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Title: Long Receptor Residence Time of C26 Contributes to Super Agonist Activity at the Human β_2 Adrenoceptor.

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Running Title: Super Agonism at the β_2 Adrenoceptor.

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Abbreviations: C26, 7-[(*R*)-2-((1*R*,2*R*)-2-benzyloxycyclopentylamino)-1-hydroxyethyl]-4-hydroxybenzothiazolone, CAS number [663925-01-7]; CHO-arrestin-β_2, PathHunter™ CHO-K1 β_2-adrenoceptor:β-arrestin-2 cells; CHO-β_2, Chinese hamster ovary cells stably transfected with the human β_2 adrenoceptor; COPD, chronic obstructive pulmonary disease; DPBS, Dulbecco's phosphate buffered saline solution; FBS, foetal bovine serum; GPCR, G protein-coupled receptors; HBSS, Hanks' balanced salt solution; LABA, long-actin β_2 adrenoceptor agonists; U2OS-GFP-β2, U2OS cells stably transfected with GFP tagged human β_2 adrenoceptor;

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Abstract

Super agonists produce greater functional responses than endogenous agonists in the same assay, and their unique pharmacology is the subject of increasing interest and debate. We propose that receptor residence time and the duration of receptor signalling contributes to the pharmacology of super agonism. We have further characterised the novel β_2 adrenoceptor agonist C26 (7-[(*R*)-2-((1*R*,2*R*)-2-benzyloxycyclopentylamino)-1-hydroxyethyl]-4-hydroxybenzothiazolone), which displays higher intrinsic activity than the endogenous ligand adrenaline in cAMP accumulation, β-arrestin-2 recruitment and receptor internalization assays. C26 recruited β-arrestin-2, and internalized the GFP-β_2 adrenoceptor at a slow rate, with $t_{½}$ values of 0.78 ± 0.1 and 0.78 ± 0.04 hr, respectively. This was compared to 0.31 ± 0.04 and 0.34 ± 0.01 hr for adrenaline-mediated β-arrestin-2 recruitment and GFP-β_2 internalization, respectively. The slower rate for C26 resulted in levels of β-arrestin-2 recruitment increasing up to 4 hr agonist incubation, at which point the intrinsic activity was determined to be 124.3 ± 0.77 % of the adrenaline response. In addition to slow functional kinetics, C26 displayed high affinity with extremely slow receptor dissociation kinetics, giving a receptor residence half-life of 32.7 min at 37°C that represents the slowest dissociation rate we have observed for any β_2 adrenoceptor agonist tested to date. In conclusion, we propose that the gradual accumulation of long-lived active receptor complexes contributes to the increased intrinsic activity of C26 over time. This highlights the need to consider the temporal aspects of agonist binding and signalling when characterising ligands as super agonists.

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Introduction

Inhaled long-actin β_2 adrenoceptor agonists (LABA) e.g. formoterol and salmeterol, which have a duration of action of approximately 12 hours have been widely used for the treatment of chronic obstructive pulmonary disease (COPD), providing symptomatic relief by inducing bronchodilation via relaxation of airway smooth muscle. More recently, a further class of β_2 adrenoceptor agonists has been developed, termed ultra LABA (e.g. indacaterol) which have duration of action after a single inhaled dose of 24 hours. 7-[(*R*)-2-((1*R*,2*R*)-2-benzyloxycyclopentylamino)-1-hydroxyethyl]-4-hydroxybenzothiazolone (from herein referred to as C26), was originally identified as a high affinity β_2 adrenoceptor agonist with predicted long duration of action. In addition to its high affinity, in a human A431 cell lysate cAMP assay C26 exhibited greater potency and a higher intrinsic activity of 140 % compared to formoterol (Beattie et al., 2010). Here we have further characterised the pharmacological profile of C26 in both binding and functional assays in comparison to the endogenous β_2 adrenoceptor agonist adrenaline and its stable analogue isoprenaline.

The binding of an agonist to a receptor produces a signalling cascade which leads to a variety of cellular, tissue and organ responses that can occur in a timescale of seconds to hours. However the initial receptor stimulation with agonist is often rapid and short lived, due mainly to desensitization of the receptor, which begins within seconds of agonist exposure and is initiated by phosphorylation of the receptor and ultimately leads to its internalization. In general, endogenous agonists of G protein-coupled receptors (GPCR), such as adrenaline for the β_2 adrenoceptor, have low affinity but high efficacy meaning that few receptors need to be activated to achieve a maximum response. In addition, although a limited number of agonists have been studied to date, they also appear to rapidly dissociate from the receptor (Sykes and Charlton, 2012) thus reducing the risk of persistent signalling and over-

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stimulation of a pathway which may be detrimental to the cell or organism, and allowing rapid re-setting of the system.

It is generally assumed that the interaction between an endogenous agonist and its receptor, as a result of strong evolutionary pressure, is likely to be as efficient as it can possibly be. If such a case were true, the maximal asymptote of the concentration-response curves of endogenous agonists, fitted to experimental data (Strange, 2008) (intrinsic activity, E_{max}), would always be equal to the system maximum. However, there are a class of synthetic ligands such as MK-677 at the GNRH receptor or R-Des-TRH at the TRH receptor (Bennett et al., 2009; Engel et al., 2006) that have been demonstrated to produce higher maximal responses than their respective endogenous ligands, leading to the term super agonist being coined. Such terminology has been met with mixed views in the pharmacology community, and the unique pharmacology of super agonists is now the subject of increasing interest and debate.

Evidence from structural studies on the β_2 adrenoceptor (Nygaard et al., 2013) demonstrated that the change in conformation upon activation of a receptor depends as much upon the presence of G protein as it does on agonist, such that agonist binding alone is not able to stabilize a fully active receptor conformation. The ‘mobile’ or ‘fluid’ receptor hypothesis describes the active receptor complex as having three entities, receptor, effector and ligand, that are able to interact in a cooperative manner (de Haën, 1976; Jacobs and Cuatrecasas, 1976). In simplest terms, a high efficacy ligand will therefore act as a positive allosteric modulator, increasing the association of the effector protein with the receptor. As allosteric interactions are reciprocal, it also follows that association of the effector molecule with the receptor can be considered to exert positive allosteric modulation of ligand binding. Conversely, dissociation of effector (or association of inactive effector complex) could be considered to negatively regulate ligand binding, thus reducing its affinity for the receptor.

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One could therefore speculate that even the fully efficacious endogenous agonists may not be able to maximally activate receptors in cellular systems where high levels of cytosolic guanine nucleotides uncouple the receptor from G proteins and drive it away from the active state (Ehlert, 2008), thus leaving scope for 'super agonism' to occur.

In this study we have used a number of functional and binding assay systems to fully characterize the pharmacology of C26. We will demonstrate that C26 displays interesting pharmacological properties, as it shares binding characteristics with low efficacy partial agonists, but appears in functional assays to be a kinetically-driven super agonist in terms of its intrinsic activity.

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Materials and Methods

Materials

PathHunter™ cell lines, lysis and Flash detection reagents were purchased from DiscoveRx (Birmingham, UK). CO_2-indepenent medium, DMEM supplemented with 4.5g/L D-Glucose, L-Glutamine and pyruvate, Hams F12 media supplemented with GlutaMAX, McCoys 5A media, heat inactivated foetal bovine serum (FBS), geneticin, hygromycin, blasticidin, penicillin, streptomycin, trypsin-EDTA, Dulbecco's phosphate buffered saline solution (DPBS), Hank's balanced salt solution without phenol red (HBSS w/o phenol red) and HEPES were all purchased from Life Technologies (Paisley, UK). ALPHAScreen cAMP detection kit, [^{3}H]-DHA and 384- and 96-well Viewplates™ were purchased from Perkin Elmer Life Sciences (Massachusetts, USA). GloSensor cAMP reagent and GloSensor cAMP plasmid were purchased from Promega (Hampshire, UK). Bovine serum albumin (BSA), Tween-20, adrenaline (epinephrine), isoprenaline, DMSO, GTP and ascorbic acid, were purchased from Sigma Aldrich (Poole, UK). C26 was synthesized in-house (Beattie et al., 2010).

Cell culture

A431 cells (human carcinoma cell line; Life Technologies, UK) were maintained in DMEM supplemented with 4.5g/L D-Glucose, L-Glutamine, pyruvate and FBS (10 % v/v). PathHunter™ CHO-K1 human β_2 adrenoceptor:β-arrestin-2 cells (CHO-arrestin-β_2) were maintained in Hams F12 nutrient mix supplemented with FBS (10 % v/v), hygromycin B (0.2 mg/mL) and Geneticin (0.5 mg/mL). Chinese hamster ovary cells stably transfected with the human β_2 adrenoceptor (CHO-β_2) prepared in-house, were maintained in Hams F-12 Nutrient Mix GlutaMAX-1, supplemented with FBS (10 % v/v) and geneticin (0.5 mg/mL). U2OS cells stably transfected with GFP tagged human β_2 adrenoceptor (U2OS-GFP-β_2) prepared in

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house (Rosethorne et al., 2015), were maintained in McCoys 5A media supplemented with FBS (10 % v/v) and geneticin (0.5 mg/mL). All cells were grown adherently and maintained at 37°C in 5 % CO_2/humidified air. For routine culture and for experiments all of the cells were harvested using trypsin/EDTA. For radioligand binding studies, membranes were prepared from CHO-β_2 cells as described in Sykes et al., (2009).

Measurement of cAMP using A431 cells endogenously expressing human β_2-adrencoceptor

A431 cells were seeded overnight in white, 96-well ViewPlates™ at 20,000 cells/well in culture medium. Spent media was removed and replaced with assay buffer (HBSS w/o phenol red, 5 mM HEPES and 0.1 % (v/v) BSA). Cells were then stimulated with a range of concentrations of agonist for 1 hr at room temperature. The incubation was terminated by the addition of lysis buffer (dH_2O, 0.3 % (v/v) Tween-20) containing 20 units/mL streptavidin coated donor beads, biotinylated cAMP and anti-cAMP acceptor beads. A cAMP standard curve (10,000 nM to 0.001 nM) was constructed in each experiment, and lysis buffer containing the bead mix was added to the standard curve at the same time it was added to the wells of the assay. The assay plate and standard curve plate were incubated in the dark, overnight at room temperature. The plate was then read on the EnVision plate reader (Perkin Elmer Life Sciences; Massachusetts, USA). The levels of cAMP produced were always within the linear part of the standard curve. For time course experiments, A431 cells were transiently transfected with the GloSensor cAMP plasmid, using Lipofectamine2000 according to manufacturers' instructions. 24 hours later, cells were stimulated with a range of concentrations of each agonist and luminescence monitored over time (every minute for 25 minutes). Responses were obtained for an EC_{80} concentration of isoprenaline (32 nM) and equi-effective concentrations of C26 (0.3 nM) and adrenaline (63 nM) (concentrations of C26 and adrenaline which produce the same amount of cAMP as the EC_{80} concentration of

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isoprenaline after 8 minute treatment in this assay). To account for the inter-assay variation in levels of cAMP which were produced in each experiment, data were normalized to the amount of cAMP produced compared to the maximum isoprenaline response.

Measuring β-arrestin-2 recruitment to the human β_2 adrenoceptor in CHO-arrestin-β_2 cells

β-arrestin-2 recruitment was monitored using an enzyme-fragment complementation assay (DiscoveRx, Birmingham, UK). CHO-arrestin-β_2 cells were seeded overnight in white, 384-well ViewPlates at 10,000 cells/well in culture medium. Spent media was removed and replaced with assay buffer (HBSS supplemented with 10 mM HEPES, 0.1 % (w/v) BSA). Cells were then stimulated with a range of concentrations of agonist for 2 hr at 37°C, after which time Flash detection reagent was added and luminescence read on the EnVision plate reader. In addition, time courses of β-arrestin-2 recruitment were performed, whereby a range of concentrations of each agonist was added to the cells and Flash detection reagent added at different times from 0 – 4 hr. To construct association curves, responses obtained for an EC_{80} concentration of isoprenaline (13.5 nM) and equi-effective concentrations of C26 (0.29 nM) and adrenaline (110 nM). To account for the inter-assay variation in levels of luminescence produced in each experiment, data were normalized to the maximum isoprenaline response.

Measuring human β_2 adrenoceptor internalization using U2OS-GFP-β_2 cells

U2OS-GFP-β_2 cells were seeded overnight in black, 384-well ViewPlates at 3,000 cells/well in culture medium. Spent media was removed and replaced with cell culture medium containing 100 mg/mL cycloheximide to inhibit protein biosysnthesis and generation of new receptors inside the cell. After 4 hr, the cycloheximide-containing medium was replaced with assay buffer (CO_2-independent medium supplemented with 5 mM HEPES, 0.01 % w/v

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ascorbic acid, 0.05 % w/v HSA and 100 mg/mL cycloheximide) containing 1 μM Hoechst to label nuclei. Cells were then stimulated with a range of concentrations of agonist for 2 hr at 37°C, after which time receptor internalization was quantified on the InCell Analyzer 2000 (GE Healthcare; Buckinghamshire, UK) using DAPI settings to visualize nuclei (5 ms exposure) and FITC settings for GFP (200 ms exposure). All images were collected using a Nikon 20x 0.45 NA objective and a large chip CCD camera (2048x2048 pixel). In addition, time courses of receptor internalization were performed, whereby an EC_{80} concentration of isoprenaline, as determined in this assay at 2 hr (80 nM), and an equi-effective concentration of C26 (0.2 nM) and adrenaline (300 nM) were added to the cells and receptor internalization monitored on the InCell Analyzer 2000 every 5 min for 2 hr, using the settings above. For agonist wash-out studies, cells were first imaged to enable monitoring of baseline levels of internalized receptors, incubated with EC_{80} concentrations of agonist (see above) for 2 hr at 37°C, and imaged again prior to washing 5 times in fresh assay buffer using an automated Biomek FX (Beckman Coulter; High Wycombe, UK) and fresh tips for each washout step. Following agonist washout, the assay plate was imaged on the InCell Analyzer 2000 every 5 min for 4 hr, using the settings above. Cellular image analysis was performed using the IN Cell Analyzer Workstation 3.7.1 (GE Healthcare; Buckinghamshire, UK) to measure the presence of vesicles (1 – 3 μm) containing the GFP-tagged β_2 adrenoceptor. To account for the inter-assay variation in levels of fluorescence produced in each experiment, data were normalized to the maximum isoprenaline response.

Common procedures applicable to all radioligand binding experiments

All radioligand experiments were conducted in 96 deep well plates. In all cases non-specific binding (NSB) was determined in the presence of 1 μM propranolol. After the indicated incubation period, bound and free radiolabel were separated by rapid vacuum filtration using a FilterMate™ Cell Harvester (Perkin Elmer Life Sciences; Massachusetts, USA) onto 96

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well GF/B filter plates and rapidly washed three times with ice cold HEPES (75 mM, pH 7.4). After drying (>4 hr), 40 µL of Microscint^{TM} 20 (Perkin Elmer Life Sciences; Massachusetts, USA) was added to each well and radioactivity quantified using single photon counting on a TopCount^{TM} microplate scintillation counter (Perkin Elmer Life Sciences; Massachusetts, USA). Aliquots of radiolabel were also quantified, to accurately determine how much radioactivity was added to each well, using liquid scintillation spectrometry on LS 6500 scintillation counter (Beckman Coulter; High Wycombe, UK). In all experiments, total binding never exceeded more than 10 % of that added, limiting complications associated with depletion of the free radioligand concentration (Scaramellini Carter, 2007).

Determination of agonist affinity (K_i)

Affinity estimates of unlabelled agonists were determined using two slightly different assay formats depending on whether the K_i determined was to be used in competition kinetic experiments or whether it was to be used to investigate GTP sensitivity. In each case, [^{3}H]-DHA competition experiments were performed at equilibrium and [^{3}H]-DHA was used at a concentration of approximately 600 pM (~25000 cpm final assay volume of 1.5 mL). Radioligand was incubated in the presence of the indicated concentration of unlabelled agonist and CHO-β_2 membranes (30 µg/ml) with gentle agitation for 180 min. K_i values determined for kinetic experiments were performed in binding buffer (HBSS supplemented with 20 mM HEPES, 0.1 % w/v BSA, 5 % v/v DMSO, 0.01 % w/v ascorbic acid and 100 µM GTP) and experiments were run at 37°C. K_i values determined to investigate GTP sensitivity were performed in assay buffer (20 mM HEPES, 1 mM $MgCl_2$, 0.1 % w/v BSA, 5 % v/v DMSO and 0.01 % w/v ascorbic acid) ± 30 µM GTPγS and the experiments were run at room temperature.

Competition binding kinetics

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To accurately determine k_{on} and k_{off} values, the K_{ob} was calculated for at least three different concentrations of [^{3}H]-DHA in binding buffer. The appropriate concentration of radioligand was incubated with CHO-β_2 membranes (30 μg/ well) in binding buffer with gentle agitation (final assay volume 1000 μL). Exact concentrations were calculated in each experiment by liquid scintillation counting. Free radioligand was separated by rapid filtration at multiple time points to construct association kinetic curves, and the resulting data were globally fitted to the association kinetic model to derive a single best fit estimate for k_{on} and k_{off} as described previously by Sykes et al., (2009).

The kinetic parameters of C26 were assessed using a competition kinetic binding assay as described by Sykes et al., (2009) in binding buffer. This approach involves the simultaneous addition of both radioligand and competitor to receptor preparation, so that at t=0 all receptors are unoccupied. Approximately 600 pM [^{3}H]-DHA (a concentration which avoids ligand depletion in this assay volume) was added simultaneously with the unlabelled compound (at t=0) to CHO-β_2 membranes (30 μg/well) in 500 μl binding buffer. The degree of [^{3}H]-DHA bound to the receptor was assessed at multiple time points by filtration harvesting and liquid scintillation counting, as described previously. NSB was subtracted from each time point meaning that t = 0 was always equal to 0. Each time point was conducted on the same 96-deep well plate incubated at 37°C with constant agitation. Reactions were considered stopped once the membranes reached the filter, and the first wash was applied within 1 sec. Three different concentrations of unlabelled competitor were tested to ensure the rate parameters calculated were independent of ligand concentration. All compounds were tested at 30, 10, and 3-fold their respective K_i and data were globally fitted to simultaneously calculate k_{on} and k_{off}.

Measuring inhibition of contraction of isolated guinea pig tracheal strips

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Male Dunkin-Hartley guinea pigs (350–700 g Charles River Margate, UK) were killed by exposure to an increasing concentration of CO_2; the trachea was removed and placed in oxygenated (95 % O_2/5 % CO_2) Krebs-Henseleit solution (118 mM NaCl, 25 mM $NaHCO_3$, 11.1 mM glucose, 4.8 mM KCl, 205 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM Na_2PO_4, 1.2 mM KH2PO4). The method used for the electrically-stimulated tracheal strip is a modification of a previously published technique (Coleman and Nials, 1989). The trachea was cut into rings of 4-5 cartilage bands in width, which were subsequently opened into strips by cutting the cartilage opposite the smooth muscle band in order to create a strip with equal lengths of trachea each side of the muscle band. Each strip was set up in a superfusion system and attached to an isometric force transducer (Fort 10, World Precision Instruments, Stevenage, Hertfordshire, UK) under a resting tension of 1 g and superfused (1 mL/min) with oxygenated Krebs-Henseleit solution at 37°C. Following a 1 hr equilibration period, phasic contractile responses were induced by electrical stimulation with 5 sec trains of square wave pulses of 10 volt, 10 Hz frequency and 0.2 ms duration every 2 min. After a 1 hr equilibration period using the above stimulation parameters, a baseline contractile response was determined over a 20 min period before the superfusion fluid was changed to Krebs-Henseleit solution containing C26 for 30 min. After this time, the superfusion fluid was changed back to compound-free Krebs-Henseleit solution for the remaining 11.5 hr of the experiment.

The onset of action of guinea pig tracheal contraction to electrical stimulation for each concentration of C26 was taken as the time from the start of compound superfusion until maximum inhibition of contraction to electrical stimulation was observed. The duration of action was defined as the time taken from the end of compound superfusion to 50 % recovery from maximum inhibition. The percentage maximum inhibition at each concentration of C26 was used to construct a concentration-response curve to calculate potency.

Data analysis

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All experiments were analysed by either linear or non-linear regression using Prism 6.0 (GraphPad Software, San Diego, U.S.A.). Competition displacement binding data and agonist concentration effect curves were fitted to sigmoidal (variable slope) curves using a four parameter logistic equation. IC_{50} values obtained from the inhibition curves were converted to K_i values using the method of Cheng and Prusoff (1973).

To monitor the rate of cAMP accumulation, the area under the curve (AUC) was fitted to the data for the initial 10 minutes of accumulation. To calculate the rate of β-arrestin-2 recruitment and GFP-β_2 internalization, data were analysed using non-linear regression, one phase exponential association to determine the rate constant *K*. The rate half-time was then calculated as 0.69/*K*. Statistical significance was determined using one-way ANOVA followed by Bonferroni multiple comparisons equation using GraphPad Prism, comparing C26 and isoprenaline to adrenaline.

For washout experiments, the data were normalised to pre-wash levels of internalized receptors and the plateau fixed to zero for all agonists. Data were analyzed using non-linear regression, one phase exponential decay to determine the rate constant *K*, and the rate half-time was calculated as 0.69/*K*. Statistical significance was determined using one-way ANOVA followed by Bonferroni multiple comparisons equation using GraphPad Prism.

Because the amount of radioactivity varied slightly for each experiment (< 5%), data are shown graphically as the mean ± range for individual representative experiments, whereas all values reported in the text and tables are mean ± S.E.M. for the indicated number of experiments. [^{3}H]-DHA association data were globally fitted in GraphPad Prism to determine a best fit estimate for k_{on} and k_{off}. Association and dissociation rates for unlabelled agonists were calculated using the equations described by Motulsky and Mahan (1984) using a global fitting model (see Sykes et al., (2009) for details).

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To evaluate the relative efficacy of agonists, data were fitted to the operational model of Black and Leff (1983). This model describes the correlation between biological response and agonist concentration [*A*] as a function of four parameters: E_m, K_A n and τ:

$$\text{Response} = \frac{[A]^n \tau^n E_m}{[A]^n \tau^n + ([A] + K_A)^n}$$

where E_m, or the operational maximum, represents the maximum possible effect in the tissue and n represents the transduction slope . K_A is the dissociation constant of the agonist and τ is the operational efficacy or the transducer ratio. When this equation was applied to data, [*A*] was varied according to experimental design, K_A was fixed to the value obtained in competition binding assays and E_m was globally fitted across all data sets, leaving n and τ as the only terms fitted individually for each agonist.

In addition, we used the following equations to calculate alternative values for agonist efficacy using experimental data from cAMP accumulation, β-arrestin-2 recruitment and β_2 adrenoceptor internalization (Strange, 2008):

$$Efficacy = \frac{K_A}{EC_{50}}$$

$$Efficacy = \frac{Emax.\, K_A}{EC_{50}}$$

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Results

Functional characterization of C26

We have previously reported that a novel 4-hydroxybenzothiazolone series of β_2 adrenoceptor agonists have both high potency and increased relative intrinsic activity compared to formoterol, in a cAMP assay using A431 cell lysates (Beattie et al., 2010). We re-visited this assay specifically to investigate the relative intrinsic activity of C26 (figure 1) with respect to the endogenous agonist adrenaline, in addition to the more stable full agonist isoprenaline, in whole cells.

Isoprenaline and adrenaline showed comparable potency and efficacy values in these cells, whereas C26 was almost 500-fold more potent in the cAMP assay. We were able to confirm that C26 does indeed have higher intrinsic activity with respect to adrenaline and isoprenaline, giving 117.8 ± 7.6 % of the isoprenaline response (p = 0.034, one-way ANOVA followed by Bonferroni post-test) (figure 2a; table 1). In addition, C26, adrenaline and isoprenaline were able to recruit β-arrestin-2 and cause GFP-β_2-adrenoceptor internalization in a concentration-dependent manner (figure 2b, c; table 1). In both assays, C26 also demonstrated greater intrinsic activity compared to adrenaline and isoprenaline, with E_{max} of 119 ± 6.7 (P = 0.027) and 121 ± 6.9 % (P = 0.0021) of the maximal isoprenaline response (one-way ANOVA followed by Bonferroni post-test), in the β-arrestin-2 recruitment and GFP-β_2-adrenoceptor internalization assays, respectively.

To determine the kinetics of the onset of cAMP accumulation, β-arrestin-2 recruitment and GFP-β_2-adrenoceptor internalization, cells were stimulated with concentrations of ligands that gave an equi-effective response to an EC_{80} concentration of isoprenaline. For cAMP accumulation, there was a slight lag between compound addition and complementation of the GloSensor-luciferase enzyme, therefore area under the curve was calculated for the first 10

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minutes of stimulation. Using this method, we determined that C26 was significantly slower than adrenaline and isoprenaline for the accumulation of cAMP (P = 0.007, one-way ANOVA followed by Bonferroni post-test). Mean AUC were 563 ± 25.6, 438 ± 20.8 and 569 ± 27.1 for adrenaline, C26 and isoprenaline, respectively. In addition, when these experiments were repeated in the presence of the phosphodiesterase (PDE) inhibitor rolipram, the increase in cAMP mediated by C26 was sustained for longer than either isoprenaline or adrenaline (figure 3b). Adrenaline and isoprenaline also recruited β-arrestin-2 and internalized the GFP-β_2-adrenoceptor at similar rates (figure 3c, d), with maximal levels being reached at approximately 2 hr after treatment. In contrast, C26 recruited β-arrestin-2 at a significantly slower rate than isoprenaline and adrenaline (P = 0.012, one-way ANOVA followed by Bonferroni post-test), as the mean $t_{½}$ values of onset of β-arrestin-2 recruitment were determined to be 0.78 ± 0.13, 0.30 ± 0.06 and 0.31 ± 0.04 hr for C26, isoprenaline and adrenaline, respectively. The slower rate for C26 resulted in levels of β-arrestin-2 recruitment increasing up to 4 hr agonist incubation, where the intrinsic activity was determined to be 126 ± 3.52 % of the maximal isoprenaline response. In addition, C26 internalized the GFP-β_2 adrenoceptor at a slower rate than isoprenaline and adrenaline (P < 0.0001, one-way ANOVA followed by Bonferroni post-test). The mean $t_{½}$ values of internalization were determined to be 0.78 ± 0.04, 0.37 ± 0.02, and 0.34 ± 0.01 hr for C26, isoprenaline and adrenaline, respectively.

Binding and kinetics of C26

As C26 demonstrated slower functional kinetics than either adrenaline or isoprenaline, but resulted in greater intrinsic activity over time, we investigated the binding kinetics of these compounds to determine if dissociation rates were contributing to this effect.

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Isoprenaline and C26 were able to produce concentration-dependent inhibition of [^{3}H]-DHA binding in CHO-β_2 cell membranes (figure 4a, b). In the case of C26 a mean pK_i of 9.81 ± 0.09 was calculated, which is consistent with the mean pK_i (9.78 ± 0.14) determined from studies by Beattie *et al.,* (2010) using [^{3}H]-CGP12177A and membranes prepared from sf9 cells expressing human β_2 adrenoceptor. As G proteins stabilise the high affinity conformation of the receptor, we also investigated the effect of including GTPγS in these assays to uncouple pre-bound receptor-G protein complexes and reveal the low affinity site. Using this approach, we showed that for isoprenaline (figure 4a), there was a shift from a two-site curve fit in the absence of GTPγS (mean pK_i values of 8.84 ± 0.45 and 6.55 ± 0.15 for high and low affinity sites, respectively), to a single-site fit in the presence of GTPγS (mean pK_i 6.59 ± 0.11, P = 0.003). In contrast, there was no shift in the inhibition of [^{3}H]-DHA binding by C26 when performed in the presence of GTPγS (figure 4b). This is similar to the data produced for the antagonist propranolol (figure 4c) where the pK_i in the absence of GTPγS was determined to be 9.14 ± 0.30 and there was no shift in the curve in the presence of GTPγS.

The kinetic parameters for C26 were then determined indirectly by monitoring how the association rate of [^{3}H]-DHA was altered in the presence of increasing concentrations of C26 (as described in Sykes & Charlton, 2010). Using this method (figure 4d), the k_{on} and k_{off} values were determined to be 3.70 ± 0.47 x10^8 (M^{-1} min^{-1}) and 0.0212 ± 0.003 (min^{-1}), respectively. In addition, the time taken for half of the bound C26 molecules to dissociate from the receptor ($t_{½}$) was calculated to be 32.7 min.

Duration of action of C26

As C26 displays very slow receptor kinetics, we postulated this would also lead to persistent signalling and a long duration of action. To test this hypothesis, we monitored the retention of

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receptors in intracellular vesicles and the relaxation effect of C26 on electrical field-induced contraction of isolated guinea pig tracheal strips following agonist washout.

Using the GFP-β_2 internalization assay, which is readily reversible, equi-effective concentrations of isoprenaline, C26 and adrenaline were incubated with U2OS GFP-β_2 cells for 2 hr. After this time the cells were washed and bathed in assay buffer whilst the level of β_2 internalization was monitored over time. The amount of internalized receptors produced by isoprenaline and adrenaline began to decrease around 15 min after agonist washout, and continued to decrease steadily until baseline levels were achieved within 2 hr post agonist washout. In contrast, even at 4 hr post agonist washout, C26 held ~80 % of internalized receptors inside the cell and the rate of decline of internalized vesicles was very slow (figure 5a). Estimated mean $t_{½}$ values representing the loss of GFP-β_2 vesicles from within U2OS cells were determined to be 44.2 ± 3.4, 1229 ± 289 and 40.0 ± 8.1 min for isoprenaline, C26 and adrenaline, respectively (span fixed to 100 %).

Following this, we assessed the duration of the relaxant effects of C26 in isolated guinea pig tracheal strips after 30 minute incubation with ligand. C26 was able to maximally inhibit the contraction of electrical field-induced contraction of isolated guinea pig tracheal strips (figure 5b), with pEC_{50} of 10.37 ± 0.04. The onset and duration of action of C26 were derived using the concentration closest to the pEC_{50} value, in this case 30 pM. At this concentration the onset of action of C26 was determined to be 5.2 ± 0.4 hr and the duration of action of more than 12 hr (figure 5c).

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Discussion

In this study we have investigated the kinetics of binding and signalling of C26, a novel super agonist at the β_2 adrenoceptor. The signalling properties of C26 were explored in three different functional assays, cAMP accumulation, β-arrestin-2 recruitment and receptor internalization. At all three levels of receptor signalling, C26 produced responses greater than the endogenous agonist adrenaline and the previously described full agonist isoprenaline, leading to its classification as a super agonist based on its intrinsic activity. In this way, C26 shares properties with other synthetic ligands that have been shown to have higher intrinsic activity than their respective endogenous ligands, such as the non-peptide growth hormone secretagogue MK-677 (Bennett et al., 2009; Holst et al., 2005) and the thyrotropin-releasing hormone analogue R-Des-TRH (Engel et al., 2006).

In addition to monitoring the intrinsic activity of C26, we also investigated its kinetics in the signalling assays. We have previously demonstrated a correlation between high efficacy agonists and an increased rate of cAMP accumulation (Rosethorne et al., 2010), which would predict a faster onset of action for C26 than for either adrenaline or isoprenaline based on their relative intrinsic activities. However, we found the opposite was the case, such that C26 produced the slowest rate of cAMP accumulation, β-arrestin-2 recruitment and receptor internalization. Maximal levels of cAMP accumulation were achieved 2-3 minutes after adrenaline and isoprenaline reached peak cAMP accumulation, and maximal levels of C26-mediated β-arrestin-2 recruitment and receptor internalization were only achieved after at least 4 hr treatment as opposed to 2 hr with adrenaline and isoprenaline. Using kinetic radioligand binding assays we demonstrated that C26 has a half-life of 32.7 min at 37°C. Compared to isoprenaline and adrenaline (0.23 and 0.14 min, respectively) this represents the slowest dissociation rate we have observed for any β_2 adrenoceptor agonist tested to date (Sykes & Charlton, 2012). As C26 demonstrates slow dissociation from the β_2 adrenoceptor,

it is likely that this compound will also be slow to reach equilibrium, thus delaying its onset of action. This is supported by the steepness of the Hill slope for C26, which often occurs under non-equilibrium conditions. We have repeated these experiments in 10-fold the assay volume (data not shown) to eliminate the possibility that the steep slope is due to ligand depletion at the lower concentrations of C26 (Carter et al., 2007). The slow binding kinetics of C26 translated into a slow onset and long duration of action, with C26 inhibition of electrically stimulated contraction of guinea pig tracheal strips being sustained for at least 12 hours after agonist wash-out. We also observed that in the presence of a PDE inhibitor the cAMP generated by C26 was sustained for longer than either adrenaline or isoprenaline, which may suggest that C26 continues to promote cAMP generation over longer periods. The slow kinetic binding and signalling appear to be properties unique to C26, as other high affinity, long acting beta2 agonists have been shown to rapidly dissociate from the receptor (Sykes and Charlton, 2012). It is believed that for many of the LABAs such as salmeterol, the high lipophilicity of these compounds contributes to their long duration of action (Anderson et al., 1994). In the case of C26, it is likely that its slow dissociation from the receptor provides a significant contribution, as it has a longer duration of action than would be predicted from its lipophilicity (calculated LogP of C26 = 2.42 ± 0.54; salmeterol = 3.90 ± 0.49; from ALOGPS v2.1), although we can't rule out a contribution of tissue retention. The persistent internalization of GFP-β_2 receptors caused by C26 even after agonist wash-out provides another potential factor that could be contributing to the long duration of action of C26. It has previously been thought that signal transduction mediated by GPCR is confined to the plasma membrane, and once receptors are phosphorylated and internalized these processes are switched off. However, recent evidence suggests that internalised receptors are capable of forming active ternary complex by accessing effectors from subcellular compartments, which contributes to the total cellular signalling. This has been demonstrated

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for a range of receptors, including the parathyroid and thyroid-stimulating hormone receptors, as well as the $S1P_1$ and β_2 adrenoceptor (Calebiro et al., 2009; Ferrandon et al., 2009; Irannejad et al., 2013; Mullershausen et al., 2009). Indeed, slow dissociation kinetics have been linked to the ability of FTY720 to cause persistent internalization of the $S1P_1$ receptor (Sykes et al., 2014). As such, the prolonged receptor internalisation caused by C26 may provide an additional mechanism of long duration of action at the β_2 adrenoceptor, which may be further exploited by targeting drugs to intracellular compartments e.g. by incorporating physiochemical properties that facilitate ion trapping (Goldman et al., 2009). The continued signalling of certain agonists from internalized compartments may contribute to the overall magnitude of intracellular signalling as well as the duration of responses, which may underlie signalling bias by directing receptors to alternative pathways/compartments or degradation. C26 may therefore be a useful tool to study this phenomenon in more detail.

A number of different approaches have been used to quantify agonist efficacy, ranging from simple calculations such as K_A/EC_{50} or $E_{max}.K_A/EC_{50}$ (Strange, 2008), to more thermodynamically complete systems such as the cubic ternary complex model (Weiss et al., 1996). However, it is more common to fit data with empirical mathematical models like the Operational model of agonism to quantify agonist efficacy (Black and Leff, 1983). This model has been used to describe super agonists that that are more efficient at transducing receptor binding events into a cellular response, such as iperoxo, which demonstrates supra-physiological efficacy compared to acetylcholine at the muscarinic M_2 acetylcholine receptor (Schrage et al., 2013). These models are of little use for C26, as both the K_A/EC_{50} and $E_{max}.K_A/EC_{50}$ calculations predict a lower efficacy for this compound than either adrenaline or isoprenaline, despite its higher intrinsic activity (Table 2). The Operational model also struggles to correctly fit the data we have obtained with C26, either over-estimating the maximal response of adrenaline and isoprenaline relative to C26, or over-estimating the

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potency based on the affinity entered into the equation. This may be because the affinity measured in this study is not an accurate representation of the "functional affinity" at a specific pathway, due to effector-dependent changes in receptor conformation (Kenakin and Christopoulos, 2013; Yan et al., 2008). Although efforts have been made to measure this using receptor-effector fusions (Rasmussen et al., 2011), it remains very difficult to accurately measure functional affinity of high full (or super) agonists.

Our data indicate that C26 was insensitive to guanine-nucleotides suggesting it either does not stabilize many receptors in the classical high-affinity state, or that it has equal affinity for the high and low affinity states of the receptor, as has been reported previously for a number of different high affinity GPCR agonists (Childers et al., 1993; Roberts et al., 2004; Schneider et al., 2009; Vanhauwe et al., 1999). One explanation for this is that ligands that do not affect the overall affinity of the receptor for the G protein, but rather accelerate guanyl nucleotides binding and dissociation will be functionally active, but unable to discriminate two receptor states in binding studies (Waelbroeck et al., 1997). This can be likened to the allosteric ternary complex model, where the G protein is defined as an allosteric modulator of ligand-receptor interactions (Leach et al., 2007). Using this model, C26 yields an α value of 1.3 ($\alpha = K_{Low} / K_{High}$; data taken from figure 4b), which indicates that the presence of the G protein on the receptor had no effect on the affinity of C26 ($K_{Low} \approx K_{High}$) (Christopoulos and Kenakin, 2002). In contrast, the presence of the G protein had a positive allosteric effect on the binding of isoprenaline to the β_2 adrenoceptor, resulting in α =194 ($K_{Low} > K_{High}$; data taken from figure 4a). Despite having an α value close to 1, C26 has higher intrinsic activity than isoprenaline, suggesting its effect is to enhance the ability of the receptor to activate the effector ($\beta > 1$), for example the ability to accelerate GDP-GTP exchange (Waelbroeck et al., 1997; Sykes et al, 2009).

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In conclusion, we have fully characterized C26, a kinetically-driven super agonist at the human β_2 adrenoceptor that displays higher intrinsic activity than either the endogenous agonist adrenaline, or the full agonist isoprenaline. We have demonstrated that this compound displays extremely slow receptor dissociation kinetics for an agonist, and propose that this contributes to the increased intrinsic activity of C26 over time. This can most easily be visualised by observing the relative intrinsic activity of an EC_{80} concentration (determined after 8 min) of each agonist over time. In this model C26 has a lower intrinsic activity at the early time points where it appears partial relative to adrenaline, versus the later time point where it has greater intrinsic activity than adrenaline (figure 6). This highlights the need to consider the temporal aspects of agonist binding and signalling when characterising ligands as super agonists.

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Authorship Contributions

Participated in research design: Rosethorne, Bradley, Sykes, Renard, Trifilieff, Fairhurst, Charlton

Conducted experiments: Rosethorne, Bradley, Gherbi, Sykes, Sattikar, Wright, Renard

Contributed new reagents or analytic tools:

Performed data analysis: Rosethorne, Bradley, Gherbi, Sykes, Sattikar, Trifilieff

Wrote or contributed to the writing of the manuscript: Rosethorne, Bradley, Gherbi, Sykes, Fairhurst, Charlton

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Figure Legends

Figure 1 Chemical structure of C26

Figure 2 Concentration-response curves for (a) cAMP accumulation in A341 cells, (b) β-arrestin-2 recruitment in CHO-β_2 cells and (c) GFP-β_2 internalization in U2OS cells using adrenaline, C26 and isoprenaline. Data are normalised to the 2 hr isoprenaline response, and expressed as means ± S.E.M. for 3-8 independent experiments, run in duplicate.

Figure 3 Time courses of cAMP accumulation in A431 cells (a), β-arrestin-2 recruitment in CHO-β_2 cells (b) and GFP-β_2 internalization in U2OS cells (c) using equi-effective concentrations of adrenaline, C26 and isoprenaline, equivalent to an EC_{80} concentration of isoprenaline taken from GloSensor data (not shown), or data in figure 2 (b) and (c), respectively. Data are expressed as means ± S.E.M. for 3 (arrestin and internalization) or 4 (cAMP) independent experiments, run in duplicate. Statistical significance was determined using one-way ANOVA followed by Bonferroni multiple comparisons equation using GraphPad Prism.

Figure 4 Inhibition of [^{3}H]-DHA binding data in CHO-β_2-adrenoceptor membranes using (a) isoprenaline, (b) C26 and (c) propranolol, in the absence or presence of 100 μM GTPγS. d) [^{3}H]-DHA competition kinetics curves in CHO-β_2-adrenoceptor membranes in the presence of either 0, 3-fold K_i, 10-fold K_i or 30-fold K_i C26. Data were fitted to the equations described in the methods to calculate k_{on} and k_{off} values for unlabelled agonists. Data in a-c are normalised to % receptors bound. All graphs show representative data (mean of duplicate or triplicate determinations ± range) from 3 independent experiments.

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Figure 5 a) Rate of loss of GFP-β_2 internalized receptors in U2OS cells following washout of agonist. Equi-effective concentrations of adrenaline, C26 and isoprenaline were used, equivalent to an EC_{80} concentration of isoprenaline taken from data in figure 2 (c). Data are expressed as means ± S.E.M. for 3 independent experiments. b) Concentration-response for the inhibition of electrical-field induced contraction by C26 in isolated Guinea-pig tracheal strips. c) Time course of inhibition of electrical-field induced contraction in isolated Guinea-pig tracheal strips using a range of different concentrations of C26. Data are expressed as means ± S.E.M from 3-5 independent experiments.

Figure 6 Relative intrinsic activities of C26 and adrenaline for cAMP accumulation after incubation with EC_{80} concentrations of each agonist. Data are normalised to the maximal adrenaline response (8 minutes), and expressed as means ± S.E.M. for 4 independent experiments, run in duplicate. Statistical significance was determined using an unpaired t-test ($P < 0.05$) at 3 versus 10 minutes.

Table 1 Potency and intrinsic activity values for β_2 adrenoceptor agonists in different functional assays.

Potency and intrinsic activity values for adrenaline, C26 and isoprenaline in cAMP accumulation in A431 cells, β-arrestin recruitment in CHO-β2 cells and internalization of GFP-β_2 in U2OS cells. Intrinsic activity was calculated as a percentage of the maximal isoprenaline response. Data are expressed as means ± S.E.M. for 3-8 independent experiments, as indicated in brackets.

* $p < 0.05$; ** $p < 0.01$; One-way ANOVA followed by Bonferroni's multiple comparisons relative to adrenaline.

	cAMP accumulation in A431 cells		**β-arrestin recruitment in CHO-β_2 cells**		**Internalization of GFP-β_2 in U2OS cells**	
Agonist	**pEC_{50} (n)**	**Intrinsic activity**	**pEC_{50} (n)**	**Intrinsic activity**	**pEC_{50} (n)**	**Intrinsic activity**
Adrenaline	7.5 ± 0.13 (5)	91.4 ± 5.7	7.5 ± 0.06 (3)	100.9 ± 1.0	7.24 ± 0.14 (4)	95.3 ± 4.9
C26	10.2 ± 0.07 (5)	117.8 ± 7.6*	9.76 ± 0.05 (6)	118.5 ± 6.7*	9.87 ± 0.08 (4)	121.0 ± 6.9**
Isoprenaline	7.8 ± 0.06 (3)	102.4 ± 1.3	8.42 ± 0.05 (6)	100.5 ± 1.2	7.65 ± 0.06 (8)	99.0 ± 1.85

Table 2 Efficacy calculations for adrenaline, C26 and isoprenaline

	cAMP accumulation in A431 cells		β-arrestin recruitment in CHO-β_2 cells		Internalization of GFP-β_2 in U2OS cells	
	K_A/EC_{50}	$E_{max}.K_A/EC_{50}$	K_A/EC_{50}	$E_{max}.K_A/EC_{50}$	K_A/EC_{50}	$E_{max}.K_A/EC_{50}$
Adrenaline	44.7	4083	44.7	4507	24.5	2339
C26	1.05	123	0.38	45.1	0.49	59.3
Isoprenaline	8.13	832	33.9	3405	5.75	570

Figure 1

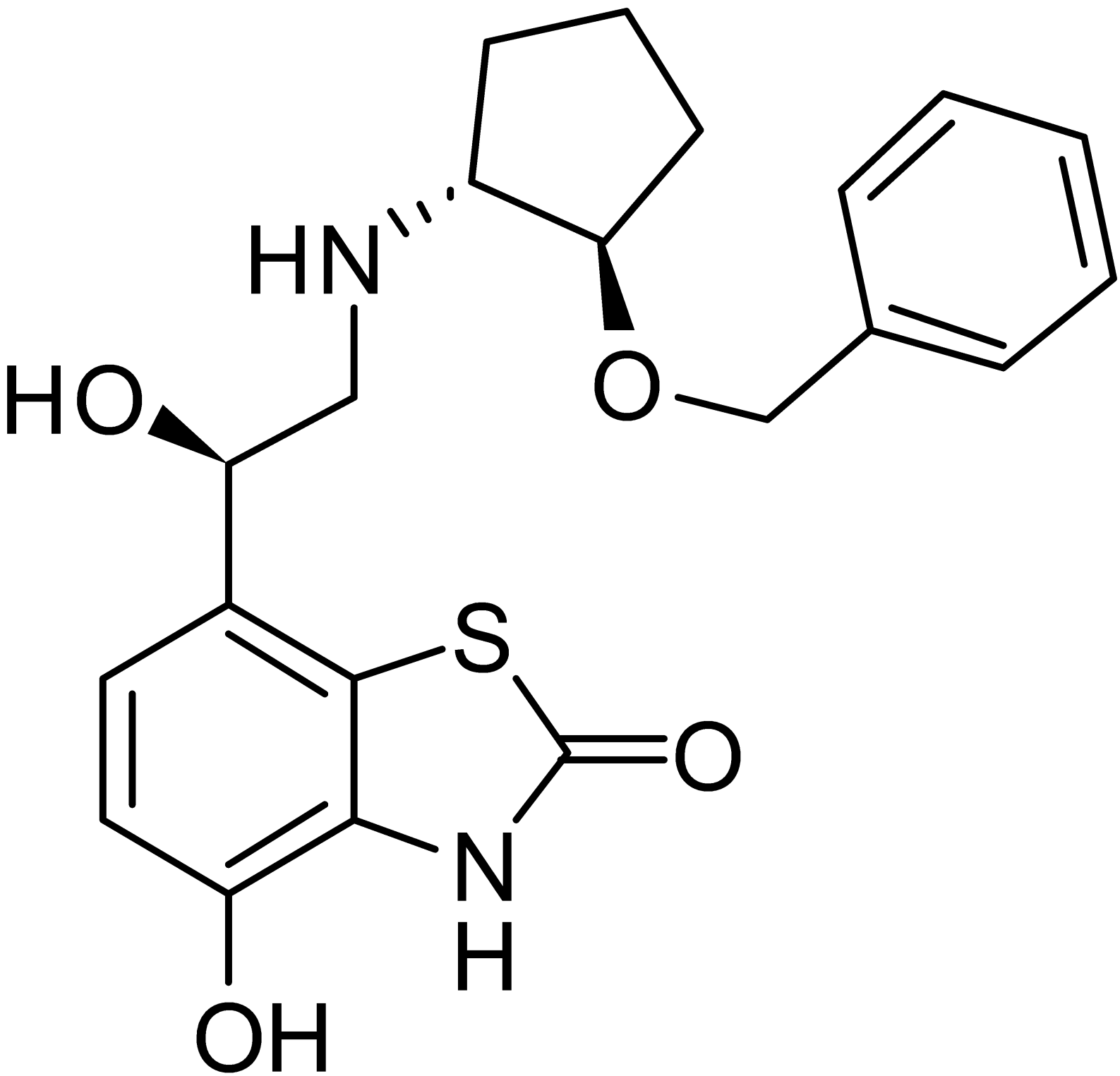


Figure 2

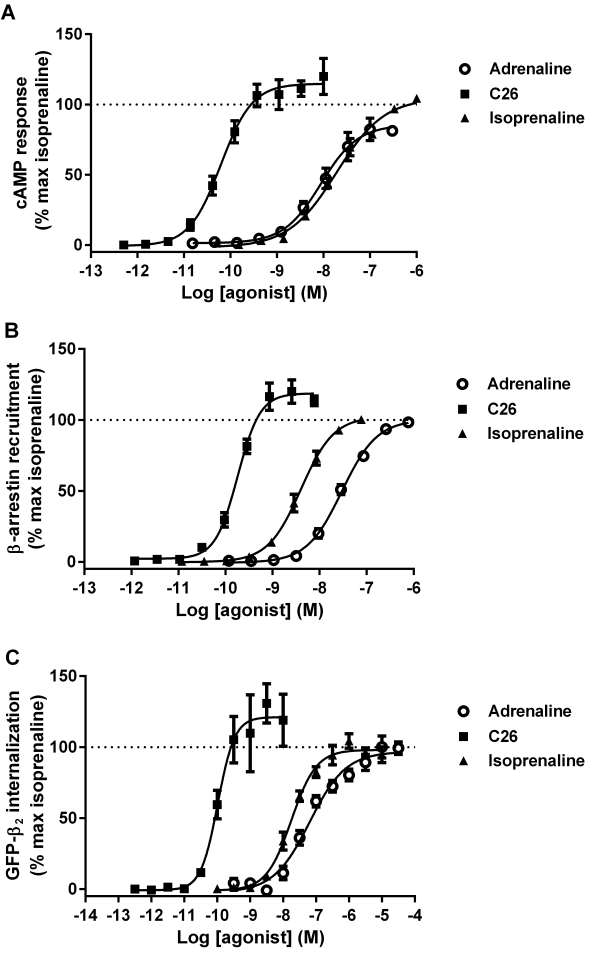

A
cAMP response
(% max isoprenaline)
150
100
50
0
-13
-12
-11
-10
-9
-8
-7
-6
Log [agonist] (M)
Adrenaline
C26
Isoprenaline
B
β-arrestin recruitment
(% max isoprenaline)
150
100
50
0
-13
-12
-11
-10
-9
-8
-7
-6
Log [agonist] (M)
Adrenaline
C26
Isoprenaline
C
GFP-β2 internalization
(% max isoprenaline)
150
100
50
0
-14
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log [agonist] (M)
Adrenaline
C26
Isoprenaline

Figure 3

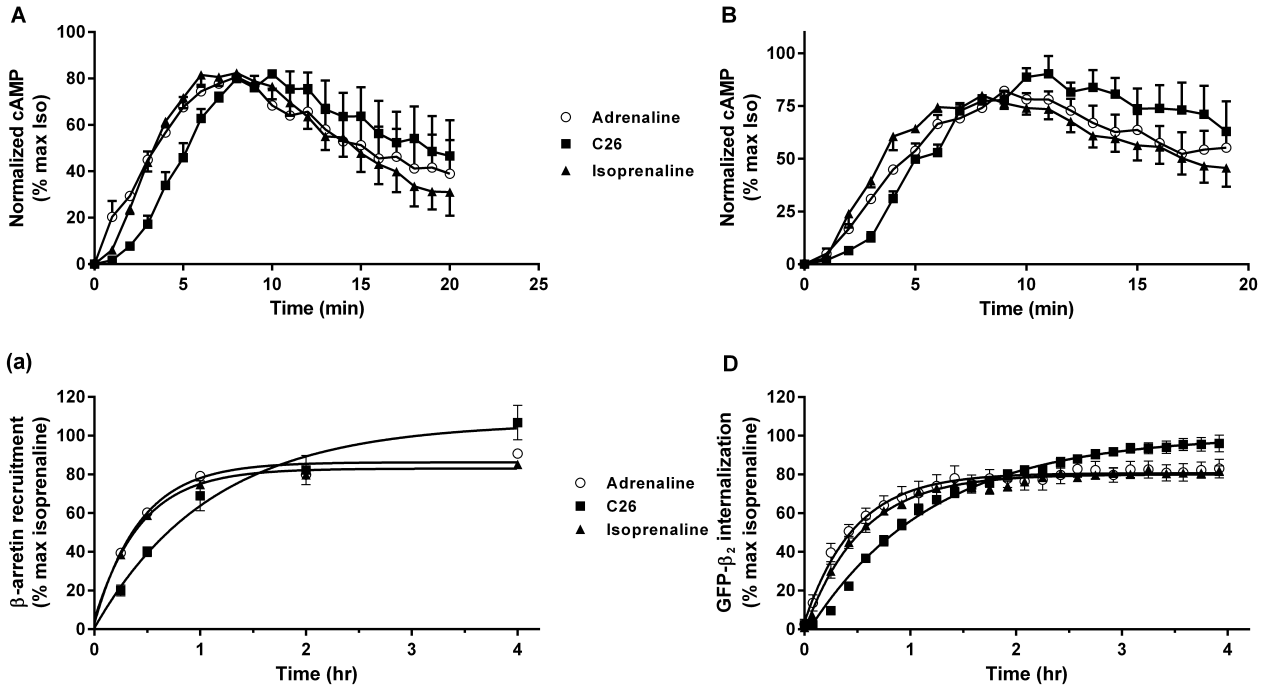

A
Normalized cAMP
(% max Iso)
Time (min)
Adrenaline
C26
Isoprenaline
B
Normalized cAMP
(% max Iso)
Time (min)
(a)
β-arretin recruitment
(% max isoprenaline)
Time (hr)
Adrenaline
C26
Isoprenaline
D
GFP-β2 internalization
(% max isoprenaline)
Time (hr)

Figure 4

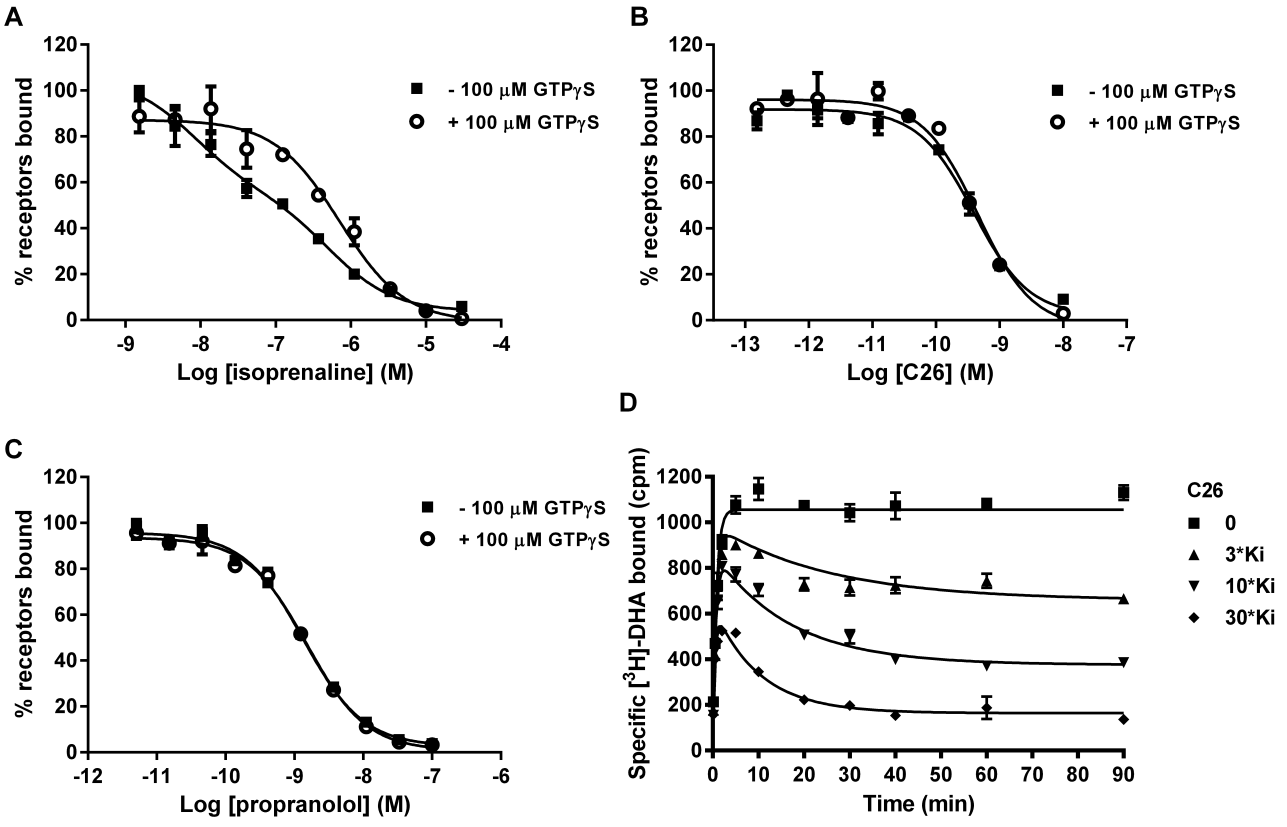

A
% receptors bound
Log [isoprenaline] (M)
- 100 μM GTPγS
+ 100 μM GTPγS
B
% receptors bound
Log [C26] (M)
- 100 μM GTPγS
+ 100 μM GTPγS
C
% receptors bound
Log [propranolol] (M)
- 100 μM GTPγS
+ 100 μM GTPγS
D
Specific [3H]-DHA bound (cpm)
Time (min)
C26
0
3*Ki
10*Ki
30*Ki

Figure 5

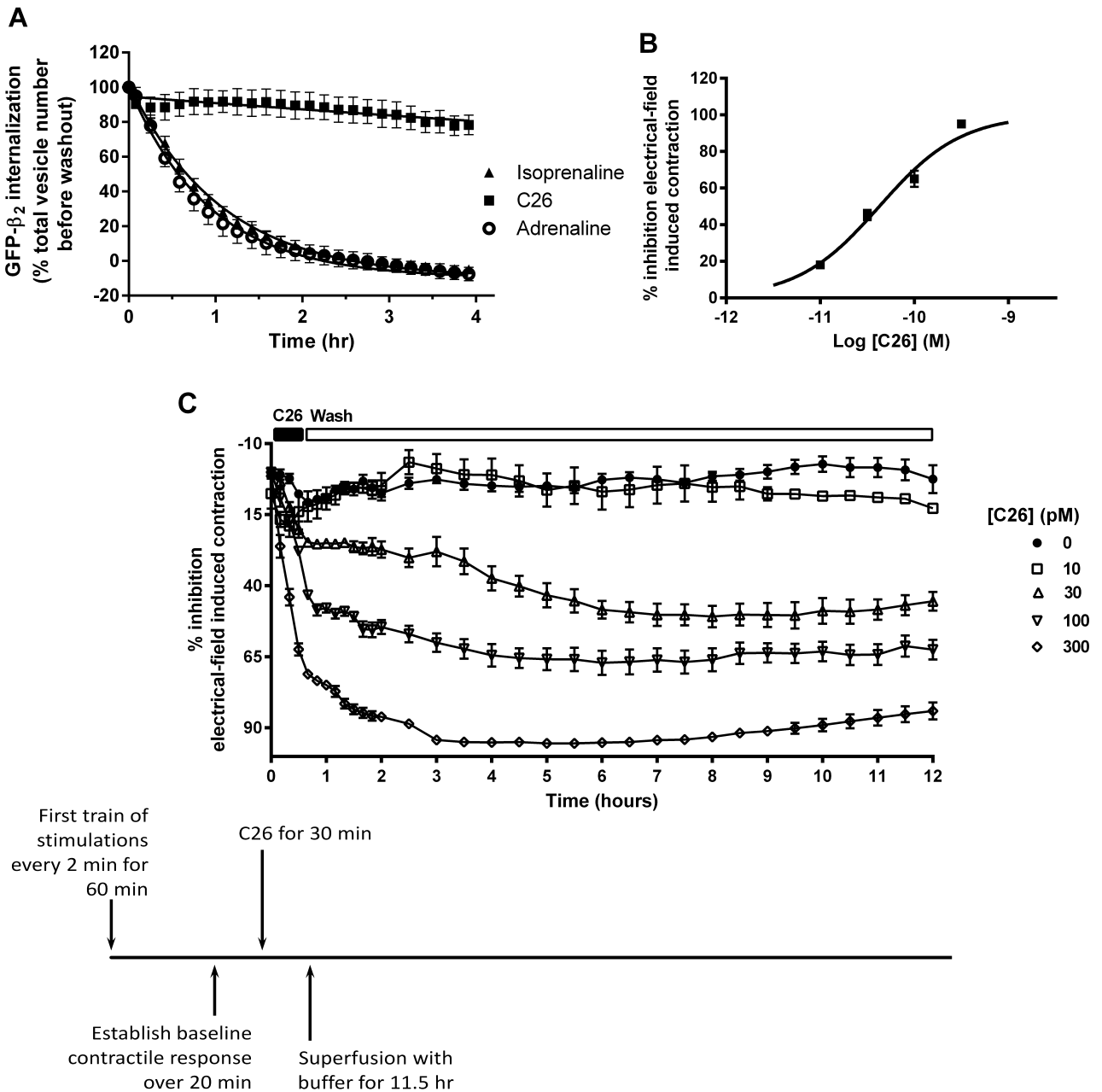
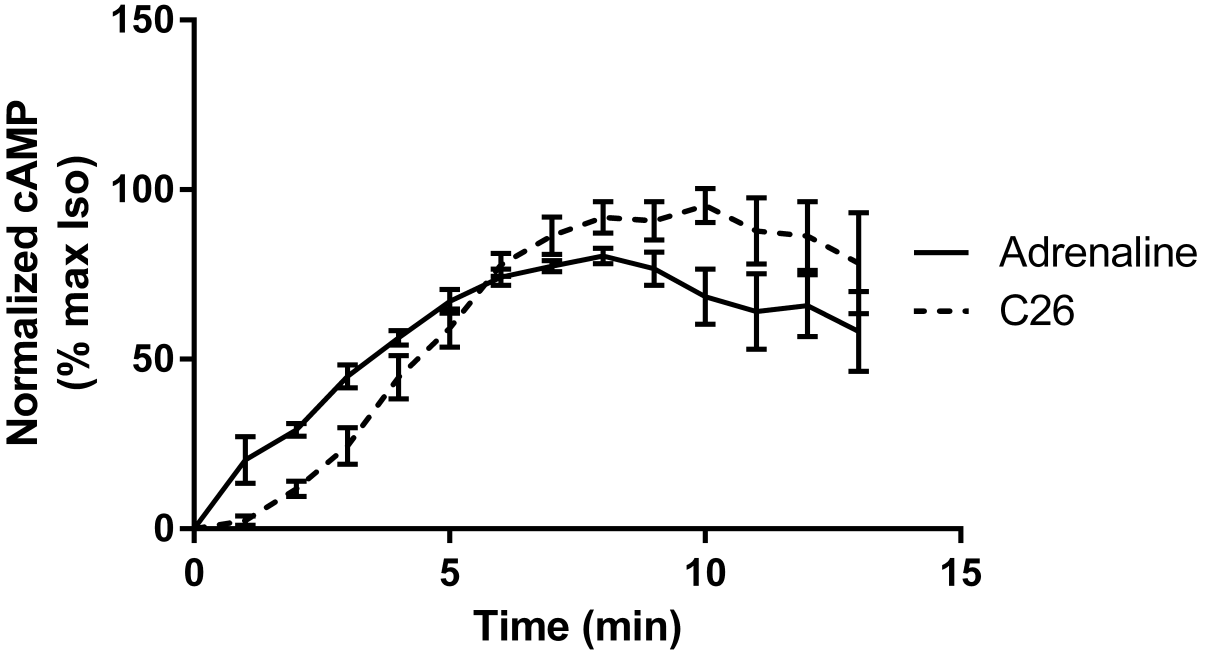

A
GFP-β2 internalization (% total vesicle number before washout)
Time (hr)
Isoprenaline
C26
Adrenaline
B
% inhibition electrical-field induced contraction
Log [C26] (M)
C
C26 Wash
% inhibition electrical-field induced contraction
Time (hours)
[C26] (pM)
0
10
30
100
300
First train of stimulations every 2 min for 60 min
C26 for 30 min
Establish baseline contractile response over 20 min
Superfusion with buffer for 11.5 hr

Figure 6


150
100
50
0
Normalized cAMP
(% max Iso)
0
5
10
15
Time (min)
Adrenaline
C26