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Original article

# A cell-based assay to assess the persistence of action of agonists acting at recombinant human $\beta_2$ adrenoceptors

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ABSTRACT

**Introduction:** The aim was to establish a robust, 96-well, cell-based assay to assess the potency and persistence of action of agonists acting at human recombinant $\beta_2$ adrenoceptors expressed in CHO (Chinese Hamster Ovary) cells and to compare this with published duration of action data in guinea pig isolated trachea and human bronchus. **Methods:** Cells were treated with either: (i) β-adrenoceptor agonist for 30 min, washed and cyclicAMP (cAMP) measured 30 min later—termed 'washed' cells or, (ii) treated with solvent for 30 min, washed, and then treated with β-adrenoceptor agonist for 30 min and cAMP measured—termed 'unwashed' cells. The 'washed' $EC_{50}$ was divided by the 'unwashed' $EC_{50}$ to determine a rightward shift concentration ratio, which was indicative of the persistence of action at the receptor. **Results:** At the $\beta_2$ adrenoceptor salmeterol, carmoterol and indacaterol were resistant to washing with a concentration ratio of $<5$, indicating a long persistence of action, whereas formoterol, isoprenaline and salbutamol were washed out with a ratio of 32, $>294$ and $>800$ respectively, suggesting a shorter persistence of action. At $\beta_1$ and $\beta_3$ adrenoceptors all compounds washed out. The persistent effects of salmeterol at $\beta_2$ following washing could be reversed by the selective $\beta_2$ antagonist ICI 118551, suggesting continued receptor activation. **Discussion:** The data presented agree well with published data assessing duration of action of $\beta_2$ agonists in human isolated bronchus and guinea pig isolated trachea. Key features are: (a) it is a 96-well format which can be used to assess many compounds in a single experiment, (b) both potency and persistence of agonist action are assessed in the same assay, (c) any effects of concentration on the persistence of action can be highlighted, and (d) it allows triage of compounds prior to tissue bath studies thus reducing the use of animal tissue.



## 1. Introduction

Inhaled $\beta_2$ adrenoceptor agonists are widely used to treat asthma and COPD, providing symptomatic relief by inducing bronchodilation via relaxation of airway smooth muscle. After the introduction of the short acting inhaled $\beta_2$ adrenoceptor agonists such as salbutamol (reviewed in Waldeck, 2002), research focussed on developing $\beta_2$ agonists which had a longer duration of action as bronchodilators. This resulted in the development of the $\beta_2$ adrenoceptor agonists salmeterol and formoterol, which when given by inhalation, produce bronchodilation for at least 12 h and thus are administered twice daily (Anderson, Linden & Rabe, 1994; Johnson, 1995a,b; Waldeck, 2002). More recently indacaterol (Battram et al., 2006; Beier et al., 2007) and carmoterol (Cazzola, Gabriella, & Lovall, 2005) have shown potential to only require once daily dosing.

*In vitro* assays to assess the functional duration of action of $\beta_2$ adrenoceptor agonists have fulfilled a central role in screening programmes to identify long acting agents. The original publication documenting the pre-clinical profile of salmeterol (Ball et al., 1991) assessed duration of action *in vitro* using superfusion of the guinea pig isolated trachea and subsequently other groups have used similar superfusion techniques in both guinea pig isolated trachea and human isolated bronchus to assess functional duration of action of salmeterol and other β-adrenoceptor agonists (Nials et al., 1994; Bergendal et al., 1996; Austin et al., 2003; Battram et al., 2006; Naline, Trifilieff, Fairhurst, Advenier, & Molimard, 2007). In a drug discovery setting a key limitation of these assays is the number of compounds that can be assessed in a single experiment. A cell-based assay may offer a valuable, higher throughput alternative.

$\beta_2$ adrenoceptors are Gs coupled receptors, whose activation leads to increased levels of cyclic adenosine monophosphate (cAMP) within cells (Johnson, 2006). This report details the development of a 96-well cell-based assay using human recombinant $\beta_1$, $\beta_2$ and $\beta_3$ adrenoceptors separately expressed in Chinese hamster ovary (CHO) cells. The functional potency and the persistence of action of $\beta_2$ adrenoceptor agonists were determined by measuring cAMP levels using a non-radioactive immunoassay. In this assay, duration of action or persistence of agonist action is expressed as a concentration ratio by

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comparing the concentration response curves to agonists in 'washed' versus 'unwashed' cells. The data from this assay agree well with data obtained using $\beta_2$ agonists in tissue bath assays with both guinea pig isolated trachea and human isolated bronchus and suggest that it is suitable for assessing the potency and persistence of action of novel compounds acting at the $\beta_2$ adrenoceptor, as an indication of their duration, in a higher throughput manner. The methodology described in this paper should be readily adaptable to other Gs coupled receptors. A preliminary report of this assay has been presented to the Cambridge meeting of the British Pharmacological Society (Summerhill et al., 2005).

## 2. Materials and methods

### 2.1. Materials

Foetal bovine serum, L-glutamine, geneticin, puromycin, trypsin, phosphate buffered saline tablets, isobutyl methylxanthine, isoprenaline-hydrochloride and salbutamol-free base were purchased from Sigma-Aldrich (Poole, Dorset, UK). Formoterol-fumarate, salmeterol-xinafoate, indacaterol-free base and carmoterol-free base were synthesised by medicinal chemistry at Pfizer (Sandwich, Kent, UK). ICI 118551 hydrochloride was purchased from Tocris Biosciences (Bristol, UK). Dulbeccos minimal essential media/NUT mix F12 and Dulbeccos phosphate buffered saline (without sodium bicarbonate) were purchased from Invitrogen (Paisley, UK). The HitHunter II cAMP Assay kit from DiscoveRx was purchased from Amersham Biosciences (Amersham, UK). White 96-well view plates were purchased from Perkin Elmer (UK). Chinese Hamster Ovary (CHO) cells expressing human recombinant $\beta_1$, $\beta_2$ and $\beta_3$ adrenoceptors were obtained from Prof. S. Liggett (Duke University) ($\beta_1$ and $\beta_2$) or Prof. J. Granneman (Wayne State University) ($\beta_3$) and were generated from full length cDNA obtained by PCR screening of a human placenta cDNA library for $\beta_1$ and $\beta_2$ (Kobilka et al., 1987; Frielle et al., 1987 and Green, Holt, & Liggett, 1992) or human genomic library for $\beta_3$ (Granneman et al., 1993) using standard molecular cloning techniques.

### 2.2. Cell culture

CHO (Chinese Hamster Ovary) cells recombinantly expressing either the human $\beta_1$, $\beta_2$ or $\beta_3$ adrenoceptor (Bmax ~500, 1500 and 120 fmol/mg protein respectively using $^{125}$I-cyanopindolol binding to membranes) were maintained in DMEM media supplemented with 10% (v/v) FBS, 2 mM L-Glutamine, 500 μg/ml Geneticin and 10 μg/ml Puromycin at 37 °C and 5% $CO_2$ at ~80% confluency. For all assays cells were seeded overnight in 96-well view plates at $2\times10^4$ cells/well in growth media containing 1% FBS. The following day cells were washed with phosphate buffered saline (PBS) using a 96-well plate washer (Skatron Skanstacker 300). The media was replaced with PBS containing 0.5 mM IBMX (isobutyl methylxanthine), a non-selective phophodiesterase inhibitor and the cells were incubated for a further 30 min prior to compound addition. All incubations were carried out at 37 °C. PBS was used rather than DMEM media since the activity to isoprenaline was observed to be markedly reduced in DMEM media ($EC_{50}$ = 57.4 +/− 35.7 nM $n$ = 3 in DMEM versus 3.6 +/− 1.2 nM $n$ = 3 in PBS).

### 2.3. Agonist stimulation

Salmeterol-xinafoate, formoterol-fumarate, isoprenaline-hydrochloride, salbutamol-free base, indacaterol-free base and carmoterol-free base were solubilised to 1 mM in 100% dimethylsulphoxide (DMSO) and then diluted in PBS or 2% DMSO/PBS to the desired concentrations. Unless otherwise stated, cells were incubated with compound for 30 min, then washed with PBS using a plate washer, the plate patted dry and the level of intracellular cAMP determined using a HitHunter II cAMP detection assay (linear detection range: 0.15–30 pmol/well cAMP) by following the kit protocol. All experiments were carried out at 37 °C.

To measure persistent receptor activation cells were treated with either (i) a range of concentrations of β-adrenoceptor agonist for 30 min, washed and cAMP measured 30 min later—termed 'washed' cells or, (ii) treated with solvent for 30 min, washed and then treated with a range of concentrations of β-adrenoceptor agonist for 30 min and cAMP measured—termed 'unwashed' cells. In both cases cells were only ever treated with a single concentration range of β-adrenoceptor agonist and the $EC_{50}$ was compared in 'washed' versus 'unwashed' cells to generate a rightward shift concentration ratio, which was indicative of the persistence of receptor activation (see Section 2.5.2). In some experiments the effect of increased washes on the persistence of agonist responses was investigated. In this protocol cells received 6 washes (with 15 min between each wash step) prior to measuring cAMP.

Agonist responses were also assessed by measuring both intracellular and extracellular cAMP. CHOh$\beta_2$ cells were incubated with increasing concentrations of either salmeterol or isoprenaline for 30 min under 'unwashed' or 'washed' conditions. At the end of the experiment the supernatant was removed to measure extracellular cAMP levels and the cells were lysed to measure intracellular cAMP levels, as described above. Total cAMP levels were calculated by adding the intracellular and extracellular cAMP levels together.

### 2.4. Effect of ICI 118551 on agonist responses

CHOh$\beta_1$ or $\beta_2$ cells were pre-incubated with several concentrations of ICI 118551 for 30 min prior to stimulation with increasing concentrations of agonist and the level of intracellular cAMP determined 30 min later, as described above. The functional affinity of ICI 118551 was estimated using Schild analysis (Arunlakshana & Schild, 1959).

### 2.5. Data analysis

#### 2.5.1. Agonist potency and efficacy

The amount of intracellular cAMP detected by the HitHunter II cAMP assay kit was quantified using a Fusion plate reader (Perkin Elmer) set to luminescent detection mode, with a count time of 1 s per well at normal counting efficiency. The maximum amount of cAMP measured from the cells was typically 1–3 pmol/well and was always within the linear portion of the cAMP standard curve (linear range: 0.15–30 pmol/well cAMP). To allow for variations in cAMP production between plates, experiments and cell lines, the data was converted into % activity by calculating it as a % of the maximum cAMP stimulated on each plate using control wells:

$$\%\ \text{activity} = \frac{(\text{compound well response}) - (\text{mean basal control wells})}{(\text{mean max control wells}) - (\text{mean basal control wells})} \times 100$$

Where basal control = 1% DMSO/PBS and max control = 100 nM formoterol ($\beta_2$) or 100 nM isoprenaline ($\beta_1$ and $\beta_3$).

Compound concentrations were plotted against the calculated % activity and a four-parameter sigmoid fit was used to generate the $EC_{50}$ (concentration of compound producing 50% of the maximum cAMP for that compound), slope, %Emin and %Emax values for both the 'unwashed' and 'washed' compound curves.

#### 2.5.2. Persistence of agonist action

The $EC_{50}$ for each agonist was determined under 'washed' and 'unwashed' conditions and where efficacy under both conditions was similar, the persistence of action was calculated by diving the 'washed' $EC_{50}$ by the 'unwashed' $EC_{50}$ and a rightward shift (RWS) concentration ratio determined. 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.