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Functional and biochemical rationales for the 24-hour long duration of action of olodaterol

Paola Casarosa, Ines Kollak, Tobias Kiechle, Angela Ostermann, Andreas Schnapp, Ralf Kiesling, Michael Pieper, Peter Sieger, and Florian Gantner.

Dept. of Respiratory Diseases Research (P.C., I.K., T.K., A.O., A.S., M.P. and F.G.), Drug Metabolism & Pharmacokinetics (R.K.) and Drug Discovery Support (P.S.), Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany

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Running Title: dissociation profile of the olodaterol/ß2-AR complex

Corresponding author: Dr. Paola Casarosa

Contact Information: Dept. of Respiratory Diseases Research, BI Pharma GmbH &
Co. KG, Birkendorferstrasse 65, Biberach an der Riss, Germany

E-mail: paola.casarosa@boehringer-ingelheim.com

Phone: +49-7351-54-93196
Fax: +49-7351-83-93196

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Abstract

β2-adrenoceptor (AR) agonists are powerful bronchodilators and play a pivotal role in the management of pulmonary obstructive diseases like asthma and COPD. Whereas these agents were first used many years ago, progress in drug development has resulted in better tolerated, long-acting β2-AR-agonists (LABAs), like formoterol and salmeterol. Although LABAs are successfully present on the market since several years, still relatively little is known on the rationale(s) behind their long duration of action. In this study we focussed on olodaterol (previously known as BI1744CL), a novel inhaled LABA, which provides a bronchodilating effect lasting 24 hr 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 /β2AR complex with a dissociation half life of 17.8 hr, due to the ternary complex formation. The tight binding of olodaterol to the hβ2-AR and stabilization of the ternary complex were confirmed in functional experiments monitoring adenylyl cyclase activity following extensive wash-out. Taken together, both binding, kinetic and functional data support the existence of a stable complex with the β2AR which, with a dissociation half life > 17 hours, might indeed be a rationale for the 24 hour 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 like asthma and COPD. β₂-AR agonists act by binding to the β₂-AR, a member of the G-protein-coupled family of receptors (GPCRs), which is present in high density in airway smooth muscle cells (aSMC). Activation of the h β₂-AR in aSMC induces increased levels of cyclic adenosine monophosphate (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 adrenoceptor 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 β Agonists (LABAs) which, 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.

Recently, concerns were raised about LABA treatment as monotherapy for asthma: for example, the salmeterol multi-centre asthma research trial (SMART) found more asthma deaths and life-threatening events in the salmeterol-treated patients (Perera,
2003), As a result, FDA added black box warning to LABAs, stating that their use is contraindicated in the absence of an asthma controller medication such as inhaled corticosteroid. However, despite all of the concerns raised by the SMART, inhaled beta2-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 the long duration of action, which 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). Whereas the long-acting bronchodilation by formoterol was found by chance in clinical studies (Lofdahl and Svedmyr, 1989), salmeterol was the result of a specific research program to design long-acting drugs through increasing lipophilicity of the β2-AR agonist salbutamol (Johnson, 1995). Although LABAs are successfully present on the market since several years, still relatively little is known on the rationale(s) behind the long duration of action. Different hypotheses have been presented in time. Based on the resistance of [3H]-formoterol to displacement by high concentrations of β agonists or antagonists, it was postulated that the stability of the formoterol/β2AR complexes may contribute to its long-lasting therapeutical action (Lemoine, 1992). On the other hand, the prolonged duration of action of salmeterol, accompanied to 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 β2AR: 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-irreversibly. 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 withdrawal of antagonists (Johnson, et al., 1993). There is some evidence to support a defined region for the exosite in the hß2-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 airway smooth muscle cells and acts as an agonist depot even after withdrawal of the drug. In this model, drug access to the active site of the ß2-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 focussed on olodaterol (previously known as BI 1744 CL), a novel, chirally pure inhaled long acting ß2 agonist (Bouyssou, et al., 2010), currently in phase III clinical trials for the treatment of COPD patients. Olodaterol was identified as part of a program aimed at the discovery of selective ß2 adrenoceptor agonists with potential for once-daily administration, as shown in clinical studies both in asthma and COPD patients (O’Byrne, et al., 2009; Van Noord JA, et al., 2009).

Here, we analyzed the ability of olodaterol to interact with lipophilic membranes as well as with the ß2-AR in order to identify potential rationales for its long duration of action shown in clinical trials.
Methods

Chemicals and reagents

MgCl₂, isoprenaline hydrochloride, salbutamol hemisulphate, CGP-20712A methanesulfonate, (±)-CGP-12177A hydrochloride, ICI 118,551 hydrochloride, guanosine 5’-[β,γ-imido] triphosphate trisodium salt hydrate (Gpp(NH)p), EGTA, Tris-HCl, NaCl and HEPES were obtained from Sigma (St. Louis, MO). [³H]-CGP12,177 was obtained from Perkin Elmer (Waltham, MA). Olodaterol hydrochloride and formoterol fumarate dihydrate were synthesized in the chemical laboratories of Boehringer Ingelheim, Biberach an der Riss, Germany. All cell culture reagents were purchased from GIBCO (Invitrogen, Carlsbad, CA).

Cell culture techniques and membranes preparation

Chinese hamster ovary (CHO) cells stably transfected with the cDNA encoding the human β₁- or β₂- adrenoceptors were previously described (Bouyssou, et al., 2010). Human airway smooth muscle cells (aSMC) were purchased from Promocell (Cat. No: C-12561), thawed at passage 2 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 β₁-2 -ARs as well as from mouse lung tissue was performed as described previously (Casarosa, et al., 2005). Briefly, cells were suspended in buffer A (15 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 0.3 mM EDTA, 1 mM EGTA), homogenized, and span down for 30 min at 48,000 x g. The pellet was resuspended in buffer B (7.5 mM Tris-HCl, pH 7.5, 12.5 mM MgCl₂, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose), aliquoted, and stored at –80 °C until use. Similarly, lungs of BALB/c mice were homogenized (Ultra-Turrax T5 M, IKA-Werke, Staufen, Germany, 24000 rpm) in ice cold buffer A and
centrifuged at 300 x g for 5 min. The supernatant was collected and centrifuged for 30 min at 40,000 x g. The sediment was resuspended in buffer B using a syringe. Protein content was measured with the BCA kit (Pierce, Thermo Fischer Scientific, Rockford, IL).

**Binding experiments**

- **Generation and purification of 3H-olodaterol**

  The precursor 6-benzyloxy-8-[2-[2-(2-bromo-4-methoxy-phenyl)-1,1-dimethyl-ethylamino]-1-hydroxyethyl]-4H-benzo[1,4]oxazin-3-one (see figure 2) was dissolved in methanol and Pd/C 105 was added to the solution; afterwards the reaction suspension was tritiated in a special apparatus for tritium gas (RC Tritec, Switzerland). After evaporating and dissolving several times and purifying by HPLC, the one-stage-synthesis yielded 50 mCi (1850 MBq) \( [3^H] \)-olodaterol with the 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 and of a solution with known concentration. Determined by HPLC the radiochemical purity was > 98 %. Chromatographic data for HPLC (for purification and analysis):

  HPLC-column: Waters, XBridge C-8 (5 µm), 4.6 x 150 mm; Mobile phase: A: water 0.05% TFA; B: MeCN 0.05% TFA; Flow rate: 1 ml/min; UV-detection: 254 nm; Temperature: 30°C.

- **Equilibrium binding experiments**

  In all radioligand experiments the binding buffer consisted of Tris-HCl 50 mM, MgCl\(_2\) 2 mM, EGTA 1 mM, pH 7.3. After the indicated incubation period, bound and free \( [3^H] \)-olodaterol or \( [3^H] \)-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 from Perkin Elmer) in pony-vials and radioactivity was quantified using liquid scintillation spectrometry on a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer). In all experiments, total binding never reached 10% of that added, limiting complications associated with depletion of the free radioligand concentration.

Saturation binding experiments were performed by incubating membranes expressing the hß2-AR (20 µg/ sample, adjusted according to the B$_{\text{max}}$ 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 non-hydrolizable GTP analog GppNHp was added (final concentration 10 µM), to induce receptor / G protein dissociation.

To ascertain which ß-AR subtypes are expressed in aSMCs, heterologous competition experiments against [³H]-CGP12,177 were performed with the ß1-selective antagonist CGP-20712A. Cells (150,000 / sample) were incubated in the presence of [³H]-CGP12,177 (final concentration approximately 1 nM) and different concentrations of unlabelled antagonist at room temperature with gentle agitation for 2 hours 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 & Prusoff (Cheng and Prusoff, 1973).

- **Kinetic studies of the interaction [³H]-olodaterol / hß2AR**

Parameters describing the association kinetics (K$_{\text{on}}$ values) for [³H]-olodaterol/ hß2-AR were determined by adding at time = 0 [³H]-olodaterol (3 different concentrations per experiment, in the range 0.05 to 1.5 nM) to membranes obtained from CHO-hß2AR cells (20 µg/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 minutes) samples (triplicates per each radioligand concentration) were filtered and radioactivity due to receptor association was quantified. Data were globally fit using the Prism equation “Association - Two or more conc. of hot” which derives a single best-fit estimate for $k_{on}$.

To determine dissociation kinetic parameters, membranes expressing the hß2-AR (20 µg/sample) were first allowed to equilibrate with multiple concentrations of [³H]-olodaterol (usually in the range 0.5 to 1.5 nM) at room temperature for at least 2 hours (300 µl per sample). Afterwards, samples were added to test tubes already containing 4 ml of binding buffer with cold olodaterol $10^{-5}$M (time 0), in order 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 fully dissociated from the hß2-AR receptor. Dissociation data were analyzed with GraphPad Prism, allowing the program to compare with the extra-sum-of-square F test the best fit between the equations “Dissociation: one phase exponential decay” vs “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:

(equation 1) $t_{1/2} = \ln(2)/K_{off}$

**pK$_a$-value and log D – profile determination**

The pK-values of olodaterol were determined by a potentiometric and/or UV-metric titration approach using the GlpKa/DPAS equipment from Sirius Analytical. Due to the lower solubility of the free base at high pH-values, the usage of an organic co-solvent (methanol) was indicated. A multiset titration with three different methanol contents was performed. The pK-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 pH 2 - 12. From the shifts of the pKa - values in the presence of n-octanol (apparent pKa – values) in comparison to the pure aqueous pKa – 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-hß1,2 cells using Lance technology (Perkin Elmer), 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/well in growth media containing 1% FBS. The following day a cAMP-wash out assay was performed, in which all the different steps were carried out at RT in medium consisting of Hank’s balanced salt solution (HBSS) with 0.1% BSA and 5 mM HEPES (pH 7.4).

Cells were treated with either of the following protocols:

(i) cells (referred to as ‘washed’ cells) were stimulated with a range of concentrations of β-adrenoceptor agonist in medium for 30 min, then washed for 1 hour (with medium exchanged every 10 mintues). After this washing step, cells were further incubated with medium containing 0.5 mM IBMX (isobutyl methylxanthine, a non-selective phophodiesterase inhibitor) in the absence of any added β agonist for 30 minutes. At the end of this incubation, the extracellular medium was discarded and intracellular levels of cAMP were quantified;

(ii) cells (referred to as control cells) were incubated with medium alone for 30 min, washed for 1 hour (with medium exchanged every 15 mintues), then stimulated with
a range of concentrations of β-adrenoceptor agonist in the presence of 0.5 mM IBMX for 30 minutes. At the end of this incubation, intracellular levels of cAMP were quantified.

In both cases cells were only ever treated with a single concentration range of β-adrenoceptor agonist: either before (referred to as washed cells) or after (control cells) the washing step. For each β agonist, the EC₅₀ 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 cells were washed the same number of times.

Data analysis

All experiments were analysed by either linear or non-regression analysis with the equations mentioned under the different assay methodology using Prism version 5.02 (GraphPad Software, San Diego, CA). Individual estimates (either Kᵋ, Kᵋᵋ, Kᵋᵋᵋ, pIC₅₀ or pEC₅₀ values) were obtained from each experiment and then averaged to provide mean data (± S.E.M).
Results

Generation and characterization of [³H]-olodaterol. In order to investigate the interactions of olodaterol with the human β2-AR as well as with lipidic membranes, a radioligand was generated as described in the material and method section and illustrated in figure 2.

To characterize the new radioligand, initial experiments were performed with membranes obtained from CHO cells selectively over-expressing the β2-AR. In these experiments, the Bₘₐₓ obtained with [³H]-olodaterol (2.22 ± 0.08 pmol/mg) was not statistically different from the Bₘₐₓ obtained with the β antagonist [³H]-CGP12,177 (2.13 ± 0.05 pmol/mg, n= 4 paired experiments), which is classically used as a non-selective radioligand for β receptors.

Similarly, radioligand binding experiments were performed with human airway smooth muscle cells and in purified membranes obtained from mouse lung homogenates (fig 3). Human aSMCs are reported to contain exclusively β₂-ARs (Green, et al., 1995). This was confirmed by displacement of [³H]-CGP12,177 with the selective β₁ antagonist CGP20,712A: a monophasic displacement was found with a pKᵢ value of 6.14±0.05 (n=2), representing its dissociation constant for the hβ₂-AR (reported pKᵢ at the hβ₂-AR: 6.11±0.05 (Baker, 2005)). Similarly to results obtained with CHO-hβ₂AR cells, the maximal number of receptor binding sites obtained in aSMCs with the 2 radioligands are in good agreement (10.304 vs 10.863 sites/cell with [³H]-CGP12,177 and [³H]-olodaterol, respectively, n=2).

As both β₁-ARs (mostly in the vasculature,(Lemoine, 1992)) as well as β₂-ARs (in smooth muscle and epithelial cells) coexist in lung homogenates, Bₘₐₓ obtained with the unselective [³H]-CGP12,177 is higher (figure 3A) compared to [³H]-olodaterol,
which, tested at low concentrations (here up to 3 nM), selectively labels the β₂-AR component (selectivity over hβ₁-AR is above 200 fold, (Bouyssou, et al., 2010)). In the presence of the β₁-selective antagonist CGP20,712A (1 µM), the maximal number of receptor binding sites obtained with [³H]-CGP12,177 and [³H]-olodaterol are in good agreement (fig 3A).

Addition of the non-hydrolyzable GTP analog guanosine-5′-[(βγ-imino)triphosphate (GppNHp), did not affect B_max values obtained with either [³H]-CGP12,177 or [³H]-olodaterol in any of the described settings (see afterwards and figure 7).

Taken together, these results indicate that [³H]-olodaterol is able to label the whole β₂-AR population expressed at the cellular membrane independently from receptor association to G proteins, similarly to the antagonist [³H]-CGP12,177.

**Phisico-chemical properties of olodaterol and potential for interaction with lipidic membranes.**

The pK-values of olodaterol were determined by a potentiometric and UV-metric titration approach as described in the materials and methods section. A pK_a of 9.3 was obtained for the protonation of the secondary amine moiety, whereas a pK_a 10.1 reflected the deprotonation of the phenolic function.

The apparent partition coefficient was then determined with different mixtures of aqueous solution and n-octanol over the range pH 2 - 12. The obtained profile is depicted in Figure 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 hβ₂-ARs, cfr fig.7), when tested at concentrations
relevant for its interaction with the β2-AR (i.e. low nanomolar range; its EC₅₀ is 0.1 nM, ref.) (Figure 4A). Similarly, unspecific binding of [³H]-olodaterol (approximately 3 nM in the presence of a large excess of the unselective β- antagonist CGP12,177) to aSMCs as well as in mouse lung homogenates was approximately 10% of total binding (8.8 and 11.2%, respectively). In order to observe any accumulation in the lipidic bilayer, the amount of membranes (150 µg/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 pmoles), olodaterol showed significant association with cellular membranes and linearity over increasing radioligand concentrations (figure 4B). However, also with these conditions the percentage of olodaterol bound to the lipidic membranes is less than 1% of free olodaterol in the aqueous solution at any concentration tested, in line with its moderate logD value.

**Olodaterol kinetics of interaction with the β₂-AR indicate fast association and biphasic dissociation**

As an alternative rationale to the microkinetic hypothesis, olodaterol interaction with the hβ₂-AR, as well as the stability of the ligand/receptor complex were investigated. For association kinetic studies, membranes obtained from CHO cells expressing the hβ₂-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 to 1.5 nM) at which binding to the hβ₂-AR is significant (i.e. 20 to 80% receptor is bound, see figure 5A) but negligible unspecific partitioning in the membranes takes place, in order to analyze a simple model of receptor-ligand interaction.
The kinetics of association followed a monoexponential relation and were fitted by nonlinear regression to the equation: “Association - Two or more conc. of hot” (see material and method section). The parameter estimate of $K_{on}$ ($7.64 \pm 0.45 \times 10^7 \text{M}^{-1} \text{min}^{-1}$, $n=3$) illustrates fast association of [³H]-olodaterol, which well correlates with the reported fast onset of action of olodaterol in an in vivo model of ACh-induced bronchoconstriction (Bouyssou, et al., 2010).

Similarly, the kinetics of [³H]-olodaterol / hß2-AR dissociation were studied at radioligand concentrations at which negligible unspecific binding to the membranes takes place (in the range 0.1 to 1.5 nM), to ensure that our analysis exclusively reflects the ligand / receptor interactions. Analysis of the dissociation kinetics revealed a biphasic behaviour (Fig 5B; Koff fast: $1.31 \pm 0.13 \text{h}^{-1}$; Koff slow: $0.039 \pm 0.008 \text{h}^{-1}$, $n=4$), with approx. 30 to 40% of the receptor pool showing very slow dissociation. Half-times ($t_{1/2}$) of 32 minutes (fast dissociation $t_{1/2}$) and 17.8 hours (slow dissociation $t_{1/2}$) were calculated according to equation (1). Given the long time necessary to achieve complete dissociation of the [³H]-olodaterol/hß2-AR complex (up to 50 hours), 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 a further proof for the existence of this stable long lasting complex between ß2-AR and olodaterol, membranes expressing the hß2-AR were preincubated with non-labeled olodaterol (3 nM) resulting in a receptor occupation greater than 90% (cfr saturation studies in figure 7). Small samples of pretreated membranes (50 µl) were diluted by adding 4 ml of binding buffer containing 3 nM [³H]-CGP12,177, thereby combining the dissociation of cold olodaterol by dilution (80-fold) with the association of an excess of competing radioligand. At different time points, membranes were filtered and radioactivity quantified (Fig. 6). According to the model built with the
The β2AR-G protein complex is responsible for the slow dissociation component of [³H]-olodaterol

In contrast to antagonists, βAR-agonists recognize two interconvertible states of the β2-AR, depending on the presence or absence of the G protein associated to the receptor (high- and low-affinity state, respectively). Keeping in mind that olodaterol is indeed a β2-agonist with an intrinsic activity close to full agonism (IA 88%, (Bouyssou, et al., 2010)), we tested whether the biphasic dissociation observed depended on two different species of [³H]-olodaterol / hβ2-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 non-hydrolyzable GTP analog Gpp(NH)p was used to induce dissociation of the G protein from the hβ2-AR and to convert all β2 receptors to the low-affinity, G protein-free binding state for agonists. Indeed, in the presence of Gpp(NH)p the dissociation of [³H]-olodaterol / hβ2-AR complex was best fitted by a monophasic equation with a Koff= 2.04 ± 0.18 h⁻¹, n=3 (dissociation t½ of 20.2 mins), resembling the fast component seen before. Taken together, these data indicate that
the slow dissociation observed with $[^3\text{H}]$-olodaterol reflects its interaction with the $\beta_2$-AR coupled to the G$\alpha$s protein, i.e. the signaling complex in its active state.

**Saturation experiments with $[^3\text{H}]$-olodaterol**

Kinetic studies indicate the presence of one association and two dissociation constants for the $[^3\text{H}]$-olodaterol /h$\beta_2$-AR complex. Given that $K_d = k_{off}/k_{on}$, this should imply the presence of two different equilibrium affinity constants. To confirm this, saturation experiments were performed in h$\beta_2$AR-expressing CHO membranes, comparing $[^3\text{H}]$-olodaterol and $[^3\text{H}]$-CGP12,177, used as a control (fig 7). As expected, analysis of $[^3\text{H}]$-olodaterol binding data indicated the model with two affinity constants as best fitting model ($p \leq 0.001$ vs 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 nM 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 $[^3\text{H}]$-olodaterol ($K_d = 1.07\pm 0.4$ nM, n=3), although Bmax was not affected (fig. 7B). The loss of high affinity binding site is in line with the loss of slow dissociation component observed in the kinetic experiments.

As expected, saturation data obtained with $[^3\text{H}]$-CGP12,177 were best fit by a model with a single $K_d$ ($K_d = 0.04\pm 0.01$ nM, n=3, fig.7C) and results did not vary in the presence of GTP (data not shown).

**CAMP measurements for stability of 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 $G_\alpha$ coupled $\beta_2$-AR. Because the $\beta_2$AR-$G_\alpha$ 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/ $\beta_2$ adrenoceptor signaling complex, a “wash-out” cAMP assay was devised. Briefly, cells were incubated 30 mins with $\beta_2$-agonists to allow binding to the $\beta_2$-AR, then a procedure consisting of 6 consecutive washing steps, of 10 mins each, was performed to remove any $\beta_2$ agonist not bound to cells. After this extensive and repetitive washing, adenylate cyclase (AC) activity in the cells was used as a measure of persistent occupancy of $\beta_2$-AR by agonists. Persistence of agonist action was then expressed as a concentration ratio by comparing the response curves to agonists in ‘washed’ versus control cells (i.e. cells which were stimulated with the $\beta_2$-agonist without any wash-out afterwards).

In order to validate this assay, several $\beta$ agonists were tested. As expected, hydrophilic compounds like isoprenaline, salbutamol and phenoterol showed significant rightward shifts in their concentration response curves following washing (Table 1 and exemplified by isoprenaline in Fig. 8A). This indicates that these compounds readily wash out of the $\beta_2$ adrenoceptor and thus have a very short duration of action. These results also prove that the extensive wash-out procedure is sufficient to eliminate cAMP generated in the cells during the first incubation with $\beta$ agonists, probably through active secretion in the extracellular medium and metabolism (PDE activity is not blocked, since no inhibitor is present during the first incubation and washing steps) and validates the measurement of AC activity after wash-out as a proof of agonist persistance.
Compounds with 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 following washout without changes in its maximal efficacy (fig. 8B). The concentration response relationships following washout for salmeterol (5.2 fold) and olodaterol (8 fold) were less shifted to the right in comparison to the control curves, (Fig. 8C and D) indicating persistent activation of the β2 adrenoceptor.

To investigate whether the long lasting effect observed with salmeterol and olodaterol is due to a stable complex with the hß2-AR resulting from slow receptor/ligand dissociation, and therefore specific for this receptor subtype, or a consequence of unspecific interaction with lipidic membranes and drug-depot formation, similar washout experiments were performed with CHO cells expressing the hß1-AR.

Following washout at the hß1-AR, the concentration response curve of isoprenaline was shifted significantly to the right compared to the control curves (Fig. 9A).

Salmeterol and olodaterol showed this time a different behavior: whereas for salmeterol wash-out induced a small shift (14 fold, fig.9B), similarly to what observed with hß2-AR, olodaterol shift was 10 times more pronounced (87 fold, see table 1 and figure 9C).

Therefore, the data indicate that the persistent effects of olodaterol at the β2 adrenoceptor 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 a multiple treatment regimen, and simplified dosing regimens are known to improve compliance (Bender, 2002). Therefore, long duration of action (preferably 24 hours) is an important feature of drugs intended to treat chronic diseases, enabling both prolonged efficacy and a simple, once-daily dosing regime that improves patient compliance (Tamura and Ohta, 2007). This strategy, which has been proven successful with the long acting muscarinic antagonist (LAMA) tiotropium (Spiriva®) (Tashkin, et al., 2008), is currently being pursued also within a second class of bronchodilators, namely the β2 adrenoceptor agonists (Cazzola and Matera, 2008).

While there are a number of mechanisms by which duration of action may be extended, four in particular appear to be exemplified by β2 adrenoceptor agonists: (1) avid biding to the receptor itself, i.e. ‘receptor kinetics’; (2) metabolic stability or low plasma clearance, i.e. ‘macrokinetics’; (3) plasmalemma diffusion kinetics, i.e. ‘microkinetics’; and (4) local binding in the vicinity of the β-adrenoceptor, i.e. ‘exosite binding’. The exosite theory, which was initially devised to explain the reassertion phenomenon observed with salmeterol (Ball, et al., 1991;Johnson, et al., 1993), postulates that the lyophilic tail of salmeterol binds to a second site within the receptor (the so-called exosite), which functions to anchor the molecule and provides the persistent receptor activation. In contrast to this hypothesis, Teschemacher and Lemoine (Teschemacher and Lemoine, 1999) demonstrated that in a cell membrane cAMP assay the persistence of action of salmeterol following washing was
dependent on the amount of salmeterol present in the solution (i.e. related to depot formation) and Bergendal et al. (Bergendal, et al., 1996) demonstrated that structural mimics of the side chain of salmeterol failed to block the relaxation and extended duration of action in the 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 behaviour. In line with these results, salmeterol possesses a very high logD (above 2.5) and was shown to significantly associate with liposomes resembling cellular membranes for 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 (see fig 1). In line with its physicochemical properties, olodaterol associates significantly with lipid bilayers only when high amounts are present in the equilibrating solution (cfr. fig. 4). It is difficult to establish the relevance of the microkinetic theory as rationale for the long duration of action observed with olodaterol, since no data are currently available on local concentrations in the lungs following inhalation. Olodaterol showed significant dose-dependent improvement in through FEV₁ at doses ranging from 2 to 20 µg (Van Noord JA, et al., 2009), corresponding to 5 to 52 nmoles, which are distributed on a wide overall surface of the lungs (approx. 70 square meters). However, due to the topical application, it is possible that in some compartments olodaterol concentration reaches levels high enough for a 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/hβ₂-AR complex. Association was fast, in line with the rapid onset of action measured in an acetylcholine-induced
bronchoconstriction model in guinea pigs (Bouyssou, et al., 2010), whereas dissociation showed a biphasic fitting.

Additional experiments performed in the presence of a non-hydrolizable analog of GTP indicated that the slow dissociating component is due to the 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 hours (cfr. figure 5B) might indeed provide a rationale for olodaterol duration of action in vivo. In our experiments performed in CHO-hβ2AR cells, which have a high receptor B_{max} and therefore a high receptor/G protein ratio, approximately 30 to 40% of the total hβ2-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 following preincubation with olodaterol. In line with the dissociation kinetic experiments, 40% of the total hβ2-AR pool showed tight binding to olodaterol and more than 24 hours were necessary to reach the new equilibrium (fig. 6).

Similar results were recently obtained with 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 hours), BI 167107 was able to stabilize the active state of the h β2AR in the presence of a nanobody mimicking Gs function, allowing crystallization. The active-state crystal structure of the agonist-bound receptor revealed rearrangements of the cytoplasmic end of transmembrane segments 5, 6 and 7 which are providing 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, as 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 following extensive wash-out. Olodaterol showed significant persistence of action at the hß2-AR (fig.8), superior to the twice-daily formoterol and similar to salmeterol. Importantly, olodaterol achieved long-lasting activation of hß2-AR with concentrations in the range 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 to 10 pmoles). Considering the low propensity of olodaterol to interact with lipidic membranes unless exposed to high amounts (figure 4), it is highly unlikely that the persistent activation of hß2-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 hß2-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 hß1-AR is significantly inferior (the EC_{50} shift is 10 times higher than the one measured at the hß2-AR, see table 1), although olodaterol’s used concentrations (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 adrenoceptor are specific to that receptor, and reflect the kinetic behaviour of this complex. On the other hand, 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
Oldolaterol has a moderate propensity to accumulate in the lipid bilayer, and therefore the microkinetic theory cannot be fully dismissed. However a second aspect, namely the tight binding of oldolaterol to the hß2-AR and formation of the ternary complex, was identified. To this end, both binding, kinetic and functional data support the existance of this complex which, with a dissociation half life of 18 hours, might indeed be a rationale for the 24 hour duration of action of oldolaterol. Additional studies investigating the oldolaterol /ß2AR interaction at the molecular level in e.g. 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, Sieger.
Contributed new reagents or analytic tools: Schnapp, Kiesling.
Performed data analysis: Pieper.
Wrote or contributed to the writing of the manuscript: Casarosa, Gantner.
Reference List


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.


Legends for Figures

Fig. 1. Chemical structures of salmeterol (A), formoterol (B) and olodaterol (C).

Fig. 2. Synthesis scheme of the radioligand [³H]-olodaterol. Reaction and purification conditions are described in detail in the “Materials and Methods” section.

Fig. 3. A: Comparison of binding profiles of [³H]-CGP12,177 and [³H]-olodaterol in mouse lung homogenates. Purified membranes obtained from mice lungs were incubated with [³H]-CGP12,177 (2 nM) in the absence and presence of the β₁-AR selective antagonist CGP20,712A (1 µM; white and grey bars, respectively) and with [³H]-olodaterol (3 nM; black bar). Bₘₐₓ 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 cold CGP12,177 (10 µM). Data are shown as means ± S.E.M. for two independent paired experiments, each performed in triplicate. The data were analyzed by using one-way ANOVA followed by Bonferroni’s multiple-comparison test. Statistical significance is denoted compared with the [³H]-CGP12,177 group (p < 0.05). B: pH-dependent lipophilicity profile of olodaterol. pKₐ-values and logD profiles of olodaterol were determined as described in detail in the “Materials and Methods” section.

Fig. 4. Interaction of [³H]-olodaterol with the lipid bilayer. A: Purified membranes obtained from mock CHO cells (i.e. not expressing any β-AR) were incubated with different concentrations of [³H]-olodaterol (up to 3 nM) using the same conditions as for saturation binding experiments with hβ₂-ARs (20 µg/sample; incubation volume =
1 ml). As a reference, specific binding of [³H]-olodaterol (3 nM) to membranes obtained from CHO-hß2AR cells is indicated with the black bar. A representative experiment out of three performed experiments is shown. **B:** Purified membranes obtained from mock CHO cells (150 µg/sample) were incubated with high concentrations of [³H]-olodaterol (10 to 180 nM) in a large volume of incubation (3 ml). The correlation between pmoles of [³H]-olodaterol bound to the membranes and the free amount in solution (pmoles) was fitted by linear regression. A representative experiment out of three performed experiments is shown.

**Fig. 5. Kinetics of association and dissociation for the [³H]-olodaterol /hß2-AR complex. A:** CHO-hß2AR membranes (20 µg/sample) were added at time = 0 to three different concentrations of [³H]-olodaterol: 0.07 nM (filled squares), 0.48 nM (empty circles) and 1.45 nM (filled circles) in a total volume of 1 ml. At the indicated time points samples (triplicates per each radioligand concentration) were filtered and radioactivity due to receptor association was quantified. Because [³H]-olodaterol concentrations somewhat varied from experiment to experiment, data are presented as mean ± S.D. from one representative of three independent experiments. D.P.M., desintegrations per minute. **B:** CHO-hß2 cell membranes (20 µg/sample) were preincubated with [³H]-olodaterol (here 1 nM), and then at time 0 dissociation was started by adding an excess of cold olodaterol (10 µM) in the absence (filled circles) and presence (empty circles) of GppNHp (10 µM). The amount of receptor-bound [³H]-olodaterol was monitored by filtering samples at the indicated time points. Data were best-fitted using a two-phase (filled circles) and a one-phase (empty circles) exponential decay, respectively (comparison with the extra-sum-of-square F test, threshold p value ≤0.01). A representative experiment out of four performed experiments, with each point performed in triplicate, is shown.
Fig. 6. Recovery of hß2-AR binding sites by [³H]-CGP12,177 following preincubation with olodaterol. CHO-hß2AR membranes (20 µg/sample) were preincubated with cold olodaterol (3 nM), then dissociation was induced at time = 0 by 80-fold dilution with binding buffer containing 3 nM [³H]-CGP12,177. The amount of receptor-bound [³H]-CGP12,177 was monitored by filtering samples at the indicated time points, i.e. after 2, 10 and 24 hours. Data are expressed as recovered percentage of total hß2-AR binding sites, defined for each group as the amount of binding obtained with [³H]-CGP12,177 in membranes that were not pretreated with olodaterol. Data are presented as mean ± S.D. from one representative of three independent experiments.

Fig. 7. Saturation binding profiles of [³H]-olodaterol to the hß2-AR in the presence and absence of Gα protein coupling. A, B: CHO-hß2AR membranes (20 µg/sample) were incubated with different concentrations of [³H]-olodaterol (5 pM to 6 nM) in the absence (A) or presence of the GTP analog GppNHp (10 µM, B). The amount of receptor-bound [³H]-olodaterol was monitored by filtering samples after overnight incubation. Data were best-fitted using a two-affinity sites (A) and a one-affinity site (B) saturation binding model, respectively (comparison with the extra-sum-of-square F test, threshold p value ≤0.001). The rejected one-Kd site fitting of the data in graph A is shown with the dotted line. C: As a control, CHO-hß2AR membranes (20 µg/sample) were incubated with different concentrations of [³H]-CGP12,177 (5 pM to 4 nM) in the absence of GppNHp. Data were best-fitted using a one- affinity site saturation binding model. Data are shown as mean ± S.D. of a representative experiment out of three performed experiments, with each point performed in triplicate.
Fig. 8. Persistence of action of β agonists in CHO cells recombinantly expressing hß2-ARs. CHO cells expressing human β2 adrenoceptors 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 section for detailed protocol description), then cAMP levels were quantified. Triplicate data points were used at each agonist concentration and expressed as % 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.

Fig. 9. Persistence of action of β agonists in CHO cells recombinantly expressing hß1-ARs. CHO cells expressing human β1 adrenoceptors were stimulated with isoprenaline (A), salmeterol (B) and olodaterol (C) under control (filled circles) and „washed“ (empty circles) conditions (see Materials and Methods section for detailed protocol description), then cAMP levels were quantified. Triplicate data points were used at each agonist concentration and expressed as % 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.
Table 1. Potency, efficacy and persistence of action of β-adrenoceptor agonists in CHO cells recombinantly expressing hß2- and hß1-ARs. 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>hß2-AR CHO cells</th>
<th>hß1-AR CHO cells</th>
</tr>
</thead>
<tbody>
<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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<tr>
<td>Salmeterol</td>
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<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>
Figure 5

A

[\(^3\text{H-olodaterol}\):
- ▐ 1.45 nM
- ▼ 0.48 nM
- ■ 0.07 nM

DPM

minutes

B

h\(\beta\)2-AR binding (%)

time (min)
Figure 6

The diagram shows the hβ2-AR binding (%) over different time periods:
- 2 hour
- 10 hour
- 24 hour

The y-axis represents hβ2-AR binding (%) ranging from 0 to 125, with the 100% line indicated by a dashed line.
Figure 7

A

![Graph with DPM vs. [3H-olodaterol] nM](image)

B

+ GppNHp

![Graph with DPM vs. [3H-olodaterol] nM](image)

C

![Graph with DPM vs. [3H-CGP12,177] nM](image)