3-[1-(1-methylthiethyl)]amino)-2-butanol hydrochloride, guanosine 5’-β,γ-imido)triphosphate trisodium salt hydrate (Gpp(NH)p), EGTA, Tris-HCl, NaCl, and HEPES were obtained from Sigma-Aldrich (St. Louis, MO). [³H]CGP12,177 was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Olodaterol hydrochloride and formoterol fumarate dihydrate were synthesized in the chemical laboratories of Boehringer Ingelheim (Biberach an der Riss, Germany). All of the cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

**Cell Culture Techniques and Membranes Preparation.** Chinese hamster ovary (CHO) cells stably transfected with the cDNA encoding the human β₂-AR or β₂-AR were described previously (Bouyssou et al., 2010).

Human aSMCs were purchased from PromoCell (Heidelberg, Germany), thawed at passage two, and used within the first 10 passages. Cells were maintained at 37°C in humidified air containing 5% CO₂.

Membrane isolation and purification from CHO cells stably expressing the human β₁-AR or β₂-AR as well as from mouse lung tissue were performed as described previously (Casarosa et al., 2005). In brief, cells were suspended in buffer A (15 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 0.3 mM EDTA, and 1 mM EGTA), homogenized, and spun down for 30 min at 48,000g. The pellet was resuspended in buffer B (7.5 mM Tris-HCl (pH 7.5), 12.5 mM MgCl₂, 0.3 mM EDTA, and 250 mM sucrose), divided into aliquots, and stored at −80°C until use. Likewise, lungs of BALB/c mice were homogenized (Ultra-Turrax T5 M; IKA-Werke, Staufen, Germany; 24,000 rpm) in ice-cold buffer A and centrifuged at 300g for 5 min. The supernatant was collected and centrifuged for 30 min at 40,000g. The sediment was resuspended in buffer B using a syringe. Protein content was measured with a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA).

**Binding Experiments.**

Generation and purification of [³H]olodaterol. The precursor 6-benzyloxy-β-2-[(3-2-bromo-4-methoxy-phenyl)-1,1-dimethylethylamino]-1-hydroxyethyl]-4H-benzo[1,4]oxazin-3-one (Fig. 2)
was dissolved in methanol, and PdC 105 was added to the solution; afterward, the reaction suspension was titrated in a special apparatus for tritium gas (Röö 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/nmol). The specific radioactive activity 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 MgCl$_2$, 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 2900TR 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 β$_2$-AR (20 μg per sample, adjusted according to the B$_{max}$ 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 β$_2$-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 IC$_{50}$ values obtained from the inhibition curves were converted to K$_i$ values using the method of Cheng and Prusoff (1973).

Kinetic studies of the interaction [3H]olodaterol/human β$_2$-AR. Parameters describing the association kinetics (K$_{on}$ values) for [3H]olodaterol/human β$_2$-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 β$_2$-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 K$_{on}$.

To determine dissociation kinetic parameters, membranes expressing the human β$_2$-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 β$_2$-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). K$_{off}$ rates obtained were transformed into t$_{1/2}$ values (dissociation half-life) using the following equation: t$_{1/2} = ln 2/K_{off}$.

pK$_a$ Value and Log D Profile Determination. The pK$_a$ 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 pK$_a$ 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 pK$_a$ values in the presence of n-octanol (apparent pK$_a$ values) in comparison to the pure aqueous pK$_a$ 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 β$_2$-AR and CHO human β$_2$-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\textsubscript{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\textsubscript{D}, K\textsubscript{off}, K\textsubscript{A}, pIC\textsubscript{50} or pEC\textsubscript{50} values) were obtained from each experiment and then averaged to provide mean data (± S.E.M.).

**Results**

**Generation and Characterization of [\textsuperscript{3}H]Olodaterol.**

To investigate the interactions of olodaterol with the human β\textsubscript{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\textsubscript{2}-AR. In these experiments, the B\textsubscript{max} obtained with [\textsuperscript{3}H]olodaterol (2.22 ± 0.08 pmol/mg) was not statistically different from the B\textsubscript{max} obtained with the β\textsubscript{2}-AR antagonist [\textsuperscript{3}H]CGP12,177 (2.13 ± 0.05 pmol/mg, n = 4 paired experiments), which is used classically as a nonselective 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\textsubscript{2}-AR (Green et al., 1995). This was confirmed by the displacement of [\textsuperscript{3}H]CGP12,177 with the selective β\textsubscript{1}-AR antagonist CGP-20712A: a monophasic displacement was found with a pK\textsubscript{A} value of 6.14 ± 0.05 (n = 2), representing its dissociation constant for the human β\textsubscript{1}-AR [reported pK\textsubscript{A} at the human β\textsubscript{2}-AR of 6.11 ± 0.05 (Baker, 2005)]. Similarly to results obtained with CHO human β\textsubscript{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 [\textsuperscript{3}H]CGP12,177 and [\textsuperscript{3}H]olodaterol, respectively, n = 2).

Because both β\textsubscript{1}-AR [mostly in the vasculature (Lemoine, 1992)] and β\textsubscript{2}-AR (in smooth muscle and epithelial cells) coexist in lung homogenates, B\textsubscript{max} obtained with the unselective [\textsuperscript{3}H]CGP12,177 is higher (Fig. 3A) compared to that obtained with [\textsuperscript{3}H]olodaterol, which, when tested at low concentrations (here up to 3 nM), selectively labels the receptor binding sites obtained with [\textsuperscript{3}H]CGP12,177 and [\textsuperscript{3}H]olodaterol in good agreement (Fig. 3A).

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

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

**Physicochemical Properties of Olodaterol and Potential for Interaction with Lipid Membranes.**

The p\textsubscript{K}\textsubscript{a} values of olodaterol were determined by a potentiometric and UV-metric titration approach as described under Materials and Methods. A p\textsubscript{K}\textsubscript{a} of 9.3 was obtained for the protonation of the secondary amine moiety, whereas a p\textsubscript{K}\textsubscript{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\textsubscript{2}-AR; Fig. 7), when tested at concentrations relevant for its interaction with the β\textsubscript{2}-AR (i.e., low nanomolar range; its EC\textsubscript{50} is 0.1 nM) (Fig. 4A) (Bouyssou et al., 2010). Likewise, unspecific binding of [\textsuperscript{3}H]olodaterol (approximately 3 nM in the presence of a large excess of the unselective 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 [\textsuperscript{3}H]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\textsubscript{2}-AR Indicate Fast Association and Biphasic Dissociation.**

As an alternative rationale to the microkinetic hypothesis, olodaterol interaction with the human β\textsubscript{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\textsubscript{2}-AR were incubated in the presence of three different concentrations of [\textsuperscript{3}H]olodaterol within each experiment. Attention was paid to select [\textsuperscript{3}H]olodaterol concentrations (in the range of 0.05–1.5 nM) at which binding to the human β\textsubscript{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\textsubscript{on} (7.64 ± 0.45 × 10\textsuperscript{7} M\textsuperscript{-1} min\textsuperscript{-1}, n = 3) illustrates fast association of [\textsuperscript{3}H] 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 [\textsuperscript{3}H]olodaterol/human β\textsubscript{2}-AR dissociation were studied at radioligand concentrations at which negligible unspecific binding to the membranes takes place.
(in the range of 0.1–1.5 nM) to ensure that our analysis exclusively reflects the ligand/receptor interactions. Analysis of the dissociation kinetics revealed a biphasic behavior ($K_{\text{off}}$ fast, $1.31 \pm 0.13$ h$^{-1}$; $K_{\text{off}}$ slow, $0.039 \pm 0.008$ 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 $[^{3}H]$CGP12,177 (2 nM) in the absence and presence of the $\beta_{1}$-AR-selective antagonist CGP-20712A (1 μM; white and gray bars, respectively) and with $[^{3}H]$olodaterol (3 nM; black bar). $B_{\text{max}}$ values (fmol/mg) were obtained by transforming the specific binding obtained under each condition [i.e., the difference between total binding and unspecific binding in the presence of a large excess of nonradioactive CGP12,177A (10 μM)]. Data are shown as mean ± S.E.M. for two independent paired experiments, each performed in triplicate. The data were analyzed by using one-way analysis of variance followed by Bonferroni’s multiple-comparison test. Statistical significance is denoted compared with the $[^{3}H]$CGP12,177 group ($p < 0.05$). **, $p < 0.05$; **, $p < 0.01$. N.S., not statistically significant. B, pH-dependent lipophilicity profile of olodaterol. $pK_{a}$ values and log D profiles of olodaterol were determined as described in detail under Materials and Methods.

**Fig. 3.** A, comparison of binding profiles of $[^{3}H]$CGP12,177 and $[^{3}H]$olodaterol in mouse lung homogenates. Purified membranes obtained from mice lungs were incubated with $[^{3}H]$CGP12,177 (2 nM) in the absence and presence of the $\beta_{1}$-AR-selective antagonist CGP-20712A (1 μM; white and gray bars, respectively) and with $[^{3}H]$olodaterol (3 nM; black bar). $B_{\text{max}}$ values (fmol/mg) were obtained by transforming the specific binding obtained under each condition [i.e., the difference between total binding and unspecific binding in the presence of a large excess of nonradioactive CGP12,177A (10 μM)]. Data are shown as mean ± S.E.M. for two independent paired experiments, each performed in triplicate. The data were analyzed by using one-way analysis of variance followed by Bonferroni’s multiple-comparison test. Statistical significance is denoted compared with the $[^{3}H]$CGP12,177 group ($p < 0.05$). **, $p < 0.05$; **, $p < 0.01$. N.S., not statistically significant. B, pH-dependent lipophilicity profile of olodaterol. $pK_{a}$ values and log D profiles of olodaterol were determined as described in detail under Materials and Methods.
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, β2-AR agonists recognize two interconvertible states of the β2-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 β2-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 β2-AR and to convert all of the /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 β2-AR complex was fitted best by a monophasic equation with a /H11005 K /H11006 off of 2.04 ± 0.18 h /H11002 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 β2-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 β2-AR complex. Given that /H9262 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 Kd). The Kd 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 Kd = Koff/Kon (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 (Kd = 1.07 ± 0.4 nM, n = 3), although Bmax 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 Kd (Kd = 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 Goαs-coupled β2-AR. Because β2-AR/Goαs 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 phenoterol 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 \( \beta_2 \)-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 \( \beta \)-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 the concentration-response curve after washout without changes in its maximal efficacy (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 \( \beta_2 \)-AR.

To investigate whether the long-lasting effect observed with salmeterol and olodaterol is due to a stable complex with the human \( \beta_2 \)-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 \( \beta_2 \)-AR.

After washout at the human \( \beta_1 \)-AR, the concentration-response curve of isoprenaline was shifted significantly to the right compared with the control curves (Fig. 9A).

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 \( \beta_2 \)-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 \( \beta_2 \)-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 (Bender, 2002). Therefore, a long duration of action (preferably 24 h) is an important feature for drugs intended to treat chronic diseases, enabling both prolonged efficacy and a simple, once-daily dosing regimen that improves patient compliance (Tamura and Ohta, 2007). 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 \( \beta_2 \)-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 \( \beta_2 \)-AR agonists: 1) avid binding to the receptor itself (i.e., “receptor kinetics”); 2) metabolic stability or low plasma clearance (i.e., “macrokinetics”); 3) plasma-membrane diffusion kinetics (i.e., “microkinetics”); and 4) local binding in the vicinity of the \( \beta \)-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

### Table 1

Potency, efficacy, and persistence of action of \( \beta \)-AR agonists in CHO cells recombinantly expressing human \( \beta_1 \)-AR and \( \beta_2 \)-AR

The average of three independent experiments performed in triplicate is shown. The rightward shift (EC\(_{50}\) shift) was calculated by dividing the washed EC\(_{50}\) by the unwashed EC\(_{50}\).

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Human ( \beta_2 )-AR CHO Cells</th>
<th>Human ( \beta_1 )-AR CHO Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC(_{50}) Control</td>
<td>EC(_{50}) Washed</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>8.88 ± 0.04</td>
<td>6.15 ± 0.07</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>8.37 ± 0.06</td>
<td>5.68 ± 0.08</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>7.15 ± 0.11</td>
<td>5.11 ± 0.15</td>
</tr>
<tr>
<td>Formoterol</td>
<td>9.83 ± 0.08</td>
<td>7.81 ± 0.05</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>10.11 ± 0.07</td>
<td>9.39 ± 0.10</td>
</tr>
<tr>
<td>Olodaterol</td>
<td>10.00 ± 0.05</td>
<td>9.1 ± 0.04</td>
</tr>
</tbody>
</table>

N.T., not tested.
expressed as percentage activity compared to 10–7 M isoprenaline (as 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.

**Materials**

**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 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.

The tight agonist binding was confirmed in experiments monitoring β2-AR reappearance after preincubation with olodaterol. 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 equilibrium (Fig. 6).

Similar results were obtained recently with 5-hydroxy-8-[2-[2-(2-methylphenyl)-1,1-dimethyl-ethylamino]-1-hydroxy-ethyl]-4H-benzo[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 longer than 40 h), BI 167107 was able to stabilize the active state of the human β2-AR in the presence of a nanobody mimicking Gs function, thus allowing crystallization. The active-state crystal structure of the agonist-bound receptor revealed rearrangements of the cytoplasmic ends of transmembrane 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 significant 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, resulting 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 inferior (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 at the β2-AR are specific to that receptor and reflect the kinetic behavior of this complex. However, functional data obtained with salmeterol suggest that membrane loading seems to play a more dominant role, in agreement with the microkinetic theory.

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.

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