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Evidence for pleiotropic signalling at the mouse β_3-adrenoceptor revealed by SR59230A

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Running title: Pleiotropic coupling of the β_3-adrenoceptor

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Abbreviations: B_{max}, maximum number of binding sites; cAMP, 3'-5'-cyclic adenosine monophosphate; CHO-K1, Chinese hamster ovary cells; CL316243, (**R,R**)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]-propyl]1,3-benzodioxole-2,2-dicarboxylate; DMEM, Dulbecco's modified Eagle's Medium; ECAR, extracellular acidification rate; FBS, foetal bovine serum; L748337, (*S*)-*N*-[4-[2-[[3-[-(Acetamidomethyl)phenoxy]-2-hydroxypropyl]amino]ethyl]phenyl]benzene sulfonamide; R_{max}, maximal relaxation; SR59230A, 3-(2-ethylphenoxy)-1-[(1,S)-1,2,3,4-tetrahydronapth-1-ylamino]-2S-2-propanol oxalate

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Abstract

This study examines the action of the β_3-adrenoceptor antagonist 3-(2-ethylphenoxy)-1-[(1,**S**)-1,2,3,4-tetrahydronapth-1-ylamino]-2**S**-2-propanoloxalate (SR59230A) at cloned mouse β_3-adrenoceptors expressed in Chinese hamster ovary cells (CHO-K1-β_3), or endogenously expressed in 3T3-F442A adipocytes or ileum. SR59230A displayed partial agonist properties compared to the β_3-adrenoceptor agonist CL316243 ((**R,R**)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]-propyl]1,3-benzodioxole-2,2-dicarboxylate) in CHO-K1-β_3 with the intrinsic activity increasing with the level of receptor expression. Functional affinity values for SR59230A at each level of receptor expression were in agreement with pK_I values determined by binding. In cytosensor microphysiometer studies, SR59230A was a full agonist for increases in extracellular acidification rates (ECAR) at all levels of receptor expression, and antagonist actions were measurable only in medium or low expressing cells. In 3T3-F442A adipocytes, SR59230A antagonized CL316243-mediated increases of cAMP (3'-5'-cyclic adenosine monophosphate) and had no agonist actions. However, in the cytosensor microphysiometer, SR59230A (acting via β_3-adrenoceptors) was an agonist with an intrinsic activity greater than CL316243. In mouse ileum, SR59230A relaxed smooth muscle, although concentration-response curves were biphasic. Relaxant effects were produced by concentrations that did not affect cAMP levels. Differences in tissue responses to SR59230A were not due to major differences in expression of Gαs. ECAR responses were not affected by pretreatment of cells with pertussis toxin indicating that signalling did not involve Gi. SR59230A therefore displays both agonist and antagonist actions at the mouse β_3-adrenoceptor. Since SR59230A only antagonized accumulation of cAMP in 3T3-

F442A adipocytes yet, in the same cells, was an agonist for ECAR, part of the agonist actions in the microphysiometer must be mediated by cAMP-independent signalling pathways.

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Introduction

β_3-Adrenoceptors are pharmacologically characterised by a set of criteria (Arch and Kaumann, 1993; Emorine et al., 1994; Strosberg and Pietri-Rouxel, 1996) that include; (a) low affinity and potency for conventional β-adrenoceptor antagonists and agonists, including radioligands; (b) low stereoselectivity of agonist and antagonist stereoisomers relative to those at typical β-adrenoceptors; (c) partial agonist activity of several β_1-/β_2-adrenoceptor antagonists such as pindolol and CGP12177A; (d) high affinity and potency of selective agonists such as CL316243 and BRL37344; and (e) antagonism by the β_3-adrenoceptor antagonist SR59230A. β-adrenoceptors exhibiting a pharmacological profile consistent with that of the β_3-adrenoceptor have been cloned from various species, including mouse, rat and human (Emorine et al., 1989; Granneman et al., 1991; Muzzin et al., 1991; Nahmias et al., 1991).

Unlike β_1- and β_2-adrenoceptors, which are intronless, all β_3-adrenoceptors so far described contain introns (Granneman et al., 1992). In the case of the mouse β_3-adrenoceptor, alternative splicing results in the expression of two splice variants, the β_{3a}- and the β_{3b}-adrenoceptor (Evans et al., 1999). Functional studies of the β_{3a}- and β_{3b}-adrenoceptor expressed in CHO-K1 cells, using either ECAR in the cytosensor microphysiometer or cAMP generation as bioassays, have shown that both the β_{3a}- and the β_{3b}-adrenoceptor couple to G_s whereas in addition the β_{3b}-adrenoceptor couples to G_i (Hutchinson et al., 2002). Both splice variants also couple to Erk1/2 by a mechanism that does not involve either the generation of cAMP or the activation of G_i, suggesting that additional pathways can be activated that are not linked sequentially to the classical pathways (Hutchinson et al., 2002). It is therefore

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possible that the study of a variety of β_3-adrenoceptor ligands may reveal differences in intrinsic activity at the different signalling pathways and thus shed light on the mechanisms involved.

SR59230A was the first selective β_3-adrenoceptor antagonist described (Manara et al., 1996), and has been shown to competitively antagonize β_3-adrenoceptor-mediated responses in a wide variety of tissues with greater preference for β_3-adrenoceptors compared to β_1-/β_2-adrenoceptors (Manara et al., 1996; Nisoli et al., 1996). However recent reports indicate that SR59230A also interacts with other β-adrenoceptors (Candelore et al., 1999; Hutchinson et al., 2001; Brahmadevara et al., 2001; Yamanishi et al., 2002), and agonist actions have been reported at the cloned human β_3-adrenoceptor (Strosberg & Pietri-Rouxel, 1997; Candelore et al., 1999) and at β_3-adrenoceptor in some rodent tissues (Horinouchi et al., 2001; Dumas et al., 1998; Brahmadevara et al., 2001).

In this study we examined the pharmacological properties of SR59230A at the cloned mouse β_3-adrenoceptor expressed in CHO-K1 cells and at the endogenous β_3-adrenoceptor expressed in mouse 3T3-F442A adipocytes or ileum. With respect to its effects on cAMP accumulation, SR59230A behaved as a classical partial agonist relative to CL316243, with agonist properties being more pronounced as levels of receptor expression increased. However at low levels of expression in CHO-K1 cells and in mouse 3T3-F442A adipocytes that endogenously express β_3-adrenoceptor, SR59230A behaved as a full agonist for ECAR, in some cases exceeding the maximal response attained by CL316243 while acting as a competitive antagonist in the same preparations for cAMP accumulation. The ECAR responses in CHO-K1 cells at all

levels of expression and in 3T3-F442A adipocytes were not affected by pretreatment of cells with pertussis toxin. These results suggest that the agonist actions of SR59230A in the cytosensor microphysiometer are not mediated by increases in cAMP levels, or by activation of Gi coupled mechanisms.

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Methods

Expression of the mouse β_3-adrenoceptors in CHO-K1 cells

The mouse β_3-adrenoceptor (β_{3a}-adrenoceptor; Evans et al., 1999) was cloned and stably transfected into CHO-K1 cells as previously described (Hutchinson et al., 2002).

Cell culture

CHO-K1 cells were grown in 50:50 Dulbecco's modified Eagle's Medium (DMEM): Ham's F-12 medium containing 10% (v/v) heat-inactivated foetal bovine serum (FBS) as previously described (Hutchinson *et al*., 2002).

3T3-F442A preadipocytes were maintained in DMEM supplemented with 10% (v/v) iron fortified calf serum, glutamine (2 mM), penicillin (100 Units/ml), streptomycin (100 μg/ml) and fungizone (2.5 Units/ml) under 5% CO_2 at 37°C. Preadipocytes were not allowed to reach more than 60% confluence under subculturing conditions. Upon reaching 90% confluence, 3T3-F442A cells were differentiated into adipocytes by culturing in the above media (substituting the iron fortified calf serum with FBS) containing insulin (5 μg/ml) for 7 days with media changes every 2 days. By day 7, >90% of the cells had differentiated into adipocytes, identified by accumulation of cytoplasmic lipid.

Mouse ileal smooth muscle strips were prepared using the following protocol. Mice (FVB male, 8-14 weeks) were anaesthetised with 80% CO_2/20% O_2 and decapitated. A 10-15 cm segment of the small intestine finishing 2-3 cm above the ileocaecal

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junction was removed and its contents flushed out with warm (37°C) Krebs-Henseleit buffer (composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 2.5, pH 7.4) containing ascorbic acid (0.1 mM) and EDTA (0.04 mM), and then with Hanks buffer containing 200 mM L-glutamine. Tissues were pinned out on a Petri dish containing Hanks balanced salt solution and the mesenteric fat carefully dissected away. The tissues were then cut open along the mesenteric fat line and pinned out as a flat tissue. The mucosa was gently scraped off with a sterile microscope slide. Tissues were then cut into segments of approximately 1 cm in length, washed in DMEM media and cultured at 37°C in 5% CO_2 in 12-well plates containing DMEM (with 200 mM L-glutamine) for 2 h.

Receptor binding assay

Membranes from mouse ileum or 3T3-F442A adipocytes were prepared and saturation experiments performed as previously described (Hutchinson et al., 2000; 2002). Competition binding experiments were performed at room temperature in a total volume of 100 µl in 96-well plates. Homogenate (10-40 µg) was incubated with [^{125}I]-cyanopindolol (500 pM) and a range of concentrations of SR59230A for 60 min in the absence or presence of (-)-alprenolol (1mM) to define non-specific binding. Experiments were performed in duplicate. All reactions were terminated by rapid filtration using a Packard Cell Harvester through GF/C filters pre-soaked for 30 min in 0.5% polyethylenimine. Filters were washed 4 times with wash buffer (50 mM Tris pH 7.4 4°C), dried, 30 µl Microscint-O added and radioactivity measured using a Packard Top Count.

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Cytosensor microphysiometer studies

The cytosensor microphysiometer (Molecular Devices Corp., California, U.S.A.) was used to measure β_3-adrenoceptor-mediated increases in ECARs as previously described (Hutchinson et al., 2002). CHO-K1 cells expressing the β_3-adrenoceptor were seeded into 12 mm transwell inserts (Costar) at $5x10^5$ cells per cup and left to adhere overnight. In experiments using 3T3-F442A adipocytes, cells ($1x10^5$) were seeded into 12 mm transwell inserts (treated with poly-D-lysine) for 1-2 days before media was changed to that containing insulin and left to differentiate in the inserts for 7 days before use in the cytosensor. Where required, cells were incubated with pertussis toxin (100 ng/ml) overnight before use. On the day of experiment, cells were equilibrated for 2 h (1 h for 3T3-F442A adipocyte cells), and cumulative concentration-response curves to CL316243 or SR59230A constructed in paired sister cells with each concentration of drug exposed to cells for 14 min (in some experiments curves were also constructed with bupranolol or (*S*)-*N*-[4-[2-[[3-[-(Acetamidomethyl)phenoxy]-2-hydroxypropyl]amino]ethyl]phenyl]benzene sulfonamide (L748337)). In other experiments with 3T3-F442A cells, concentration-response curves to either CL316243 or SR59230A were conducted in the presence/absence of (-)-propranolol. All drugs were diluted in modified RPMI1640. All results are expressed as a percentage of the maximal response to CL316243 or SR59230A in a given experiment.

cAMP accumulation studies

CHO-K1 cells ($1x10^5$ cells per well) were grown in 12-well plates in DMEM/Ham's F-12 medium containing 0.5% (v/v) FBS for 2 days. 3T3-F442A adipocytes ($1x10^5$ cells per well) were differentiated as above. On the day of the experiment, medium was replaced with one containing 3-isobutyl-1-methylxanthine (1 mM) and ascorbic

acid (1 mM) for 2 h before exposure to CL316243 or SR59230A for 30 min. To examine the antagonist effect of SR59230A on CL316243-mediated responses SR59230A was incubated with cells for 10 min before the addition of a single submaximal concentration of CL316243 for 20 min (in 3T3-F442A cells, (-)-propranolol was investigated for antagonist actions on CL316243 using the same protocol). cAMP was extracted as described previously (Hutchinson et al., 2002) and measured using a commercial kit (Amersham Pharmacia TRK 432). All experiments were performed in duplicate with *n* referring to the number of independent experiments.

Segments of mouse ileum were prepared as described above. Following an initial equilibration period of 2 h, media was replaced with one containing 3-isobutyl-1-methylxanthine (1 mM) and ascorbic acid (1 mM) for 1 h. Tissues were exposed to drugs for 30 min. Following treatment of ileum segments, tissues were blotted dry and snap frozen in liquid nitrogen and stored at -70°C. cAMP was extracted by homogenisation of tissues in 0.8 ml ice-cold 75% (v/v) ethanol containing EDTA (1 mM). Samples were centrifuged (2,000 x g, 2 min, 4°C), the supernatant transferred to a new tube and cAMP measured as above. Data were normalized for protein (Lowry et al 1951).

Detection of Gαs by immunoblotting

Total cell lysates from mouse ileum were obtained by homogenisation of frozen issue samples in RIPA buffer (1 x phosphate buffered saline, 1% v/v Igepal CA-630, 0.5% v/v sodium deoxycholate, 0.1% v/v sodium lauryl sulfate, 1mM phenylmethy sulfonyl fluoride, 1mM sodium orthovanadate, 1 μg/ml aprotinin) with a Dounce homogeniser.

Monolayer cells were lysed directly with RIPA buffer. Cell debris was removed by centrifugation at 10,000 x g (10 min, 4ºC) and protein concentrations determined (Lowry et al 1951). Total cell lysates (40µg protein) were electrophoresed on 12% polyacrylamide gels and electrotransfered to Hybond-P membranes (pore size 0.45µm, Amersham). After blocking with 5% nonfat dry milk in Tris buffered saline for 1h at room temperature, membranes were incubated with anti-Gαs antibody (1:1000 dilution; Upstate) overnight at 4ºC, and detected using a secondary antibody (HRP linked anti-rabbit IgG) diluted 1:2000 and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Organ bath studies

Organ bath studies were performed as previously described (Hutchinson et al., 2001). Tissues were mounted on hooks suspended in jacketed organ baths in 6 ml Krebs-Henseleit solution containing ascorbic acid (0.1 mM) and EDTA (0.04 mM) and equilibrated for 30 min. Tissues were contracted with carbachol (1 µM, 10-15 min) before cumulative concentration-response curves to either CL316243 or SR59230A were constructed at increments of 0.5 log units until a stable state was observed. At the end of each concentration-response curve, tissues were maximally relaxed with papaverine (10 µM) and all responses expressed as a percentage of this response.

Data Analysis

[^{125}I]-cyanopindolol saturation binding isotherms were analyzed via nonlinear regression using GraphPad PRISM 3.0 (GraphPad Software, San Diego, CA) using a one-site mass action model to derive estimates of the radioligand dissociation constant (K_D) and maximal density of receptor binding sites (B_{max}). Competition binding

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isotherms were analyzed according to a one-site mass action model via non-linear regression using PRISM in order to derive estimates of competitor potency estimates (IC_{50} values), which were then converted to K_I values (competitor-receptor dissociation equilibrium constant) (Cheng and Prusoff 1973).

For the functional assays of receptor-mediated cAMP accumulation or changes in ECAR, empirical estimates of agonist potency and intrinsic activity were derived from fitting the data to the Hill equation using PRISM:

$$E = Basal + \frac{Range}{1 + \left(\frac{[EC_{50}]}{[A]} \right)^{n_H}} \qquad (1)$$

where E is the effect, [A] is the concentration of agonist, Basal is the minimal asymptotic effect in the absence of agonist, Range is the maximal response range (i.e., the difference between the minimum response in the absence of ligand and the maximum response in the presence of ligand), n_H is the Hill slope and EC_{50} is the midpoint location (potency) parameter. Additional analyses of cAMP experiments performed in CHO cells were undertaken to determine functional estimates of the affinity of SR59230A as an agonist. The "comparative method" (Barlow et al., 1967), was used whereby the SR59230A concentration-response curve was compared to that of a reference full agonist (CL316243) curve constructed in the same cell line. For the analysis, CL316243 data were fitted to equation (1), whereas the SR59230A concentration-response data were fitted to the following form of the operational model of agonism (Black and Leff, 1983):

$$E = \frac{E_m \cdot \tau^n \cdot [A]^n}{(K_A + [A])^n + \tau^n \cdot [A]^n} + Basal \qquad (2)$$

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where E_m is the maximum possible response of the cell above Basal, K_A is the agonist-receptor equilibrium dissociation constant, n is the slope of the transducer function linking occupancy to response, and τ is the operational definition of intrinsic activity. For this latter analysis, the parameters E_m and n in Equation (2) were shared with the values of Range and n_H, respectively, from Equation (1), thus allowing the estimation of K_A and τ for SR59230A (Leff et al., 1993). The parameters Range and n_H are good estimates of E_m and n, respectively, for a full agonist in a given receptor-transducer system. Thus, the full agonist, CL316243, provided the reference parameters E_m and n for the determination of the SR59230A K_A and τ values. This analysis requires global curve-fitting with parameters shared between multiple datasets, and was performed using a pre-release version of GraphPad PRISM 4.0.

In functional experiments where SR59230A was an antagonist of CL316243-mediated responses, K_B values were calculated according to the method of Furchgott (1972). Because these experiments tested the effects of one antagonist concentration, the derived potency estimate is referred to as an apparent K_B value.

In practice, all affinity and potency parameters were estimated as negative logarithms. All results are expressed as mean ± s.e.mean. Statistical significance was determined using 2-way ANOVA or Student's t-test. Probability values less than or equal to 0.05 were considered significant.

Drugs and reagents

CL316243 and SR59230A were gifts from Dr T. Nash (Wyeth-Ayerst) and Dr L. Manara (Sanofi-Midy) respectively. L748337 was a gift from Dr M. Candelore (Merck) and bupranolol from Schwarz Pharma (Berlin, Germany). Drugs and reagents were purchased as follows: EDTA (AJAX Chemicals, Melbourne, VIC, Australia); G418 (CalBiochem Corp, La Jolla, CA, U.S.A.); L(+)-ascorbic acid (Merck, Frankfurt, Germany); [^{125}I]-cyanopindolol (2200 Ci mmol^{-1}, NEN Life Science Products, Boston, MA, U.S.A.); carbachol (carbamylcholine chloride), IBMX, insulin (from bovine pancreas), papaverine hydrochloride, (-)-propranolol, poly-D-lysine (Sigma Chemical Company, St. Louis, MO, U.S.A.). Cell culture medium and supplements were obtained from Trace Biosciences (Castle Hill, NSW, Australia) except for iron fortified calf serum (CSL, Australia). All other drugs were of analytical grade.

Stock solutions of SR59230A were prepared in 50% distilled water, 25% ethanol and 25% DMSO. IBMX was dissolved in DMSO. All the remaining drugs were prepared in distilled water.

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Results

[^{125}I]-Cyanopindolol binding

We have previously reported the generation of CHO-K1 cells expressing the β_{3a}-adrenoceptor at three different levels of receptor density (Hutchinson et al., 2002). [^{125}I]-cyanopindolol binding occurred in a saturable manner in membranes from cells expressing receptors at high, medium or low levels (Hutchinson et al., 2002), and the radioligand binding parameters for each of these expression levels are shown in Table 1. Additional [^{125}I]-cyanopindolol saturation binding assays were undertaken with membranes from cells that endogenously express the mouse β_3-adrenoceptor. As shown in Table 1, the radioligand had comparable affinities for the native β_3-adrenoceptors in the FVB ileum, differentiated 3T3-F442A adipocytes and for the cloned β_{3a}-adrenoceptor in the CHO-K1 cells. However, the maximal density of binding sites of the native receptors most closely approximated that in the low-expressing CHO cells (Table 1).

Subsequent binding experiments were performed using SR59230A in competition with [^{125}I]-cyanopindolol. These data are summarized in Table 2, where it can be seen that SR59230A had higher pK_i values in CHO cells expressing recombinant β_3-adrenoceptors compared to the cells/tissues that endogenously express β_3-adrenoceptors. However when conducted in the presence of GTPγS (10μM) the pK_I values were reduced to values that were similar to the pK_A values obtained in the cyclic AMP functional experiments (Table 2) suggesting that the higher pK_I values were due to G-protein trapping.

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Agonist and antagonist effects of SR59230A at the cloned mouse β_3-adrenoceptor expressed in CHO-K1 cells

SR59230A increased cAMP accumulation in a concentration-dependent manner in CHO-K1 cells expressing the β_{3a}-adrenoceptor at all levels of expression investigated (Figure 1). However, the intrinsic activity of SR59230A was significantly lower than that of the β_3-adrenoceptor agonist, CL316243, in all instances (Figure 1, Table 3) and there were marked differences in the maximal levels of cAMP attained with the different expression levels. Because SR59230A behaved as a partial agonist relative to CL316243, operational model-fitting of the data was undertaken, allowing for the determination of functional estimates of the affinity of SR59230A at the cloned β_{3a}-adrenoceptor at each level of receptor expression (Table 2). These values were in good agreement with the pK_i values for SR59230A determined from radioligand binding assays.

In experiments that monitored changes in ECAR in CHO-K1 cells, SR59230A also displayed agonist behaviour, causing robust concentration-dependent increases in ECAR in all three β_3-adrenoceptor clones examined. However, in stark contrast to its partial agonist effects in the cAMP assays, SR59230A had an intrinsic activity equal to (CHO medium, CHO low) or significantly ($p < 0.05$) greater (CHO high) than that for CL316243, determined using sister cells studied in parallel (Figure 2, Table 4). As expression levels were lowered, the concentration-response curve for each agonist was shifted to the right (Figure 2, Table 4) and absolute maximal responses to both SR59230A and CL316243 also decreased (data not shown).

To examine the antagonist effect of SR59230A on CL316243-mediated increases in ECAR, CHO-K1 cells expressing low or medium levels of receptor were exposed to SR59230A (300nM) for 1 h prior to construction of CL316243 concentration-response curves (Figure 3). This concentration of SR59230A was chosen since, based on the binding results, it was appropriate for antagonism of β_3-adrenoceptor-mediated responses while producing a submaximal agonist response relative to CL316243 (Figure 3). In medium and low-expressing cells, SR59230A caused the expected initial increase in ECAR, but subsequent CL316243 concentration-response curves were shifted to the right in a parallel manner with no change in the maximal response compared to CL316243 concentration-response curves constructed in the absence of SR59230A. This shift was used to determined apparent pK_B values for SR59230A in both of the cell lines (Table 2). Similar experiments could not be performed in CHO-K1 cells expressing the highest levels of β_{3a}-adrenoceptors because low concentrations of SR59230A that would not be expected to appreciably antagonize CL316243, produced significant increases in ECAR in their own right (data not shown).

In untransfected CHO-K1 cells, no effects were observed to either CL316243 or SR59230A (up to 10μM) on cAMP accumulation or ECAR (data not shown).

Investigation of agonist effects on extracellular acidification rates by other β_3-adrenoceptor antagonists

To determine if the agonist properties of SR59230A were unique to this compound or if other agonists acting at the β_3-adrenoceptor were also capable of having agonist effects on ECAR, concentration-response curves were constructed with a “human

active" β_3-adrenoceptor antagonist L748337 and a non-specific β-adrenoceptor antagonist bupranolol, in cells expressing high levels of receptor (Figure 4, Table 5). L74337 was a full agonist with respect to both CL316243- or SR59230A-mediated responses, while bupranolol, had no agonist effect with respect to ECAR (preliminary experiments also indicated that propranolol and carvedilol both had agonist activity on ECAR). Bupranolol acted as an antagonist, capable of antagonizing both CL316243, SR59230A or L748337 mediated increases in ECAR.

Agonist and antagonist effects of SR59230A at the endogenous mouse β_3-adrenoceptor expressed in 3T3-F442A adipocytes

In order to determine whether differences in signalling efficiency of SR59230A for the cAMP pathway and ECAR were confined to cloned β_{3a}-adrenoceptors expressed in a recombinant system, subsequent experiments investigated the same bioassays with cells that natively express the mouse β_3-adrenoceptor. When cAMP accumulation was investigated in differentiated 3T3-F442A adipocytes, SR59230A produced no response, in contrast to the marked increase of cAMP levels following CL316243 treatment (pEC_{50} 7.91 ± 0.16; n=4) (Figure 5). However, SR59230A did bind to the β_3-adrenoceptor in adipocytes, as it was able to concentration-dependently inhibit cAMP production in response to a submaximal concentration of CL316243 (30nM), with a pIC_{50} value of 7.69 ± 0.41 (n=3) (Figure 5). Although differentiated 3T3-F442A adipocytes express all three β-adrenoceptor subtypes (Feve et al., 1991; El Hadri et al., 1996; 1997), CL316243-mediated increases in cAMP levels were not inhibited by the β_1-/β_2-adrenoceptor antagonist (-)-propranolol (300nM), the β_1-adrenoceptor antagonist CGP20712A (300nM) or the β_2-adrenoceptor antagonist

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ICI118551 (300nM) (data not shown), confirming that CL316243-mediated increases in cAMP are due to actions at the β_3-adrenoceptor.

When β_3-adrenoceptor-mediated changes in ECAR were monitored, a different profile of activity was observed. SR59230A caused concentration-dependent increases in ECAR in differentiated 3T3-F442A adipocytes with an intrinsic activity significantly ($p < 0.05$) greater than that of CL316243, determined in sister cells studied in parallel (Figure 6; Table 4). To further examine the effect of SR59230A on CL316243-mediated increases in ECAR, 3T3-F442A cells were exposed to SR59230A (300nM) for 1 h prior to construction of CL316243 concentration-response curves. SR59230A caused an initial increase in ECAR and subsequent construction of the CL316243 concentration-response curve showed that SR59230A shifted the CL316243 concentration-response curve to the right, with an apparent pK_B value close to that obtained in similar experiments conducted in the transfected CHO cells (Table 2). Maximum responses to CL316243 were not changed by SR59230A (300nM). To determine if the agonist actions of SR59230A were due to actions at β_1-/β_2-adrenoceptors that are also expressed in 3T3-F442A adipocytes, concentration-response curves to either CL316243 or SR59230A were conducted in the presence/absence of (-)-propranolol (300nM). Addition of (-)-propranolol did not alter basal ECAR and subsequent CL316243 or SR59230A concentration-response curves were not affected by (-)-propranolol (Figure 6).

Agonist effects of SR59230A on extracellular acidification rate are independent of Gi

Agonist activation of the β_3-adrenoceptor can produce signalling through Gi (Soeder et al., 1999; Chaudhry et al., 1994; Gerhardt et al., 1999; Hutchinson et al., 2002) and

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although there is evidence that the β_{3a}-adrenoceptor variant used in the present studies couples solely to Gs (Hutchinson et al., 2002) it is feasible that SR59230A may activate the Gi pathway. Pertussis toxin pretreatment of CHO-K1 cells expressing high, medium or low levels of β_{3a}-adrenoceptor had no effect on the ECAR response to SR59230A (Figure 7). Although 3T3-F442A adipocytes express both β_{3a}-adrenoceptors and the pertussis toxin-sensitive β_{3b}-adrenoceptor (Hutchinson et al., 2002; Hutchinson, unpublished observations), Pertussis toxin pretreatment had no significant effect on responses to SR59230A suggesting that the ECAR response to SR59230A is not mediated by Gi.

SR59230A-mediated relaxation of mouse ileum is independent of increases in cAMP levels

The findings in the cell lines suggested that SR59230A could produce agonist responses via the β_3-adrenoceptor in recombinant and natively-expressing cells by a mechanism unrelated to its ability to signal via the cAMP pathway. This hypothesis was further investigated using mouse ileum. SR59230A produced a concentration-dependent relaxation of mouse ileum that was biphasic (Figure 8). The first phase (pEC_{50} 7.97 ± 0.30, n=6) occurred at concentrations similar to those reported previously for (-)-isoprenaline (Hutchinson et al., 2001) and CL316243 (pEC_{50} 7.57 ± 0.53, n=5), and caused a response that was approximately 40% of the maximum relaxation to papaverine. The second phase failed to reach a maximum within the concentration range used. Investigation of cAMP accumulation in this tissue provided no evidence for an involvement of this second messenger in the actions of SR59230A. cAMP accumulation in ileum (n=6-7) was significantly increased compared to basal levels (68.07 ± 15.45 pmol cAMP/mg protein) by the direct activator of adenylate

cyclase, forskolin (100μM; 1392.40 ± 195.98 pmol cAMP/mg protein; ***p<0.0001), the β-adrenoceptor agonist (-)-isoprenaline (10μM; 177.82 ± 36.79 pmol cAMP/mg protein; **p<0.01) the β_3-adrenoceptor agonist CL316243 (10μM; 144.49 ± 23.11 pmol cAMP/mg protein; **p<0.01), but not by SR59230A (10μM; 75.90 ± 18.43 pmol cAMP/mg protein; NS).

Comparison of Gαs levels in cells and tissues by immunoblotting

Since differences in signalling observed between CHO cells, 3T3-F442A adipocytes and ileal smooth muscle could be due to differences in levels of Gαs proteins, Western blots were carried out using an antibody that recognises both the long and short isoforms of Gαs. In all tissues the dominant isoform present was the short Gαs isoform and it was expressed at similar levels in CHO cells, 3T3-F442A adipocytes and ileal smooth muscle (Figure 9) suggesting that the different signalling properties observed do not result from major differences in Gαs levels.

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Discussion

This study reveals that the β_3-adrenoceptor "antagonist", SR59230A, possesses agonist properties at cloned and natively expressed mouse β_3-adrenoceptors. The striking disparity between the effects of SR59230A and CL316243 when ECAR is used as the bioassay rather than cAMP accumulation provides strong evidence for pleiotropic coupling of the β_3-adrenoceptor to distinct signalling pathways that are differentially activated by the two ligands.

In initial experiments, SR59230A competed with [^{125}I]-cyanopindolol binding (Table 2) with affinity appropriate for binding to β_3-adrenoceptors (Candelore et al., 1999; Hutchinson et al., 2002). However, pK_I values in cells expressing recombinant β_3-adrenoceptors were greater than those determined in 3T3-F442A cells and mouse ileum. Since the binding assays were conducted in membrane homogenates, it is likely that receptor-G protein complexes would be present (Christopoulos and El-Fakahany, 1999) and assuming that total cellular G protein content was not limiting, this would cause an overestimate of apparent agonist affinity that would be greatest in cells expressing high receptor levels (Christopoulos and El-Fakahany, 1999). The reduction in pK_I values following addition of GTPγS supported this view.

The affinity of SR59230A was also determined using the concentration-response curve to the full agonist, CL316243, in cAMP assays, and operational model-fitting of SR59230A concentration-response curves allowed functional estimates of agonist affinity to be obtained (Table 2). The pK_A estimates were in agreement with pK_i estimates determined from binding assays, suggesting that the impact of G protein-

trapping in the membrane-based binding assays was minimal and restricted to the high expressing cells.

Interestingly, the use of SR59230A as an antagonist of CL316243-mediated increases in ECAR to estimate affinity yielded the largest discrepancies compared to the other two methods. The pK_B values for SR59230A were some 10-fold higher than pK_i or pK_A values from binding or cAMP assays (Table 2). It is possible that agonist properties of SR59230A during the initial 1 hr equilibration led to a partial desensitisation of CHO-K1-β_3 cells to CL316243. This would underestimate CL316243 potency in the presence of SR59230A, and hence an overestimation of the apparent affinity. Further experiments are required to examine this possibility.

SR59230A increased cAMP in CHO-K1-β_3 cells and was a partial agonist relative to CL316243 (Figure 1, Table 3), with the effect being dependent upon receptor expression; the reduction in receptor density across the three cell lines was associated with a corresponding reduction in agonist potency and intrinsic activity of both SR59230A and CL316243, as predicted by classical receptor theory (Christopoulos and El-Fakahany, 1999). In contrast, no agonist effects of SR59230A were detected on cAMP accumulation in mouse ileum or 3T3-F442A adipocytes, which endogenously express the mouse β_3-adrenoceptor. Given that our binding experiments show that these cells express β_3-adrenoceptors at similar levels to CHO-K1-β_3 low-expressing cells (Table 1), the agonist activity of SR59230A in CHO-K1-β_3 cAMP assays and absence of such activity in ileum or 3T3-F442A cells suggests that stimulus-response coupling in adipocytes and ileum is significantly weaker than in CHO-K1-β_3 cells. Accordingly, a partial agonist such as SR59230A would lose its

agonist properties under conditions of reduced stimulus-response coupling relative to the full agonist CL316243. The differences were not due to marked differences in the expression of Gαs in the different cells as immunoblots showed similar levels of the predominant Gαs small isoform in CHO cells, 3T3-F442A cells and ileum. Importantly, SR59230A antagonized CL316243-mediated increases in cAMP accumulation in 3T3-F442A cells (Figure 5) confirming that SR59230A still bound to β_3-adrenoceptors in these cells.

In contrast to cAMP accumulation, a strikingly different signalling profile was observed for SR59230A but not CL316243 when function was measured using the cytosensor microphysiometer. Although SR59230A had significantly lower potency than CL316243, it was full agonist for ECAR, with an intrinsic activity somewhat greater than CL316243 (Figure 2, Table 4). This was not restricted to CHO-K1-β_3 cells since the maximal ECAR response to SR59230A in 3T3-F442A adipocytes was greater than that for CL316243 (Figure 6). The ECAR response to SR59230A was not due to low levels of β_1-/β_2-adrenoceptors in differentiated 3T3-F442A adipocytes (El Hadri et al., 1997), since the β_1-/β_2-adrenoceptor antagonist (-)-propranolol failed to shift the concentration-response curves to either CL316243 or SR59230A in these cells.

These observations led us to consider mechanisms to account for such an effect. A higher intrinsic activity may indicate higher intrinsic activity for SR59230A than CL316243. However cAMP assays showed that SR59230A is a lower intrinsic activity agonist than CL316243. This property would be retained if the ECAR was linked solely to cAMP, i.e., downstream signal amplification could lead to

enhancement of the intrinsic activity of SR59230A, but not to a greater maximal response than CL316243 if both agonists activate the same signal transduction cascade. It is also possible that CL316243 activates G_s and G_i to mediate the responses detected as a change in ECAR, whereas SR59230A only activates G_s. The net result would be a greater maximal response to agonist signalling solely through the stimulatory pathway. However, the mouse β_{3a}-adrenoceptor, as used here, cannot couple to G_i (Hutchinson et al., 2002). It is more likely, therefore, that SR59230A couples to another signalling pathway, independently of its effects on cAMP, that is either not utilized or only weakly recruited by CL316243. In contrast to linked intracellular signalling cascades, which predict increases in both potency and intrinsic activity of agonists with increases in signal amplification, the summation of multiple independent signalling pathways that converge to a common endpoint such as ECAR can account for an enhanced response range to an agonist without postulating the need for increased agonist potency. This was also in accord with our experimental observations. If ECAR was linked to upstream changes in cAMP accumulation, the degree of signal amplification required to increase the SR59230A maximal response from that of a partial agonist in the cAMP assay to a full agonist in the cytosensor assay would also cause significant enhancement in agonist potency. Yet, a comparison of pEC_{50} values obtained in the CHO-K1 cells (Tables 3 and 4) clearly indicate that this is not the case.

Experiments using mouse ileum also provided evidence for relaxation responses to SR59230A not involving cAMP. SR59230A caused relaxation of the ileum significantly greater than that caused by CL316243 (Figure 8), but only the latter agent caused increases in cAMP. Horinouchi & Koike (2002) also suggested that β_3-

adrenoceptor-mediated relaxation of gastrointestinal tissues occurs independently of cAMP.

Currently, the pathway stimulated by SR59230A to increase ECAR is unknown. The β_3-adrenoceptor also signals through G_i to inhibit cAMP production, or to activate Erk1/2, c-Src, p38 MAPK, and the nitric oxide synthase pathway to increase cGMP production (Soeder et al., 1999; Lindquist et al., 2000; Chaudry et al., 1994; Cao et al., 2000; 2001; Gerhardt et al., 1999; Hutchinson et al., 2002; Trochu et al., 1999; Mizuno et al., 2002). Since the response was insensitive to pertussis toxin, Gi would not appear to be involved but it is feasible that SR59230A may activate one or more of the remaining signalling pathways. In guinea-pig duodenum and gastric fundus endogenously expressing β_3-adrenoceptors, SR59230A is an agonist with a potency similar to isoprenaline (Horinouchi and Koike, 2001). At high concentrations it causes relaxation of hypoxic pulmonary vasoconstriction in rats (Dumas et al., 1998) and rat aortic rings (Brahmadevara et al., 2003). We are further investigating the mechanism of SR59230A-mediated increases in ECAR.

Other β-adrenoceptor antagonists have agonist actions at β_3-adrenoceptors including tertatolol, alprenolol, propranolol, oxprenolol, pindolol, cyanopindolol, nadolol and carteolol (reviewed in Arch, 2000). We have shown here that in CHO-K1-β_3 cells, the β_3-adrenoceptor antagonist L748337 also has agonist actions on ECAR, whereas bupranolol is a classical antagonist of CL316243, SR59230A or L748337-mediated responses. Clearly the agonist effects are not confined to SR59230A and further analysis of other β-adrenoceptor antagonists may provide information on structural requirements for pleiotropic signalling.

Several reports suggest that receptors exist in multiple active states corresponding to different conformations with specific pharmacological and functional properties, and that different ligands and/or G-proteins affect these states in different ways (reviewed in Kenakin, 2002). For the human β_3-adrenoceptor transfected in CHO-K1 cells it has been suggested that three different states exist: one state favouring β_3-adrenoceptor signalling towards adenylate cyclase, another favouring Erk1/2, and other receptor states not discriminating between the two signalling pathways (Gerhardt et al 1999). This may also occur in mouse CHO-K1-β_3 cells since pEC_{50} values for ECAR, cAMP accumulation and Erk1/2 activation are markedly different when the receptor is stimulated with CL316243 (Hutchinson et al., 2002).

In conclusion, SR59230A displayed agonist properties at the mouse β_3-adrenoceptor that were increased with the level of receptor expression. However SR59230A displayed higher intrinsic activity in ECAR than cAMP accumulation bioassays. These differences were further accentuated in 3T3-F442A cells where SR59230A acted as a classical competitive antagonist for cAMP responses produced by CL316243, but as a full agonist with an intrinsic activity greater than CL316243 in the cytosensor microphysiometer. SR59230A is therefore capable of producing agonist responses in 3T3-F442A cells by a mechanism independent of cAMP.

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Footnotes

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Figure 1: The effect of the β_3-adrenoceptor "antagonist" SR59230A on cAMP accumulation in cells expressing the β_3-adrenoceptor at (a) high, (b) medium or (c) low receptor levels. The results are expressed as the amount of cAMP produced (pmol) per well. Basal and CL316243 stimulated cAMP accumulation is also indicated. Each point shows mean ± s.e.mean (n=3-5).

Figure 2: Comparison of the agonist effects of SR59230A in the cytosensor microphysiometer to increase extracellular acidification rates performed in parallel with CL316243-mediated increases in extracellular acidification rates in cells expressing the β_3-adrenoceptor at (a) high, (b) medium or (c) low receptor levels. The results are expressed as a percentage of the maximal response produced to the highest concentration of CL316243 in each experiment. Each point shows mean ± s.e.mean (n=4-8).

Figure 3: Interactions between CL316243 and SR59230A in cells expressing the cloned mouse β_3-adrenoceptor at (a) medium or (b) low receptor levels to increase extracellular acidification rates in the cytosensor microphysiometer. The results are expressed as a percentage of the maximal response produced to the highest concentration of CL316243 in each experiment. Each point shows mean ± s.e.mean (n=4-6).

Figure 4: (a) CL316243, the β_3-adrenoceptor "antagonists" SR59230A or L748337, but not the β-adrenoceptor antagonist bupranolol, increase extracellular acidification rates in cells expressing the cloned mouse β_3-adrenoceptor at high receptor levels. The results are expressed as a percentage of the maximal response produced to the highest concentration of CL316243 in each experiment. Each point shows mean ± s.e.mean (n=4). Bupranolol (100nM) competitively antagonises (b) CL316243- (c) SR59230A- or (d) L748337-mediated increases in extracellular acidification in cells

expressing the cloned mouse β_3-adrenoceptor at high receptor levels. The results are expressed as a percentage of the maximal response produced to the highest concentration of ligand in each experiment. Each point shows mean ± s.e.mean (n=4).

Figure 5: (a) SR59230A has no effect on cAMP levels in differentiated 3T3-F442A adipocytes in comparison to the increase mediated by CL316243. (b) However, in cells pre-treated with SR59230A before stimulation with CL316243 (30nM), SR59230A antagonised CL316243-mediated increases on cAMP levels. The results are expressed as the amount of cyclic AMP produced (pmol) per well. Each point shows mean ± s.e.mean (n=3-4).

Figure 6: (a) SR59230A and CL316243 both increase extracellular acidification rates in 3T3-F442A cells. The results are expressed as a percentage of the maximal response produced to the highest concentration of CL316243 in each experiment. Each point shows mean ± s.e.mean (n=8). (b) SR59230A (300nM) competitively antagonises CL316243-mediated increases in extracellular acidification in 3T3-F442A adipocytes. (-)-Propranolol (300nM) failed to antagonize (c) CL316243 or (d) SR59230A mediated increases in extracellular acidification rates in 3T3-F442A adipocytes. The results are expressed as a percentage of the maximal response produced to the highest concentration of CL316243 in each experiment. Each point shows mean ± s.e.mean (n=4).

Figure 7: The effect of pertussis toxin pretreatment (100ng/ml, 16 h) on extracellular acidification rate responses in CHO-K1 cells expressing (a) high, (b) medium and (c) low levels of mouse β_{3a}-adrenoceptors and also in (d) 3T3-F442A cells that endogenously express mouse β_3-adrenoceptors. The results are expressed as a percentage of the maximal response produced to the highest concentration of

CL316243 in control treated cells in each experiment. Each point shows mean ± s.e.mean (n=3-4).

Figure 8: SR59230A and CL316243 both relax mouse ileum precontracted with carbachol in a concentration-dependent manner. Each point shows mean ± s.e.mean (n=5-6).

Figure 9: Immunoblot of Gαs small and large isoforms in CHO-K1 cells expressing high, medium and low levels of β_{3a}-adrenoceptors, 3T3-F442A adipocytes and mouse ileum. The dominant isoform present was the small isoform which was expressed at similar levels in all tissues.**Table 1:** [^{125}I]-cyanopindolol saturation binding parameters for cloned and native mouse β_{3a}-adrenoceptors expressed in membranes derived from different cellular backgrounds. Parameters derived from nonlinear regression analysis represent the mean ± s.e. mean (n=3).

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Table 1: [^{125}I]-cyanopindolol saturation binding parameters for cloned and native mouse β_{3a}-ARs expressed in membranes derived from different cellular backgrounds. Parameters derived from nonlinear regression analysis represent the mean ± s.e. mean (n=3).

Membrane source	**pK_D** [a]	**B_{max}** [b] **(fmol/mg protein)**
CHO High [c]	9.04 ± 0.09	1118 ± 48
CHO Medium [c]	9.05 ± 0.04	587 ± 97
CHO Low [c]	9.46 ± 0.14	115 ± 6
FVB Mouse ileum	9.02 ± 0.14	159 ± 49
3T3-F442A	9.08 ± 0.09	166 ± 28

[a] Negative logarithm of the radioligand equilibrium dissociation constant

[b] Maximal density of binding sites

[c] Data taken from Hutchinson et al. (2002)

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Table 2: SR59230A affinity estimates derived from radioligand binding and/or functional assays in cells expressing the cloned or native mouse β_3-adrenoceptors. Parameters derived from nonlinear regression analysis represent the mean ± s.e. mean from the number of experiments indicated in parentheses.

Cell type	pK_I [a]	pK_A [b]	pK_B [c]
CHO High	8.24 ± 0.07 (4)	6.93 ± 0.48 (3)	n.d.
+ GTPγS 10μM	6.99 ± 0.14 (4)		
CHO Medium	7.88 ± 0.39 (4)	6.95 ± 0.48 (3)	8.23 ± 0.11 (8)
+ GTPγS 10μM	7.51 ± 0.41 (4)		
CHO Low	7.90 ± 0.33 (4)	6.53 ± 0.33 (3)	7.95 ± 0.15 (4)
+ GTPγS 10μM	6.93 ± 0.41 (4)		
FVB Mouse ileum	5.54 ± 0.28 (3)	n.d.	n.d.
3T3-F442A	5.79 ± 0.47 (3)	n.d.	7.91 ± 0.33 (4)

[a] Negative logarithm of the SR59230A dissociation constant as determined from [^{125}I]-cyanopindolol competition binding.

[b] Negative logarithm of the SR59230A dissociation constant as determined from operational model fitting of cyclic AMP functional data (Figure 1)

[c] Negative logarithm of the SR59230A apparent dissociation constant, as determined using a single concentration of antagonist against CL-mediated changes in extracellular acidification rate (Figures 3,6).

n.d. Not determined

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Table 3: Agonist potency (pEC_{50}) and intrinsic activity (IA) values for effects on cAMP accumulation in CHO-K1 cells expressing the mouse β_3-adrenoceptor at different receptor levels. Values are mean ± s.e.mean of the indicated number of experiments.

CHO-K1 β_3-adrenoceptor expression level		**pEC_{50}**	**IA[a]**	**n**
High	CL316243	9.74 ± 0.16	1.00 ± 0.19	5
	SR59230A	6.93 ± 0.49	0.75 ± 0.08	3
Medium	CL316243	9.15 ± 0.27	1.00 ± 0.02	4
	SR59230A	6.95 ± 0.48	0.39 ± 0.02	3
Low	CL316243	7.52 ± 0.35	1.00 ± 0.13	4
	SR59230A	6.53 ± 0.74	0.59 ± 0.06	3

[a] Defined as a fraction of the absolute maximal response to CL316243 in each individual experiment

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Table 4: Agonist potency (pEC_{50}) and intrinsic activity (IA) values for effects on ECAR at the cloned or the native mouse β_3-adrenoceptor. Values are mean ± s.e.mean of the indicated number of experiments.

Cell type	pEC_{50} CL316243	IA[a]	pEC_{50} SR59230A	IA[a]	n
CHO High	10.53 ± 0.07	1.00 ± 0.04	7.77 ± 0.10	1.15 ± 0.05	8
CHO Medium	9.83 ± 0.34	1.00 ± 0.07	7.05 ± 0.12	1.08 ± 0.05	4
CHO Low	9.11 ± 0.13	1.00 ± 0.04	6.12 ± 0.11	0.96 ± 0.10	8
3T3-F442A	8.34 ± 0.10	1.00 ± 0.04	5.66 ± 0.88	1.30 ± 0.11	8

[a] Defined as a fraction of the absolute maximal response to CL316243 in each individual experiment

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Table 5: Agonist potency values (pEC_{50}) for two β_3-adrenoceptor "antagonists" and pK_B values of bupranolol-mediated antagonism of agonist effects on ECAR at the cloned mouse β_3-adrenoceptor at high levels. Values are mean ± s.e.mean of the n=4.

Ligand	**pEC_{50}**	**pK_B**
CL316243	11.09 ± 0.12	
+ bupranolol (100nM)	9.31 ± 0.05	9.07 ± 0.21
SR59230A	7.12 ± 0.14	
+ bupranolol (100nM)	6.27 ± 0.05	7.84 ± 0.22
L748337	8.16 ± 0.09	
+ bupranolol (100nM)	6.49 ± 0.09	8.59 ± 0.10

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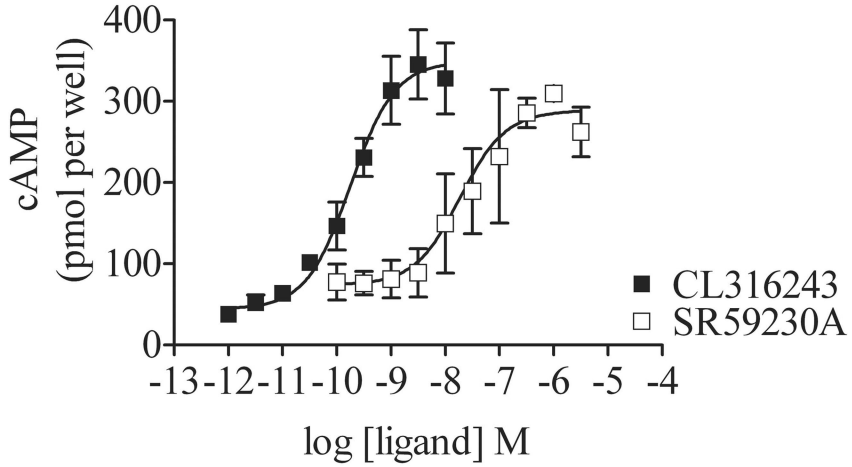
(a)



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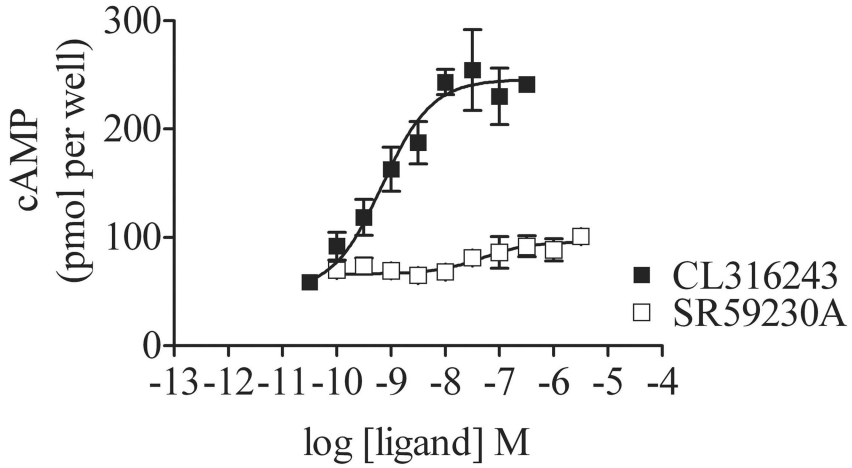

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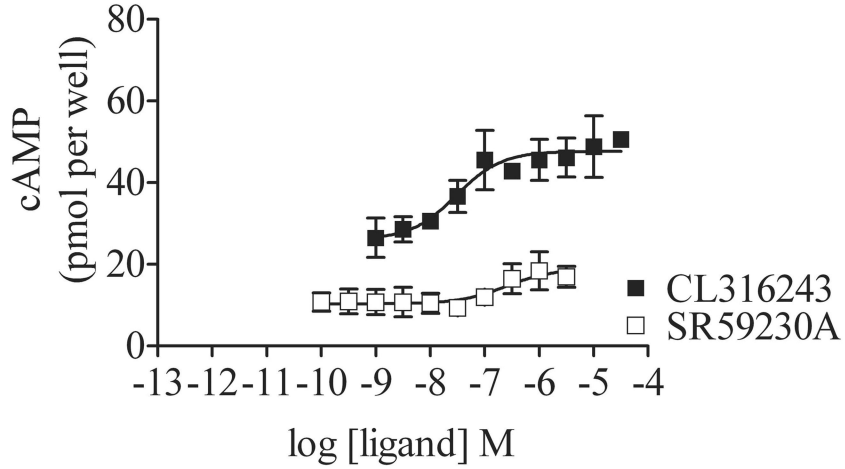

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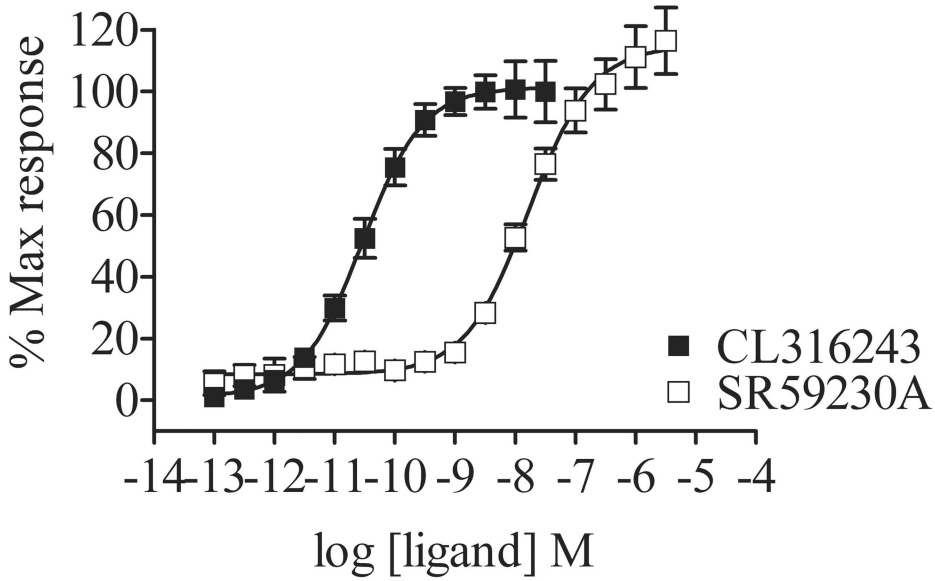

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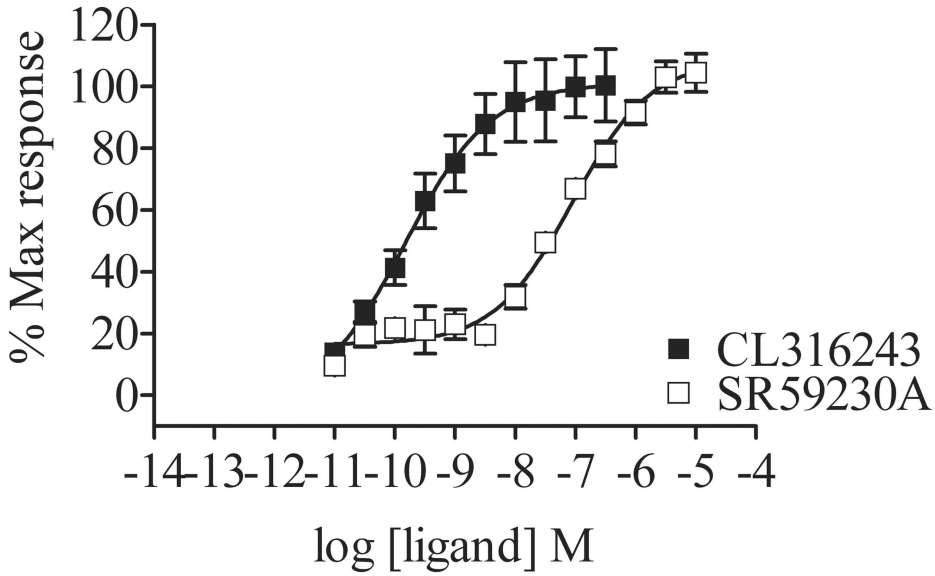

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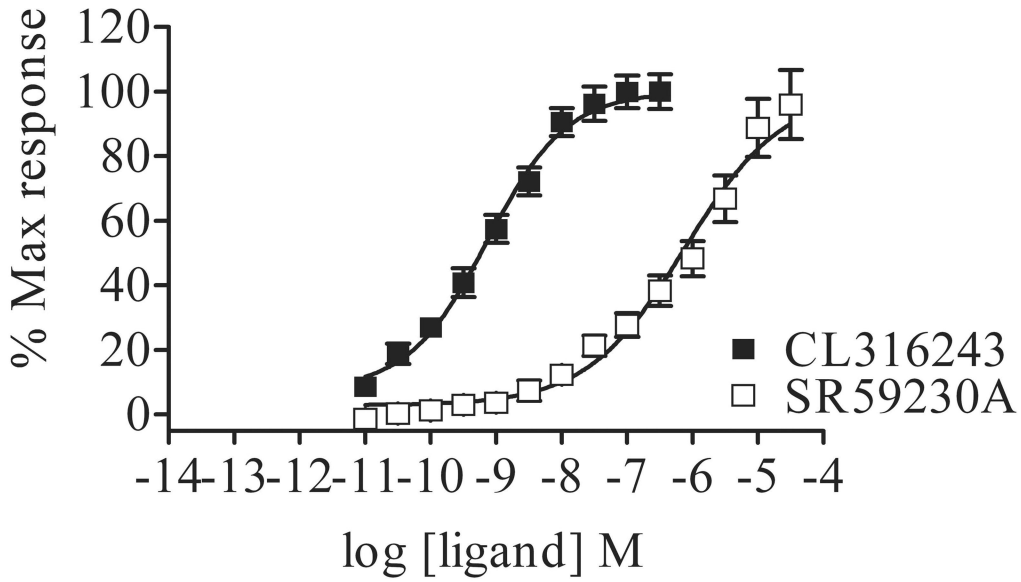

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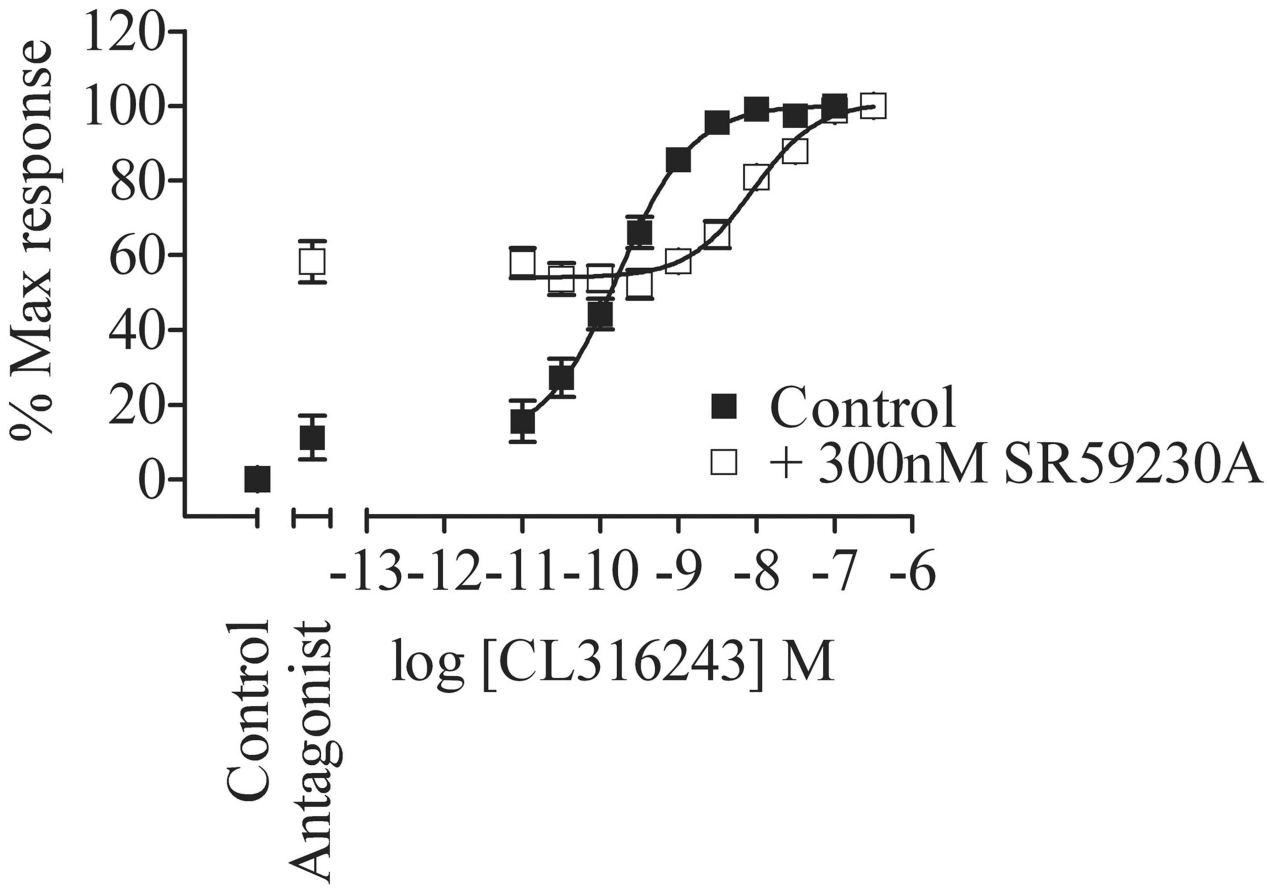

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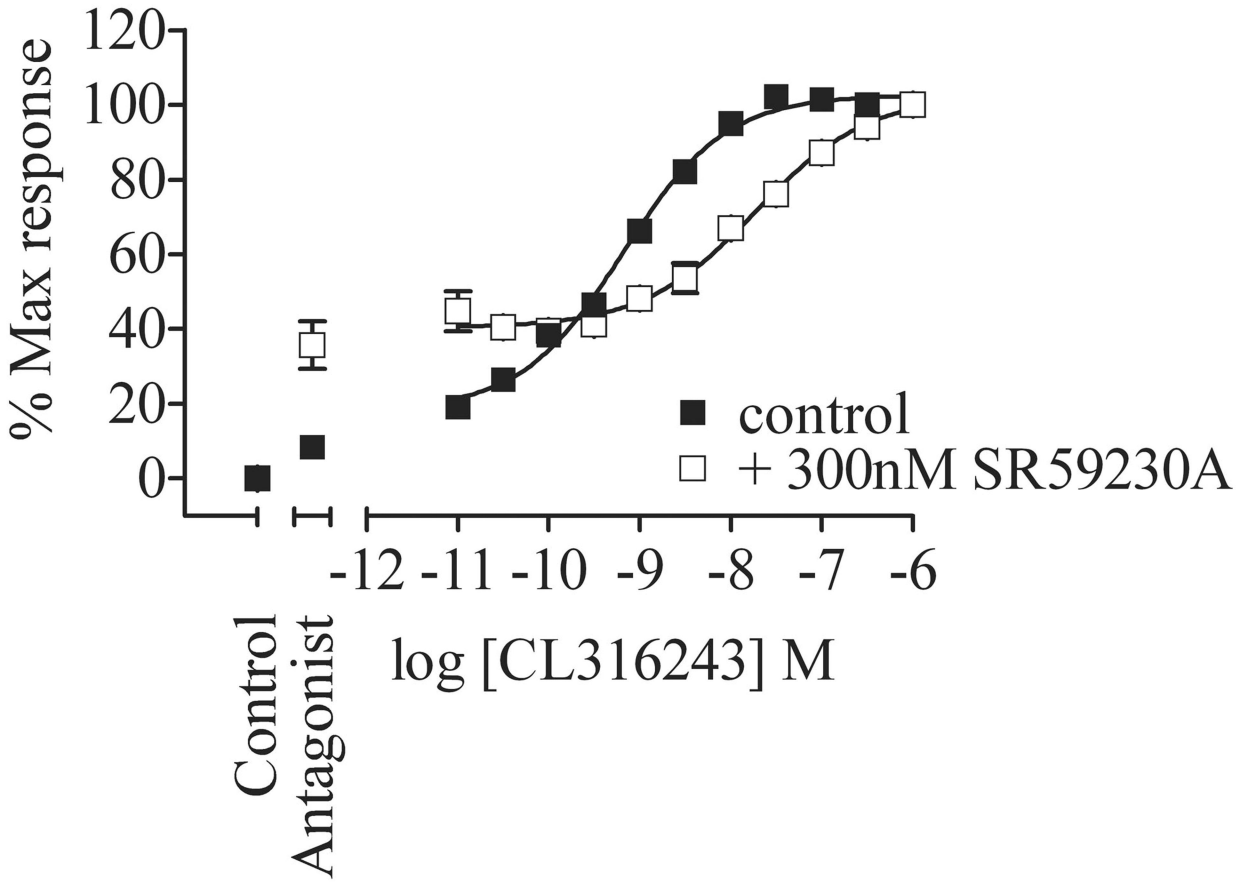

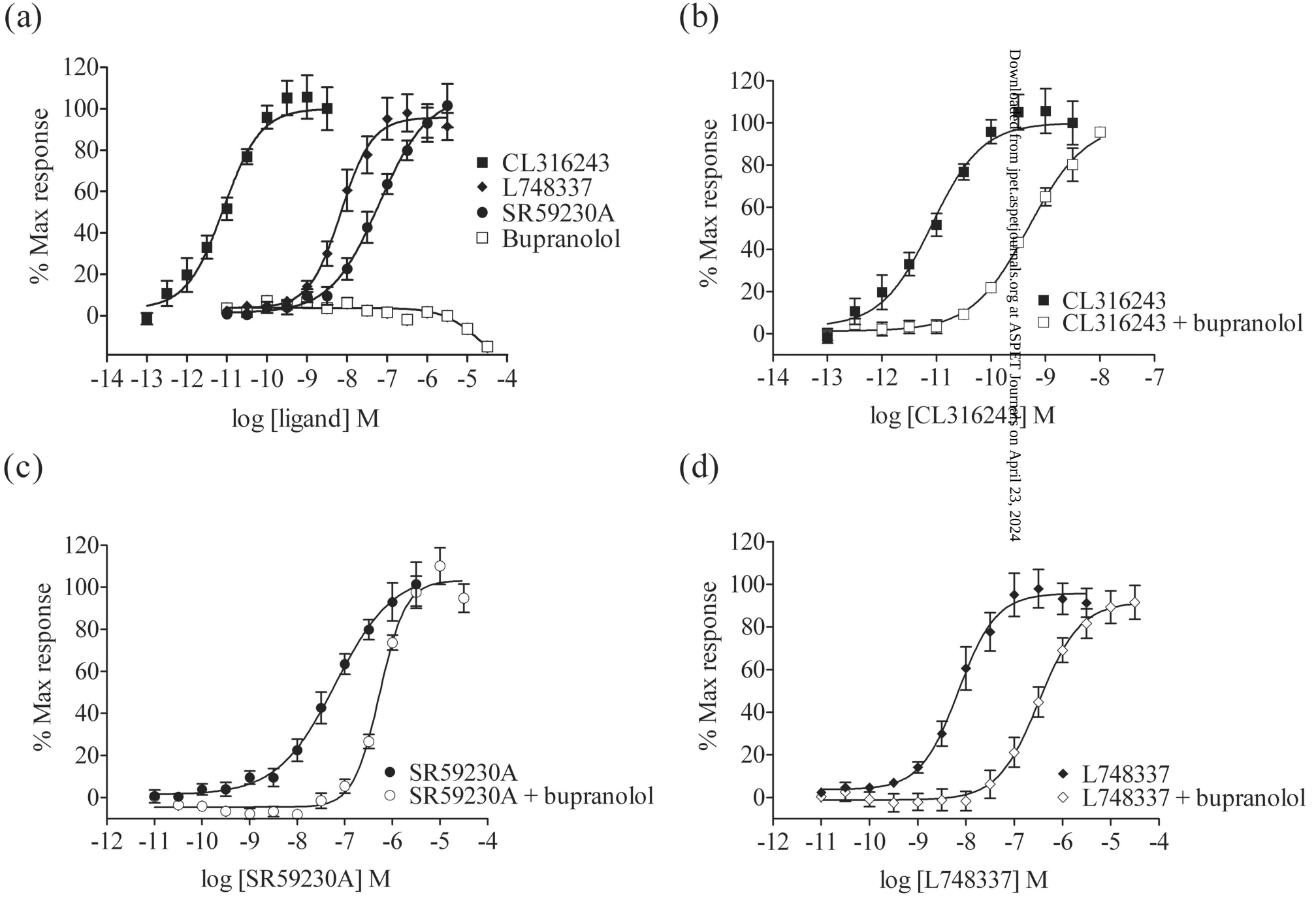

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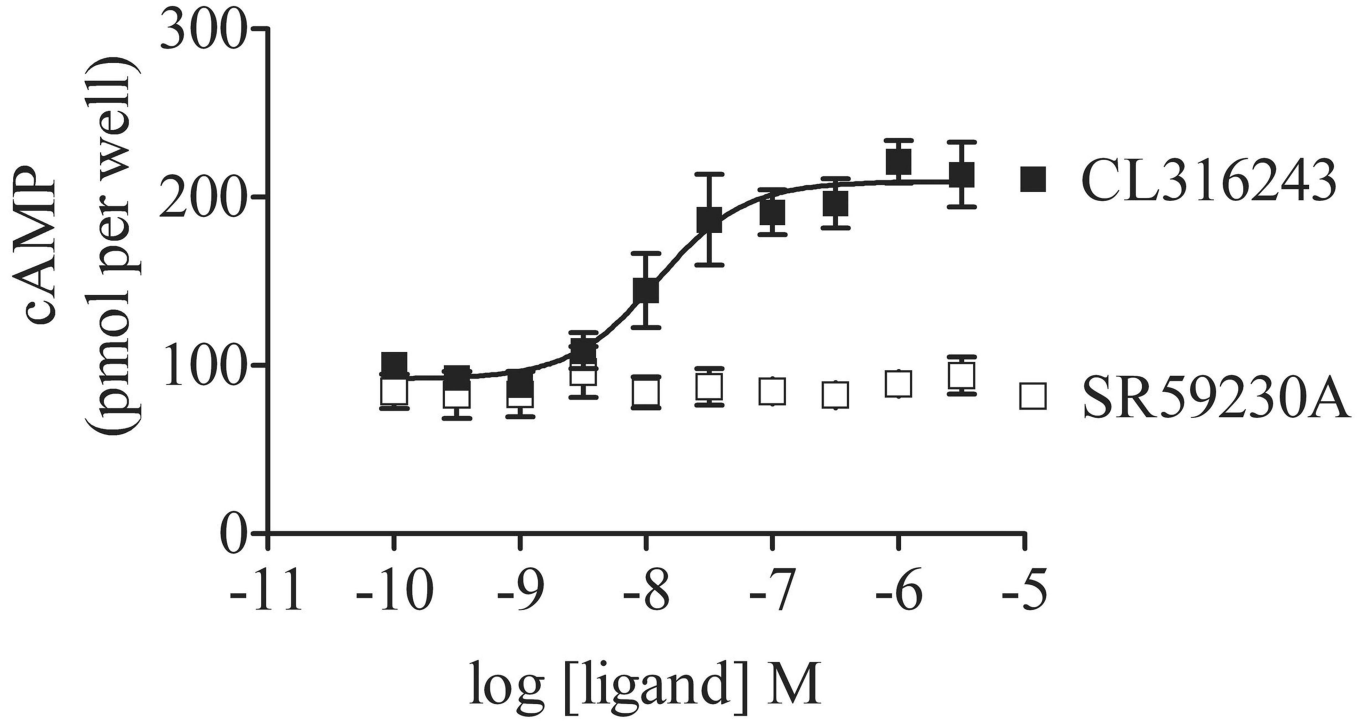

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