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Divergent agonist selectivity in activating β_1 and β_2 -adrenoceptors for G protein and arrestin coupling

Ida Casella¹, Caterina Ambrosio¹, Maria Cristina Grò, Paola Molinari and Tommaso Costa
Dipartimento del Farmaco, Istituto Superiore di Sanità, Rome, Italy

Synopsis

The functional selectivity of adrenergic ligands for activation of β_1 and β_2 adrenoceptor (AR) subtypes has been extensively studied in cAMP signalling. Much less is known about ligand selectivity for arrestin-mediated signalling pathways. Here we used resonance energy transfer methods to compare the ability of β_1 and β_2 AR to form a complex with the G proteins β_1 subunit or β -arrestin 2 in response to a variety of agonists with varying degrees of efficacy. The profiles of β_1/β_2 AR selectivity of the ligands for the two receptor-transducer interactions were sharply different. For G protein coupling, the majority of ligands were more effective in activating the β_2 AR, whereas for arrestin coupling the relationship was reversed. These data indicate that the β_1 AR interacts more efficiently than β_2 AR with arrestin but less efficiently than β_2 AR with G protein. A group of ligands exhibited β_1 AR selective efficacy in driving the coupling to arrestin. Dobutamine, a member of this group, had 70% of the epinephrine effect on arrestin via β_1 AR, but acted as competitive antagonist of epinephrine via β_2 AR. Thus, the structure of such ligands appears to induce an arrestin-interacting form of the receptor only when bound to the β_1 -adrenoceptor subtype.

Short Title: *BRET analysis of adrenoceptor-transducer interactions*

Key words: G protein-coupled receptors, β adrenoceptors, β -arrestin 2, ligand efficacy, resonance energy transfer, protein-protein interactions.

Abbreviations used: RET, resonance energy transfer; BRET, bioluminescence RET; E_{max} , maximal effect; bDOC, *bis*-desoxycoelenterazine; DMEM, Dulbecco's modified Eagle's medium; RGFP, *Renilla* green fluorescent protein; RLuc, *Renilla* luciferase; GPCR, G protein-coupled receptor; HEK293, Human embryo kidney 293 cells; PBS, Phosphate buffered saline; MEF, mouse embryo fibroblast; β_{arr2} , β -arrestin 2; β_1 AR and β_2 AR, β_1 - and β_2 -adrenoceptors. Abbreviations, together with generic names, structures and PubChem links of all compounds are provided in supplemental table s0.

Correspondence :

Tommaso Costa,
Istituto Superiore di Sanità, Dipartimento del Farmaco
Viale Regina Elena, 299, 00161, Roma, Italy.
Tel. 0039-0649902386, Fax: 0039-0649902014
Email: tomcosta@iss.it

Footnotes

¹These authors contributed equally to the experiments

Introduction

Although β_1 and β_2 adrenoceptors subtypes are structurally alike [1, 2] and similarly interact with the G protein Gs, which controls adenylyl cyclase activity and cAMP-mediated signalling, many studies show that there are marked differences among subtypes in activating downstream signalling cascades [3] and eliciting functional responses [4-6].

One element underlying the functional diversity between β_1 and β_2 receptor subtypes is the different topology of membrane location and cAMP signalling patterns that these two proteins display when coexisting on the surface of the same adult myocardiocyte [7]. Selective compartmentalization into specialized membrane microdomains [8, 9] and the differential interaction of the receptors C-termini with PDZ domains containing proteins [10-12] may be key factors that determine such location-dependent differences in signalling properties of the receptors.

However, there is also evidence that the interactions of β adrenoceptors subtypes with distinct signal transducers, such as Gs, Gi, and β -arrestins, may differ, thus generating signalling diversity. Striking differences among the three β AR subtypes were reported for the interaction with arrestins. It has been observed that the β_3 AR subtype does not interact with arrestin [13], nor undergoes GRK-mediated phosphorylation in response to agonist occupation [14], whereas the β_1 AR was found less efficient than the β_2 AR in interacting with arrestin [15].

Arrestins were originally considered molecular devices specifically designed to "arrest" receptor-G protein signalling. It is now clear, however, that these proteins, particularly β -arrestin 1 and 2, are full-fledged signal transducers [16, 17], and the broadness of the signalling network they can regulate has been recently exposed by comprehensive phosphoproteomic analysis [18, 19].

Several studies have focused on agonists that acting on the same receptor subtype can display differential efficacy for the interaction with the alternative transducers G protein and arrestin [17]. This transducer-dependent difference in agonist efficacy, commonly called biased agonism or ligand-directed signalling [3, 20, 21], has been investigated for the β_2 AR subtype, where a number of agonists with a slight preference for arrestin interactions were reported [22, 23]. However, an evaluation of how ligands differentially activate β_1 and β_2 adrenoceptors in promoting G protein or arrestin interactions is not available.

In this study we used resonance energy transfer (RET) to compare the differential ability of the two adrenoceptor subtypes to form a complex with each transducer in response to occupation by 45 distinct adrenergic structures. Our data show that the β_1 AR/ β_2 AR selectivity of adrenergics is strikingly different between arrestin and G protein interactions.

Experimental.

Reagents and drugs

Cell culture media, reagents and foetal calf serum (FCS) were from Invitrogen; restriction enzymes from New England Biolabs; pertussis toxin from List Biologicals; coelenterazine and bis-desoxycoelenterazine (bDOC, sold as coelenterazine 400a) from Biotium Inc. All other biochemicals were purchased from Sigma Aldrich. A clonal murine cell line (2B2 cells) previously isolated from embryos carrying the targeted ablation of *Gnas* exon 2 [24] was kindly made available to us from Dr. M. Bastepe (Harvard Med. School, Boston MA) and O.H. Onaran (Ankara University, Turkey). Adrenergic ligands were purchased from Bachem, Tocris, or kindly donated by Dr. Ijzermann. Details on source, structures, and abbreviations for the ligands used in this paper are listed in Supplemental data, table s0

Plasmid constructs

Adrenergic receptors β_1 AR and β_2 AR fused to the Rluc were obtained by inserting the PCR fragments encoding for each receptor cDNAs into Renilla Luciferase Vector (Packard), upstream the Rluc CDS. Resulting β_1 AR-Rluc and β_2 AR-Rluc chimeras were then transferred into a neomycin resistance retroviral expression vectors, (pQC series, Clontech).

RGFP-tagged-transducers were made by linking the amplified RGFP coding sequence (from Prolume) to the second codon of either bovine the β_1 subunit of the heterotrimeric G protein

(G β_1), through a 21-mer linker peptide (EEQKLISEEDLGILDGGSGSG), or to the β arrestin-2 (β arr2) cDNA sequence, through a 13-mer peptide corresponding to the *c-myc* epitope (EEQKLISEEDLGRT). Both constructs were transferred into hygromycin resistant retroviral vectors (pQH), which were used to generate the viral supernatants used for cells transduction. Retroviral vectors expressing the long form of the human G alpha protein G α_{sL} , were obtained by transferring the G α_{sL} coding sequence from pcDNA3.1 plasmid (Missouri S&T cDNA Resource Centre), to the retroviral vector pQP, bearing the puromycin resistant gene coding sequence. We also prepared a luminescent- β arrestin-2 chimera by a similar procedure: Rluc cDNA was fused to the β arrestin-2 coding sequence using a 21-mer linker peptide (GDLGELSREEQKLISEEDLRT), and subcloned into neomycin resistant retroviral vector. This construct was used to prepare cells co-expressing luminescent arrestin and a membrane-targeted variant of RGFP, which carries at the C-terminus the farnesylation-palmitoylation consensus sequence of hRAS [25].

Cell lines.

HEK293 cell lines were cultured in DMEM and 2B2 cell lines in a 50% mixture of DMEM and F12, both media added with 10% FCS. Cell lines stably co-expressing each luminescent adrenoceptor in association with either RGFP-tagged G β_1 or RGFP- β arr2 transducers were obtained by infecting cells sequentially with retroviruses encoding for the single fusion proteins followed by selection with G418 (500 μ g/ml) in combination with hygromycin B (100 μ g/ml). 2B2 cell lines additionally expressing exogenous G α_{sL} were obtained by viral transduction and puromycin selection as well. Clones expressing different ratios of chimeric proteins were isolated after low density plating of virally transduced cells, and 2-3 weeks culture in the presence of the proper antibiotics.

Expression levels of luminescent and fluorescent chimeric proteins

To measure the receptor density of chimeric receptors we used [¹²⁵I] (-)-pindolol (Amersham). Cell monolayers were detached using Ca²⁺/Mg²⁺-free PBS (PBS⁻) containing 1mM EDTA, and pelleted at 600 x g. After resuspension in PBS⁻, duplicate aliquots of ~100,000 cells were incubated with the radioligand (10pM) with or without 12 log-spaced concentrations of unlabeled pindolol in a total volume of 1ml. The reaction was conducted for 90 min at 20°C and terminated by rapid filtration onto GF/B glass fiber microplates (Filtermate 196, Packard). Radioactivity was counted and binding constants computed as described before [26]. The B_{max} (fmol/ μ g proteins \pm S.E.M., n=3) measured in the main cell lines used in this study are as follows. In 2B2 cells coexpressing RGFP- β arr2: β_2 AR, 1.43 (\pm 0.2); β_1 AR, 1.22 (\pm 0.12). In HEK293 cells coexpressing RGFP-G β_1 : β_2 AR, 2.6 (\pm 0.73); β_1 AR 5.3 (\pm 0.85). The K_d values (pM \pm S.E.M., n=6) for pindolol are 386 (\pm 77) at β_2 AR and 918 (\pm 138) at β_1 AR. Further characterization of the level of fusion proteins and their ratios in the cell lines and additional clones generated in the study was performed by measuring the intrinsic luminescence and fluorescence of cell membrane preparations or whole cell extracts, according to transducer localization, as reported before [25]. Enriched plasma membranes were prepared as in [26].

Cholera toxin-catalyzed ADP-rybosilation

Cholera toxin labeling of α -subunits was performed using 100 μ g of membrane proteins prepared from 2B2-KO and 2B2-G α_s reconstituted cells as described [26]. Proteins were then separated by SDS-polyacrylamide gel electrophoresis. The radioactivity of the bands corresponding to G α subunits was quantified on vacuum dried gel with a microchannel array detector counter (Packard Instant Imager). To ADP-ribosylate endogenous subunits cell monolayers were exposed to pertussis toxin (50 ng/ml) for 18 hr prior to harvesting.

BRET recording of receptor-transducer interactions

The usage of Renilla photoproteins as reporters of protein-protein interactions has been documented previously [27]. Luminescence was recorded using 96-well white plates (Packard View-plate) using a plate luminometer (VICTOR light, PerkinElmer) equipped with two independent automatic injectors.

G protein coupling assays were done on membrane prepared from HEK293 or 2B2 cells expressing each luminescent receptor plus RGFP-G β_1 ; membranes were incubated in PBS, containing 2.5 μ M coelenterazine for 10 minutes. Aliquots (5 μ g / 75 μ l) of suspension were rapidly distributed into a white 96-well OptiPlate (PerkinElmer) already preassembled with 25 μ l of PBS containing or not adrenergic ligands at 4X the final desired concentration. The plate was counted in the luminometer after 3 minutes of further incubation. Receptor-arrestin interactions were measured on monolayers of intact 2B2 cells: after over night growth in 96-well OptiPlate, cell culture media was replaced with 90 μ l of PBS containing 2.5 μ M bis-desoxycoelenterazine, and incubated for 2 minutes prior to the addition of 10 μ l of serial dilutions of ligands (10X the final in PBS), which had been prepared in a companion plate. Luminescence was recorded after ten additional minutes of incubation. Maximal effects for all ligands were determined using a single saturating concentration (10-100 μ M). In addition, agonists displaying a significant effect in at least one of the 4 assays were further characterized by construction of concentration-response curves, using 7 or 11 half-log spaced concentrations of each ligand in duplicate wells. In both types of assays, epinephrine was used as reference ligand and included in every test plate to account for inter-assay variability of the estimated parameters.

Kinetics experiments were performed as described previously [25].

The direct effects of adrenergics on the luciferase activity of RLuc were measured as described before [25]. Since, several compounds had significant inhibitory activity (20-40%) on the enzyme at 1 mM, concentrations greater than 100 μ M were avoided in the determination of BRET ratios. Consequently, for a few ligands (e.g. dopamine, its N-Methyl analog, MAPE, sulfonterol) the concentration-response curves in some assays did not reach a true experimentally determined plateau level. In such cases, the E_{\max} value was extrapolated through the fitting routine (see below), by constraining the a parameter of the curve to be \leq that for epinephrine. Because such parameter estimates are not obtained with the same degree of experimental confidence as the others, they were not included in the final comparisons of intrinsic activities (fig. 6 B, C) and are marked by stars in the final tables.

Data analysis.

RET ratios were determined as the ratios of high energy (donor) and low energy (acceptor) emissions, sequentially recorded with different filters [25]. Using coelenterazine, light was recorded through 450/20 nm and 510/20 nm windows, and RET ratios, corrected for spectral overlap, are calculated as: $(\text{cps}_{510} \times T_{450} / \text{cps}_{450} \times T_{510}) - 1$, (where T is the maximal transmittance in the two filter sets). With bis-desoxycoelenterazine, light was recorded through short-pass (450 nm cut-off) and long-pass (490 nm cut-off) filters and $\text{RET ratio}_{(\text{bDOC})} = \text{cps}_{\text{LP}} / \text{cps}_{\text{SP}}$. The relative E_{\max} of all ligands (intrinsic activities) were computed as fraction of the E_{\max} for epinephrine, after subtraction of the RET signal recorded in the absence of ligand. Concentration-response curves were analyzed by nonlinear curve fitting to the general logistic function $y = (a - d) / [1 + (x/c)^b] + d$ (where y and x are RET ratio and ligand concentration; a and d , upper and lower asymptotes, c is the ligand concentration yielding half-maximal RET change, and b is the slope factor at c). From best-fitting parameters, the EC_{50} (c) and E_{\max} ($a - d$) of each ligand were obtained. The significance of the difference of fitted parameters among ligands were assessed according to the extra sum of squares principle [28].

Agonist-induced changes of RET kinetics were fitted to an exponential change function:

$$y = \begin{cases} Y_0 & \text{if } \Delta t < 0 \\ Y_0 + \sum_{i=1}^n Y_i [1 - \exp(-\Delta t / \tau_i)] & \text{if } \Delta t \geq 0 \end{cases}$$

Where, Δt is the time difference (s) from ligand injection, Y_0 is the baseline RET signal, Y_i and τ_i are amplitude and time constant (s) of n exponential components.

Results

RET analysis of receptor-G β subunit interaction in membranes and role of G α subunits

Receptor-G protein efficacy was measured using a previously described cell-free BRET assay [25], to eliminate the inhibitory effect of endogenous arrestins on receptor-G protein interaction. We used membranes from HEK293 cells co-expressing either β_2 AR-Rluc or β_1 AR-Rluc and RGFP-G β_1 . The agonist-induced interaction between receptor and the β_1 subunit of heterotrimeric G proteins (G β_1) in membranes, results in enhancement of the RET signal, denoting a diminution of distance between the receptor's C-terminus and the G β_1 N-terminus. As discussed before [25], it is not possible to discriminate whether such a change is due to the rearrangement of a pre-associated supramolecular complex or to micro association reactions among proteins that diffuse along the plane of the membrane (and may perhaps be confined within specialised microdomains).

The interaction kinetics of β -adrenoceptors was similar to that previously observed for opioid receptors ($t_{1/2}$, 10-15 s), reaching a plateau within 1 min from agonist addition, with no apparent differences between β_2 and β_1 AR subtypes (data not shown). The rank order of catecholamine's potency for RET enhancement (isoproterenol > epinephrine >> norepinephrine in β_2 AR vs. isoproterenol > norepinephrine \geq epinephrine in β_1 AR) follows the typical pattern established in physiological studies (fig. 1 A, B). Both agonist effect and block by antagonist displayed the distinctive enantioselectivity expected for β_2 AR (fig. 1C, D). Thus the interaction receptor-G protein described by the resonance signal appears to maintain the characteristics of native adrenoceptors, despite the presence of fused reporter tags on both partners.

As previously observed for opioids [25], this signal results from a trimolecular interaction that requires G α subunits. To investigate which G α subunits are involved, we used a MEF cell line (2B2) established from transgenic mouse embryos that carry a targeted deletion of the G α_s gene [24]. Several lines co-expressing β_1 AR-Rluc or β_2 AR-Rluc and RGFP-G β_1 were engineered. In addition, we reintroduced a functional "dark" G α_s subunit in this host, by transducing β_2 AR/G β_1 -2B2 cells with a retroviral vector coding for the "long" spliced-variant form of the *GNAS* gene (G α_{sL}).

The activation of the β_2 AR was compared in membranes from 2B2 and 2B2-G α_s cells, both of which were treated or not with pertussis toxin overnight. Agonist-induced enhancement of RET was reduced by 80% in membranes of cells lacking G α_s subunits, but a smaller signal still persisted. Pertussis toxin treatment abolished the agonist response in cells lacking G α_s subunit, but produced only slight inhibition (~ 20%) in the presence of G α_s (fig.2 AB). Similar pertussis toxin sensitive signals were observed in 2B2 cells expressing β_1 AR (data not shown). These results indicate that while G α_s accounts for the majority of the interaction reported by the resonance signal, a smaller interaction supported by pertussis sensitive G α_i protein exists in both receptor subtypes.

To confirm this indication, we measured direct interactions of β_2 AR-Rluc with endogenous α subunits using agonist-induced enhancement of cholera toxin-catalysed ADP-ribosylation in the same 2B2 and 2B2-G α_s membranes. In the absence of G α_s , isoproterenol enhanced [32 P] ADP-ribose incorporation into the 40 kDa band corresponding to G α_i , and the effect was inhibited by the beta blocker ICI-118551. In membranes reconstituted with G α_s , the agonist primarily increased the labelling of a 46/48 kDa doublet corresponding to G α_{sL} , but no clear effect on G α_i labelling was detected in this case, suggesting that in the presence of G α_s the interaction with G α_i may be negligible (fig.2 C). The agonist-induced enhancement of cholera toxin labeling of the 40 kDa band in 2B2 membranes was abolished following treatment of the cells with pertussis toxin (50 ng/ml, 18h), suggesting that the labeled protein is a member of the G α_{iO} family of G proteins (data not shown). Such results also indicate that the ability of the luciferase-fused receptor to interact with endogenous α -subunits in the membrane is preserved.

As observed in the opioid receptor system, the adrenergic RET signal was rapidly suppressed by guanine nucleotides. We used this allosteric signal quenching reaction to compare the

relative potency of GDP to inhibit epinephrine-induced interactions at the two receptor subtypes. The IC_{50} for GDP was 10-fold lower at β_1 than at β_2 receptor (fig. 3), suggesting that the β_2 AR subtype can form a much more stable complex with $G\alpha\beta\gamma$ than the β_1 AR, despite the similarity in interaction kinetics.

Receptor-arrestin interaction: kinetics and role of $G\alpha$ subunits

Our objective was to determine the intrinsic activity of agonists for each adrenoceptor-arrestin coupling without the influence of the concurrent receptor-G protein interaction. Thus, 2B2 $G\alpha_s$ -KO cells co-expressing each luminescent receptor type and fluorescent β -arrestin 2 (β arr2) were prepared. In addition, β_2 AR-arrestin coexpressing cells were also transduced with dark $G\alpha_s$, to evaluate if and to what extent the presence of $G\alpha_s$ might modify the interactions. Both β_1 AR and β_2 AR interact rapidly with arrestin, approaching steady-state within 5 min following agonist injection to the cell monolayer. However, the initial rate of the β_2 AR subtype is somewhat delayed by a slower transient of RET increase in the first 30 s (Fig. 4). Modelled by mono-exponential functions, the half-time of epinephrine-induced β_1 AR-arrestin interaction was smaller (35 ± 7 s) than that of β_2 AR-arrestin (76 ± 9 s). Partial agonists displayed reduced maximal effects with slower rates in both β_1 AR and β_2 AR (fig. 4AB), whereas lowering the molar concentration of agonist primarily reduced amplitudes and not rates, for concentrations > 30 nM (fig. 4C).

We also found that for β_1 AR RET kinetics, a two-component exponential model (with $t_{1/2}$ ranging 3-7 s and 30-100 s) often afforded significant reductions of the fit-standard error. This reminds the biphasic kinetics described before for β_2 AR-- β arr2 interaction in single cell imaging FRET studies [29]. However, we failed to find a consistent relationships between agonist efficacy and $t_{1/2}$ or amplitudes of the two components. For β_2 AR kinetics, the s-shaped inflection in the initial time course prevented significant improvements by fitting a sum of exponentials, unless two components with amplitudes of opposite sign were allowed in the model. Although improving fitting statistics, the physical interpretation of such a model is unclear. For this reason and because estimating both amplitudes and rates from multiexponential fits of experimental data is a notorious ill-posed problem [30], all comparisons of kinetic parameters in this study are based on mono-exponential approximations of the data. Regardless of the complexity, the data in fig. 4 indicate that the maximal enhancement of RET at steady state is a good descriptor of ligand efficacy for both β_1 and β_2 receptor subtypes. Thus, this parameter was chosen to assess ligand intrinsic activities for receptor-arrestin coupling in subsequent studies.

As observed for G protein, concentration-response curves of catecholamines for arrestin coupling displayed the typical signature of β_1/β_2 adrenoceptor pharmacology (catecholamine potencies and agonist stereoselectivity in fig. 5). Interestingly, the EC_{50} of the three catecholamines for β_1 AR were smaller at arrestin than at G protein, whereas this trend was reversed for β_2 AR (see Table 2 and supplemental table s0).

To verify that the C terminal Rluc extension of adrenoceptors does not modify the intrinsic ability of the receptor to interact with arrestin we also developed for the β_2 AR subtype an indirect BRET system capable of detecting arrestin binding to intact wild type receptors. We took advantage of a membrane-targeted RGFP variant (mtRGFP) previously used to monitor receptor internalization [25]. This protein is localized in close proximity of luminescent receptors thus producing a high RET signal, which fades away as agonist binding triggers receptor endocytosis. Exploiting the same principle, we could detect the docking of luminescent arrestin to "dark" wild-type β_2 AR through the increase of proximity-induced RET between the arrestin-tethered Rluc donor and the membrane-anchored RGFP acceptor. In HEK293 cells co-expressing mtRGFP plus Rluc- β arr2, and further transduced with wild-type β_2 AR, the intrinsic activity of agonists was in good agreement with that measured using the conventional assay in B2B cells (supplementary fig. s1), indicating that Rluc tagging does not change receptor efficacy for arrestin. The correspondence between the two assays also suggests that the bulk of BRET signal that we measure in this study is generated by an arrestin-receptor complex present on the cell surface rather than segregated into endocytic

vesicles. In fact, unlike the direct interaction between luminescent receptor and fluorescent arrestin, the indirect RET between receptor-bound luminescent arrestin and membrane-anchored RGFP is interrupted as the receptor-arrestin complex undergoes endocytosis [25]. The role of $G\alpha_s$ on receptor-arrestin interaction was evaluated by comparing the relative E_{max} of a series of adrenergic agonists in β_2AR - $\betaarr2$ cells expressing or not the co-transfected $G\alpha_{sL}$ gene. The correlation between the intrinsic activities of ligands measured in the two cell lines was tight (supplementary fig. s2), indicating that the coupling with $G\alpha$ subunits does not manifestly change the efficiency of ligands in inducing receptor-arrestin interaction. Similarly, there was no influence of functional $G\alpha_i$ subunits, when agonists intrinsic activities for β_2AR or β_1AR were compared in cells that had been exposed or not to pertussis toxin (data not shown), nor did pertussis toxin alter significantly the kinetics of the RET signal triggered by full agonists in β_2AR - $\betaarr2$ or in β_1AR - $\betaarr2$ cells (Fig.4D). Thus, taken collectively, these data show that the interaction receptor-G proteins does not appear to modify the binding of receptor to arrestin reported by RET, neither directly via protein-protein associations, nor indirectly via activation of signalling pathways.

Effect of apparent receptor-transducer stoichiometry on intrinsic activity

To investigate how the relative E_{max} of ligands may depend on the level of expression of receptor- $G\beta$ interacting partners, for each receptor subtype, we selected clones with a different stoichiometry of the two chimeric proteins, as determined by the luminescence and fluorescence of the tethered reporters. The stoichiometry of expression had no detectable effect on the relative intrinsic activity of agonists for both receptor subtypes (supplemental fig. s3). Interestingly, however, the overexpression of "dark" $G\alpha_s$ subunit significantly increased the intrinsic activity of the partial agonist clenbuterol for the β_2AR (sup. fig. s2 D). The cell lines used in the study had comparable level of $G\alpha_s$ expression in immunoblots (data not shown). Nonetheless the final data were collected using at least two different clones for each receptor subtype, to minimize the chance that unknown differences in endogenous G protein subunits could bias the final results.

For the study of arrestin coupling we could not obtain lines showing a significant variation in the expression of fluorescent arrestin. Thus, cell lines expressing equal levels of luminescent β_2 or β_1 adrenoceptors and fluorescent-arrestin were selected and used for the pharmacological analysis. However, to evaluate how a change of expression stoichiometry could affect the data, we generated a cell line expressing 2.5-fold higher level of β_2AR luminescence, with no difference in fluorescence expression. The raised receptor/arrestin ratio in cells expressing a higher level of receptor, increased slightly the intrinsic activity of a number of partial agonists (supplemental fig. s4). This indicates that in the β_2AR and β_1AR cell lines used for the comparison the receptor/arrestin ratio is safely below the critical 1:1 proportion, where even small differences (easily masked by experimental noise in the determination of intrinsic luminescence and fluorescence) might affect the comparison of relative E_{max} of ligands across receptors. (More discussion on this point is in supplemental appendix s5).

β_1AR and β_2AR ligand intrinsic activities for G protein and arrestin interactions

Using the BRET interaction assays described above, we compared the intrinsic activity of 45 adrenergic ligands, including antagonists and agonists endowed with varying degrees of efficacy. The entire data set is available as a spreadsheet (supplemental table s0). Mean intrinsic activity data pooled from both concentration-response curves and determinations at a single saturating concentration are also reported in table 1, while EC_{50} are in table 2.

To mark the ligands exhibiting the largest β_1/β_2AR difference in maximal complex formed with each transducer we plot the net difference between relative intrinsic activities (β_1AR minus β_2AR) of all ligands. This index arbitrarily sets β_1AR -selectivity as positive and β_2AR -selectivity as negative numbers (fig. 6A).

For receptor-G protein interaction, cimeterol is the ligand with the highest level of β_2AR selectivity, followed by MAPE and N-methyl-dopamine (although E_{max} values of the latter two ligands may be underestimated, given the very low potency for β_1AR -G protein interaction).

Other structures with significant β_2 AR preferential effects include well known β_2 AR agonists, such as albuterol, clenbuterol and terbutaline. A direct plot of G protein intrinsic activity for the two receptor subtypes (Fig. 6C) shows an overall β_2 AR preference in promoting G protein coupling for the majority of ligands, but no marked reversal of the ordering of efficacy. Only CGP-12177 and xamoterol (β_2 AR antagonist and partial agonist, respectively) show significant β_1 AR preference (Fig. 6C and table 1). This preserved ranking of efficacy across β_1 and β_2 receptor subtypes for G protein coupling largely agrees with a recent study based on cAMP signalling measurements [31].

For receptor-arrestin interaction, the trend is reversed. The direct comparison of intrinsic activity data shows an inverse pattern compared to G protein (Fig. 6B). Many ligands with β_2 AR preference on G protein show β_1 AR-preference on arrestin. Even two notorious β_2 AR agonists, such as cimeterol or clenbuterol, gain a slightly greater β_1 AR intrinsic activity in promoting receptor-arrestin interaction (Fig. 6A and table 1).

Such a switch towards β_1 AR preference as the transducer shifts from G protein to arrestin is also apparent on examination of ligands EC_{50} (table 2). For G protein coupling most ligands exhibit significantly smaller EC_{50} at the β_2 AR subtype, but the ratio of potency in favour of β_2 AR is drastically reduced for arrestin coupling (table 2 and supplemental table s0). Most impressive is the change for clenbuterol, with a 200-fold shift towards the β_1 AR as we move from G protein to arrestin. Also interesting is the potency of norepinephrine: the EC_{50} ratio shows only a 2-fold β_1 AR selectivity for G protein coupling, but raises to 160 fold for arrestin interaction (table 2).

Note that although carvedilol was reported to induce translocation of arrestin to the membrane via β_2 AR [32, 33], in this study we only see a minor effect of carvedilol at β_1 AR and none at β_2 AR for arrestin coupling (Table 1). It would be interesting to investigate whether this discrepancy only reflects a technical difference, or underlies an important functional separation between the biological events that are monitored by the two types assay.

Agonists with restricted β_1 AR-arrestin efficacy.

In addition to the global shift towards β_1 AR observed for most structures, a number of agonists (particularly, dobutamine, synephrine analogs, dopamine, and ritodrine) exhibit a clear reversal in the relative order of β_2/β_1 AR efficacy for arrestin. This is best illustrated in fig. 6D, where such ligands are compared with other agonists that maintain similar relative effects at both subtypes. In contrast to albuterol (which shows similar E_{max} in both subtypes), the maximal receptor-arrestin complex induced by such ligands decreases to negligible levels at β_2 AR while correspondently increases at β_1 AR (fig. 6D). The loss at β_2 AR efficacy with high β_1 AR efficacy for arrestin in such ligands suggests that they can act as competitive antagonists of β_2 AR-arrestin interactions. This was further investigated using dobutamine.

The concentration-response curves shown in fig. 7 indicate that dobutamine is a strong partial agonist (IA = 0.5-0.6 relative to epinephrine) for both β_1 AR- and β_2 AR-G protein interaction, and even slightly stronger (IA = 0.75) for β_1 AR-arrestin interaction. In contrast, dobutamine produces no effect on the β_2 AR-arrestin interaction. However, the concentration-response curves of epinephrine in the presence of increasing concentrations of dobutamine show a typical rightward shift of EC_{50} indicating competitive antagonism (fig. 7). The pA_2 constant computed from Schild plots analysis of such experiments (6.52 ± 0.3) is in good agreement with the EC_{50} of dobutamine determined for β_2 AR-Gprotein interaction (-6.85 ± 0.2) (see table 2). Although dobutamine is a racemic mix of two enantiomers [34], which were not tested in resolved form here, the inability of racemic dobutamine to form a detectable β_2 AR-Arrestin complex suggests that both enantiomers have no efficacy on this interaction.

Discussion

We have compared the ability of the structurally similar β_1 and β_2 adrenoceptors to bind the major transduction proteins that mediate their biological effects, G proteins and β -arrestin 2, in response to occupation by 45 different ligand structures. The interaction was quantified as maximal enhancement of RET ratio induced by each ligand (relative to the reference agonist epinephrine).

This enhancement of RET ratio reflects two equiprobable mechanisms: (1) intramolecular rearrangement of a pre existing complex that results in tighter receptor-transducer association or (2) the new formation of a receptor-transducer complex. Any of the two mechanisms might be involved in G protein coupling, whereas the second is most likely involved in arrestin coupling. In both reactions the “molecular efficacy” of the ligand is given by the free energy change that couples ligand’s and transducer’s binding sites on the receptor molecule [35]. This free-energy change and the maximal ratio transducer-bound vs. unbound receptor (i.e. the E_{\max} in RET) are predicted to be linearly related according to the first mechanism, and are also virtually linear according to the second, unless the receptor exceeds the transducer concentration. However, theoretical analysis (given in-depth in appendix s4) also shows that any deviation from 100% RET efficiency of the reporter system introduces significant non linearity between the optical signal and the molar fraction of transducer-bound receptor. Consequently, the differences of intrinsic activities between two receptor subtypes not only reflect divergences in ligand molecular efficacy, but also in the stoichiometry and the affinity/stability of transducer-receptor complexes.

For this reason, experiments were designed to assess how the stoichiometry between receptor and transducer can influence the observed differences. In the G protein system, ligand’s intrinsic activity was not affected by a wide range of receptor/ $G\beta_1$ expression ratios, except when the abundance of $G\alpha_s$ was enhanced by overexpression. Such a result is consistent with the idea that in this system the change of RET reflects internal rearrangements of a preformed receptor- $G\alpha\beta\gamma$ complex [25], although it cannot rule out alternative mechanisms. The effect of $G\alpha_s$ overexpression may tell that this subunit is the limiting factor in the stability of functional receptor-transducer complexes that can be preassembled in the membrane. In the arrestin system, we used cell lines with identical expression levels of luminescent receptors and fluorescent arrestin, to ensure results independent of differences in relative expression. Doubling receptor expression of the β_2 subtype at equal arrestin levels shifted only slightly the E_{\max} of partial agonists, indicating that small differences of expression in the cell lines used for the analysis cannot affect the comparison.

Even if the role of expression stoichiometry can be discounted, changes of ligand E_{\max} between the two receptors reflect both a difference in ligand molecular efficacy and a difference in receptor-transducer affinity. The first involves particular ligands individually, the second affects all ligands, but to a variable extent according to their efficacy, because of the hyperbolic relation between the fraction of transducer-receptor complex and the optical RET signal (supplemental appendix s5). The exact distinction of “individual” and “global” differences is obviously impossible in the presence of experimental noise. However, the large panel of ligands examined in this study helps to identify general trends resulting from a difference in transducer-receptor affinity, as such a difference alters the linearity but not the ordering of ligand efficacies. In contrast, individual ligand divergences that significantly change the ranking of intrinsic activities between receptor subtypes more likely reflect a true diversity in the conformational perturbations that that ligand’s structure can transmit on the two receptor molecules.

According to this interpretation algorithm, most of the β_1/β_2 adrenoceptors differences in ligand intrinsic activities for G protein coupling, primarily reflect a molecular difference of G protein interaction between the two receptors, rather than individual divergences of efficacy among ligands. When compared on the same plot (fig. 6C), the bulk of E_{\max} data deviates from the identity line and is bended towards the β_2 AR axis, with largest differences occurring in the mid range (0.4-0.6) of intrinsic activity. This is the pattern expected if we assume that the β_2 AR receptor can establish a more stable interaction than the β_1 AR subtype with G protein

subunits. This conclusion agrees with our finding that the potency of GDP to quench epinephrine-induced coupling is ten-fold greater at β_1 AR than at β_2 AR. It also agrees with previous work where β_1/β_2 adrenoceptor subtypes differences were assessed in conventional signalling assays [36]. Only a few ligands that appear to deviate significantly from this general trend (e.g. CGP12177 and xamoterol) are structures possibly endowed with the ability to induce a better G protein-interacting conformation when occupying the β_1 AR rather than the β_2 AR binding site.

In contrast, the mirror-like trend in the differences of ligand intrinsic activities observed for arrestin coupling (fig.6B) indicates that the default β_2 AR -preference existing for G proteins is abolished or reversed for arrestin. This suggests that the β_1 subtype interacts with the arrestin/GRK system more efficiently than the β_2 AR. Note that this inversion cannot be attributed to the stronger coupling of β_2 AR to $G_{\alpha s}$ (which thus may “compete” against arrestin), because in this study arrestin interactions were recorded under a G_s -null background. A greater arrestin affinity and/or phosphorylation efficiency of the β_1 subtype may be responsible for this global shift. Although for arrestins there is no allosteric inhibitor of transducer-receptor interaction (like GDP) to support this finding, the faster kinetics observed for β_1 AR-arrestin interaction would be in line with such a conclusion.

Our data seem at variance with other studies that reported a reduced ability of β_1 AR to undergo internalization and down-regulation compared to β_2 AR [15, 37-39]. However, while in previous work the comparison was based on events downstream the arrestin-receptor complex, here we directly measured the formation of that complex. Moreover, the comparison of two alternative ways to measure β_2 AR-arrestin interactions (suppl. fig. s1), suggests that the arrestin-receptor complex responsible for the bulk of RET signal is probably on the cell surface. Thus, it is possible that despite a stronger interaction with arrestin, the β_1 AR -arrestin complex might be less efficient in progressing along the subsequent steps of endocytosis and recycling than the β_2 AR. More experiments are necessary to clarify this surprising paradox.

Some agonists display levels of β_1 AR efficacy for arrestin coupling which are greater than expected from the general trend. Besides xamoterol and CGP12177 (already displaying β_1 -preference in G protein coupling), the most obvious is a cluster of agonists (e.g. dobutamine, synephrine analogs, ritodrine and dopamine) that show reversal of β_2/β_1 AR efficacy profiles, thus producing considerable levels of receptor-arrestin complex via β_1 AR but undetectable levels via β_2 AR. As demonstrated for dobutamine, these ligands are competitive antagonists of the β_2 AR-arrestin interaction induced by a full agonist. The dobutamine K_i for blocking epinephrine on β_2 AR-arrestin is very close to its EC_{50} as an agonist on β_2 AR-G protein interaction. Thus, the loss of β_2 AR efficacy on arrestin is not due to reduced binding affinity for the β_2 AR-arrestin complex. The most logical conclusion is that these ligands are capable of inducing a proper “arrestin-fitting” conformation of the receptor when binding to the β_1 AR subtype, but fail to do so when occupying the site of the β_2 AR subtype.

While data derived from the direct measurement of protein-protein association are the best to gauge the conformational change that each ligand structure can transfer to the receptor-transducer interface, they cannot predict the relative effect of such a change on downstream signalling. In fact, a complex signalling network can generate strong non linearity between receptor-transducer complex formation and resulting biological responses. Thus, the profile of β_1/β_2 AR selectivity that we have here registered at the molecular level might be considerably altered at the stage of arrestin-mediated functional responses.

With such a caveat in mind, it is however interesting to note that dobutamine and dopamine are clinically-relevant sympathomimetics, considered to exert β_1 AR -mediated inotropic effects via cAMP signalling. Yet we find that the transducer where such drugs show β_1 AR selectivity of efficacy is arrestin, not G_s . This suggests a role of arrestin in mediating adrenergic control of the myocardial contractile response. Indeed, a recent study has demonstrated that arrestins can mediate enhancement of cardiomyocyte contractility via angiotensin 1 receptors [40], and rapid changes of local free Ca^{2+} , apparently mediated by β -arrestin-2/ERK via β_2 AR receptors, were described in hippocampus neurons [32]. The efficacy profile of “dobutamine-like”

agonists identified in this study predicts a peculiar pattern of receptor-transducer output in cells expressing both β_1 and β_2 adrenoceptors: G protein responses are activated via both subtypes, but arrestin signalling can only occur through the β_1 AR, as these ligands block the influence of endogenous catecholamines on β_2 AR-arrestin interaction. One obvious question is whether such an unbalance in β_1/β_2 AR –mediated signalling might be related to the adverse effects that dobutamine has shown in clinical trials of heart failure patients [41]. Interestingly, a functional distortion in the relative balance of β_1/β_2 AR –mediated signalling, resulting from the loss of compartmentation, was found in myocardiocytes from failing hearts [7].

In conclusion, we have shown that the β_1/β_2 adrenoceptor efficacy profiles of adrenergics are diametrically different for G protein and arrestin. This primarily reflects an inversion in the strength of receptor-transducer interactions (β_2 AR > β_1 AR for G proteins and β_1 AR \geq β_2 AR for arrestins). We have also identified a group of β_1 -selective arrestin agonists, which can induce formation of the receptor-arrestin complex only when bound to the β_1 subtype.

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Legends to Figures

Figure 1. Catecholamine preference and stereoselectivity for adrenoceptors – G protein coupling. Concentration response curves for ligand-induced enhancement of RET ratio in membranes prepared from cells co-expressing fluorescent $G\beta_1$ and luminescent β_2AR (**A,C,D**) or β_1AR (**B**). All data were fitted using a general logistic function (*solid lines*) as described in *Experimental*. (**A,B**) Data are means (\pm S.E.M.) of three experiments. See supplemental table s0 for the means of EC_{50} estimates. (**C**) Data show means from two experiments with ranges given by dashes, and were replicated in an additional experiment using different enantiomers concentrations. Log EC_{50} values (mean \pm S.E.M., $n=3$) are -8 ± 0.22 and -6.8 ± 0.21 for the (-) and (+) isomer, respectively. (**D**) Concentration-dependent inhibition by propranolol (propr) stereoisomers of RET enhancement induced by epinephrine (EPI). A representative experiment performed in the presence 20 nM agonist is shown (IC_{50} , 1.4 nM for the (-) and 96 nM for the (+) isomer). The experiment was repeated using 100 nM EPI or 10 nM ISO with similar results. The average (+)/(-) IC_{50} ratio from the three experiments is 85 ± 18 . Abbreviations: ISO, (\pm) isoproterenol; NE, (\pm) norepinephrine; EPI, (\pm) epinephrine.

Figure 2. Interaction of β_2AR -Rluc with endogenous $G\alpha$ subunits. Membranes of 2B2 cell lines expressing β_2AR -Rluc and RGFP- $G\beta_1$ with (**A**) or without $G\alpha_{sL}$ (**B**) were assayed for RET enhancements in the absence (basal) or presence of the agonist isoproterenol (ISO, 1 μ M) and the antagonist ICI 118551 (ICI, 10 μ M). The same membranes were ADP-ribosylated in vitro with cholera toxin (*see Experimental*) in the absence or presence of the ligands as indicated (*see lane numbering in D*), and separated by SDS-polyacrylamide gel electrophoresis. (**C**) Autoradiogram of the dried gel. (**D**) Radioactivity counted in the 41 kDa bands ($G\alpha_i$, *left histogram*) and in the 46/48 kDa doublet ($G\alpha_s$, *right histogram*) using a Packard Instant Imager.

Figure 3. Different potency of GDP in inhibiting G protein coupling at β_1 and β_2 adrenoceptors. (**A**) Membranes from cells expressing β_1AR - $G\beta_1$ (solid circles) or β_2AR / $G\beta_1$ (open circles) were assayed for RET in the presence of 10 μ M epinephrine (EPI) and

increasing concentrations of GDP (*x-axis*). Data are averaged from two separate experiments (ranges shown by dashes) and are expressed as fraction of the effect measured in the absence of nucleotide. **(B)** Membranes were prepared from two cell clones expressing different levels of β_2 AR-Rluc and RGFP-G β_1 and the effect of GDP was measured as in panel A. Data are averages of triplicate determinations in a single experiment. To test for a difference of IC₅₀ in the two clones, data were first fitted with no constraints, and then refitted by forcing a common parameter *c*. The second fit was not significantly worse ($p = 0.37$) as determined by extra-sum-of-square statistics [28] indicating that the IC₅₀ of GDP is similar in the two curves. Thus GDP potency does not depend on the magnitude of RET signal measured in the two membranes.

Figure 4. Kinetics of the enhancement of RET ratios in 2B2 cell lines expressing luminescent β_1 AR or β_2 AR, and fluorescent β -arrestin 2. Micro wells containing cells expressing β_2 AR **(A)** or β_1 AR **(B)** were injected at time 0 with the indicated agonists (all 10 μ M) and the progress of luminescence was recorded continuously at 0.5 s intervals. Values of $t_{1/2}$ (s) from mono-exponential fits (solid lines) are: in **(A)**, 76 ± 7 for epinephrine and 161 ± 21 for terbutaline; in **(B)**, 32 ± 3 for epinephrine, 72 ± 5 for terbutaline, and 143 ± 15 for clenbuterol. **C** Different concentrations of epinephrine (as indicated) were added at time 0 to cells expressing β_2 AR; $t_{1/2}$ (\pm S.E.M. from the fit) are: $54 (\pm 9)$ at 10 μ M, $85 (\pm 11)$ at 0.1 μ M, $57 (\pm 7)$ at 100 nM, and $173 (\pm 21)$ at 30 nM. **D.** Micro wells containing cells expressing β_2 AR (*triangles*) or β_1 AR (*squares*), pre-treated (*open symbols*) or not (*solid symbols*) with 100 ng/ml pertussis toxin (PTX) for 18 hr, were injected at time 0 with 10 μ M epinephrine. Computed $t_{1/2}$ (s) are: β_2 AR, $87 (\pm 9)$ control and $76 (\pm 7)$ PTX; β_1 AR, $39 (\pm 4)$ control and $37 (\pm 5)$ PTX. Data in **A** are means (\pm S.E.M. *grey bars*) of 4 experiments. All other plots are representative of experiments repeated at least three times, (although using different time sampling or type of partial agonists), which yielded comparable results.

Figure 5. Catecholamine preference and stereo selectivity for adrenoceptors – arrestin coupling. Concentration response curve for catecholamine-induced enhancement of RET ratio in 2B2 cell lines co expressing fluorescent β -arrestin 2 and luminescent β_1 AR (*top*) or β_2 AR (*down*). Data are from a representative experiment where the four catecholamines were compared in the same microplate. The log EC₅₀ values for isoproterenol enantiomers are: at β_1 AR, -8.1 (-) and -6.8 (+); at β_2 AR, -7.4 (-) and -6.1 (+). Refer to table 2 and Supplemental table s0 for means of catecholamine EC₅₀ values. Abbreviations as in fig. 1.

Figure 6. β_1/β_2 adrenoceptors selectivity profiles of ligand intrinsic activity for G protein and arrestin. **(A)** The shift of intrinsic activity (*y-axis*) was computed as the net difference between β_1 AR and β_2 AR intrinsic activities measured for G proteins coupling (*grey*) and arrestin coupling (*black*) using the cumulative data shown in table 1. Error bars were calculated from the sum of variances of the data. Positive numbers indicate net β_1 AR preference and negative numbers β_2 AR preference. Data are shown in histogram form, sorted according to decreasing net difference. Asterisks mark ligands for which plateau E_{max} values could not be reached with the highest concentration, in at least one of the 4 assays (see *Experimental*) Ligands abbreviations are as listed in table s0 of supplemental materials. **B** and **C.** β_1 intrinsic activities plotted as a function of β_2 AR intrinsic activities for arrestin **(B)** and G protein **(C)** coupling assays. Data are ordered according to increasing β_2 AR effects. Dashed lines are the line of identity, whereas the solid lines were traced using a polynomial function to approximate the general trend of the data points. Ligands marked with arrows (*grey triangles*) showing most evident variations from the general trend are discussed in the text. **(D).** The β_1 AR and β_2 AR-arrestin intrinsic activities of the group of ligands exhibiting highest β_1 AR selectivity (arrows in **B**) are replotted with those of ligands having similar effects on both receptors. Note the reversal in rank ordering of efficacy. With respect to e.g. albuterol, the relative E_{max} is progressively increasing at β_1 AR while correspondently fading to undetectable levels at β_2 AR.

Figure 7. Competitive antagonism of dobutamine for β_2 AR-arrestin interaction. **A-C** Concentration-response curves of dobutamine (DOB) and epinephrine (EPI) for the interactions indicated in the plots. Note that dobutamine is a relatively effective partial agonist in the three assays. **D** Concentration-response curves of epinephrine in the absence or presence of increasing concentrations of dobutamine (as indicated in the box). The inset is a Schild plot of the data (CR, concentration ratio i.e. ratio of epinephrine EC_{50} in the presence vs. absence of dobutamine). The pA_2 value computed from the plot is 6.5 ± 0.32 . Data were averaged from two independent experiments using the indicated concentrations of dobutamine, and were replicated in one additional experiment with different concentrations of antagonist.

Table 1 Ligand intrinsic activities (E_{\max} minus basal as fraction of epinephrine values). Weighed means from both single saturating concentrations experiments and concentration-response curves (the latter were given double weight in the final mean calculation)

<i>Ligand</i>	β_2 AR-Gprot.		β_1 AR-G prot.		β_2 AR-arrestin		β_1 AR-arrestin	
	Mean	(\pm S.E.M.)	Mean	(\pm S.E.M.)	Mean	(\pm S.E.M.)	Mean	(\pm S.E.M.)
adrenalone	1.00	0.05	0.87*	0.05	0.94	0.03	0.87*	0.07
AH 3021	0.79	0.02	0.47	0.05	0.30	0.05	0.52	0.05
AH 3474A	-0.02	0.03	-0.01	0.01	0.00	0.01	0.02	0.03
Albuterol	0.70	0.05	0.39	0.07	0.30	0.02	0.37	0.07
Alprenolol	0.05	0.02	0.09	0.03	0.00	0.01	0.05	0.02
Atenolol	0.03	0.09	-0.01	>0.01	0.00	0.01	-0.01	0.02
Bisoprolol	-0.03	0.021	-0.01	0.02	0.00	0.01	-0.02	0.02
Butoxamine	-0.02	0.01	0.02	>0.01	0.00	0.01	0.02	0.02
C78 (Tulobuterol)	0.38	0.03	0.20	0.02	0.03	0.01	0.30	0.04
Carvedilol	-0.01	0.01	0.10	>0.01	0.00	0.01	0.04	0.02
CGP 12177	0.01	0.04	0.26	0.05	0.00	0.02	0.33	0.07
Cimeterol	0.85	0.01	0.37	0.09	0.50	0.05	0.67	0.06
Clenbuterol	0.50	0.06	0.25*	0.06	0.14	0.02	0.38	0.05
Dichloroisoproterenol	0.22	0.06	0.10	0.04	0.00	0.01	0.24	0.04
Dobutamine	0.58	0.04	0.55	0.04	0.02	0.02	0.72	0.06
Dopamine	0.70*	0.10	0.57*	0.10	0.04	0.02	0.58*	0.06
Du 21117	0.92	0.04	0.79	0.046	0.62	0.06	0.89	0.04
Du 28663	0.89	0.07	0.59	0.03	0.59	0.07	0.64	0.08
epinephrine	1.00	–	1.00	–	1.00	–	1.00	–
fenoterol	0.81	0.08	0.81	0.03	0.80	0.07	0.87	0.04
hexoprenaline	0.95	0.10	1.01	0.05	0.95	0.08	0.96	0.06
ICI 118551	-0.06	0.04	-0.04	0.04	0.00	0.01	0.01	0.02
ICI 215001	-0.03	0.07	0.06	0.04	0.00	0.01	0.12	0.04
ICI 89406	0.03	>0.01	0.07	0.01	0.00	0.01	0.11	0.03
isopropyl- <i>nor</i> -synephrine	0.53	0.01	0.56	0.04	0.02	0.01	0.71	0.09
isoproterenol	1.00	0.02	1.03	0.01	0.95	0.09	0.94	0.05
labetalol	0.06	0.05	0.01	0.03	0.00	0.01	0.03	0.025
MAPE	0.58	0.04	0.16*	0.04	0.02	0.01	0.07	0.03
NAB 277 (Clenproperol)	0.58	0.04	0.31	0.11	0.13	0.02	0.37	0.04
N-methyl-dopamine	0.91	0.08	0.59*	0.08	0.89	0.05	0.67*	0.08
<i>nor</i> -epinephrine	1.01	0.04	1.00	0.03	0.84	0.03	0.95	0.09
<i>nor</i> -metanephrine	0.24	0.01	0.11	0.03	0.00	0.01	0.02	0.02
orciprenaline	0.79	0.08	0.65	0.07	0.41	0.08	0.95	0.06
pindolol	0.01	0.02	0.11	0.02	0.00	0.01	0.07	0.04
practolol	0.02	0.04	-0.01	0.04	0.00	0.01	0.05	0.03
pronethalol	0.01	0.07	0.04	0.06	0.00	0.01	0.08	0.04
propranolol	-0.01	0.03	0.03	0.02	0.00	0.01	0.02	0.03
ritodrine	0.61	0.08	0.51	0.06	0.04	0.01	0.40	0.05
SKF 42469	0.87	0.02	0.62	0.02	0.79	0.07	0.73	0.05
sotalol	0.01	0.05	-0.03	0.03	0.00	0.01	0.01	0.02
sulfonterol	0.35	0.03	0.39*	0.06	0.01	0.01	0.15	0.04
terbutaline	0.80	0.07	0.50*	0.06	0.43	0.03	0.54*	0.06
<i>tert</i> -butyl- <i>nor</i> -synephrine	0.49	0.01	0.35	0.05	0.02	0.02	0.41	0.07
timolol	-0.03	0.03	0.00	>0.01	0.00	0.01	0.05	0.04
xamoterol	0.18	0.04	0.34	0.03	0.00	0.01	0.19	0.04

Notes:

* Data labeled with stars indicate E_{\max} estimates based on curve-fitting extrapolations, since the maximal concentration allowed in the assay did not reach a true plateau in the concentration-response curve (see *Experimental*).

Table 2 Comparison of the potency of adrenergic ligands (expressed as log₁₀ molar value of the EC₅₀) for inducing β₂AR or β₁AR coupling to G protein and arrestin. Only ligands for which a comparison can be made are presented here. The complete data set is given in supplemental table s0.

Ligands	Receptor-G protein interaction							Receptor-Arrestin interaction						
	β ₂ AR			β ₁ AR			β ₂ /β ₁ AR E ₅₀ ratio	β ₂ AR			β ₁ AR			β ₂ /β ₁ AR E ₅₀ ratio
	Log(E ₅₀)	err ^a	n	Log(E ₅₀)	err ^a	n		Log(E ₅₀)	err ^a	n	Log(E ₅₀)	err ^a	n	
adrenalone	-6.5	0.03	2	-4.80*	0.27	2	0.019*	-5.4	0.11	2	-4.5*	0.10	2	0.13*
AH 3021	-6.9	0.32	2	-5.81	0.15	2	0.075	-6.4	0.38	2	-6.0	0.06	2	0.42
Albuterol	-7.2	0.08	2	-5.47	0.49	2	0.020	-6.7	0.06	3	-5.9	0.09	2	0.15
C78 (Tulobuterol)	-7.3	0.18	4	-5.4	0.19	2	0.014	<i>no crc^b</i>			-5.6	0.25	2	–
CGP 12177	<i>no crc^b</i>			-7.6	0.08	2	–	<i>no crc^b</i>			-6.9	0.75	3	–
Cimeterol	-8.4	0.27	2	-7.3	0.12	2	0.068	-7.3	0.06	3	-7.4	0.04	2	1.19
Clenbuterol	-8.6	0.07	3	-5.2	0.41	2	0.0003	-7.6	0.06	3	-6.4	0.27	3	0.06
Dobutamine	-6.8	0.17	3	-6.3	0.06	6	0.31	<i>no crc^b</i>			-6.4	0.18	2	–
Dopamine	-5.1	0.34	2	-4.6*	0.31	3	0.39*	<i>no crc^b</i>			-4.2*	0.65	2	–
Du 21117	-6.6	0.42	2	-5.7	0.22	3	0.11	-5.8	0.06	3	-6.0	0.10	2	1.62
Du 28663	-6.6	0.38	2	-5.1	0.37	2	0.03	-6.0	0.05	3	-5.2	0.20	2	0.18
epinephrine	-7.3	0.07	20	-6.3	0.05	26	0.103	-7.0	0.04	15	-6.9	0.04	15	0.75
fenoterol	-7.9	0.01	2	-5.6	0.44	2	0.004	-7.2	0.08	3	-6.4	0.03	3	0.13
hexoprenaline	-7.4	0.39	2	-5.7	0.09	2	0.020	-7.2	0.06	3	-5.4	0.32	2	0.02
Isopropyl-norsynephrine	-6.7	0.32	2	-5.8	0.22	2	0.130	<i>no crc^b</i>			-6.1	0.17	2	–
isoproterenol	-7.6	0.15	4	-7.2	0.26	3	0.48	-7.4	0.01	4	-8.0	0.12	2	3.83
N-methyl-dopamine	-5.9	0.22	2	-4.8*	0.24	2	0.1*	-5.0	0.07	3	-4.6*	0.04	2	0.37*
nor-epinephrine	-6.5	0.18	6	-6.8	0.09	8	2.1	-5.2	0.03	3	-7.4	0.23	2	158
orciprenaline	-6.4	0.50	2	-5.7	0.22	2	0.18	-5.6	0.11	2	-5.0	0.22	2	2.5
ritodrine	-7.0	0.02	2	-5.4	0.28	2	0.025	<i>no crc^b</i>			-5.7	0.13	2	–
SKF 42469	-7.8	0.12	3	-5.7	0.09	3	0.008	-7.1	0.18	4	-6.1	0.22	2	0.11
terbutaline	-6.6	0.28	2	-5.0*	0.12	2	0.023*	-5.9	0.06	2	-5.0	0.20	3	0.13
<i>tert</i> -butyl-norsynephrine	-7.0	0.11	2	-5.3	0.15	2	0.023	<i>no crc^b</i>			-5.7	0.52	3	–

Notes: (a) *err* is the range of values for $n=2$ or S.E.M. for $n>2$; (b) *no crc* means that the ligand effect was not sufficient to obtain a reliable concentration-response curve. Data extrapolated from computer fittings are labeled with asterisks as in table 1.

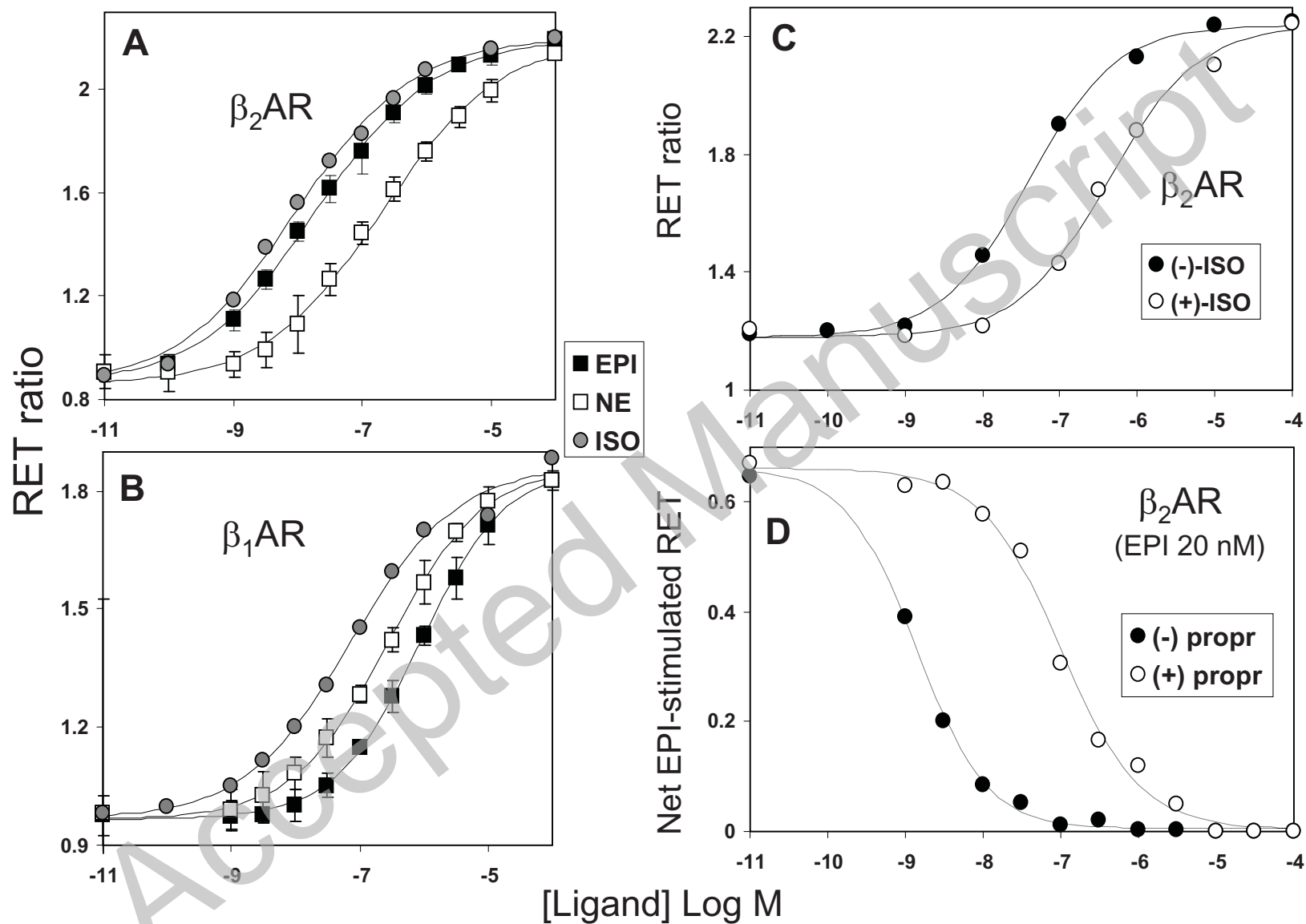


Figure 1

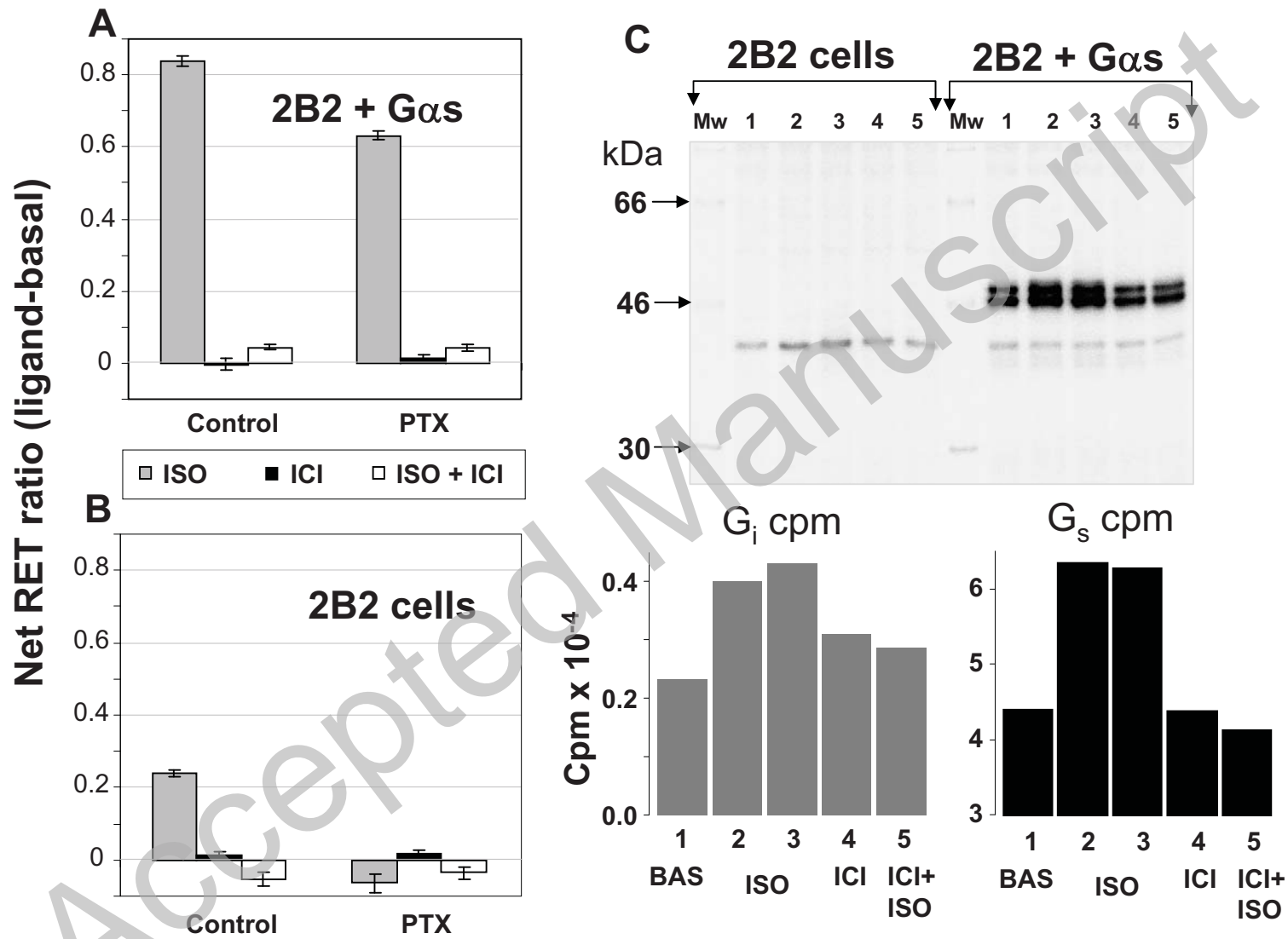


Figure 2

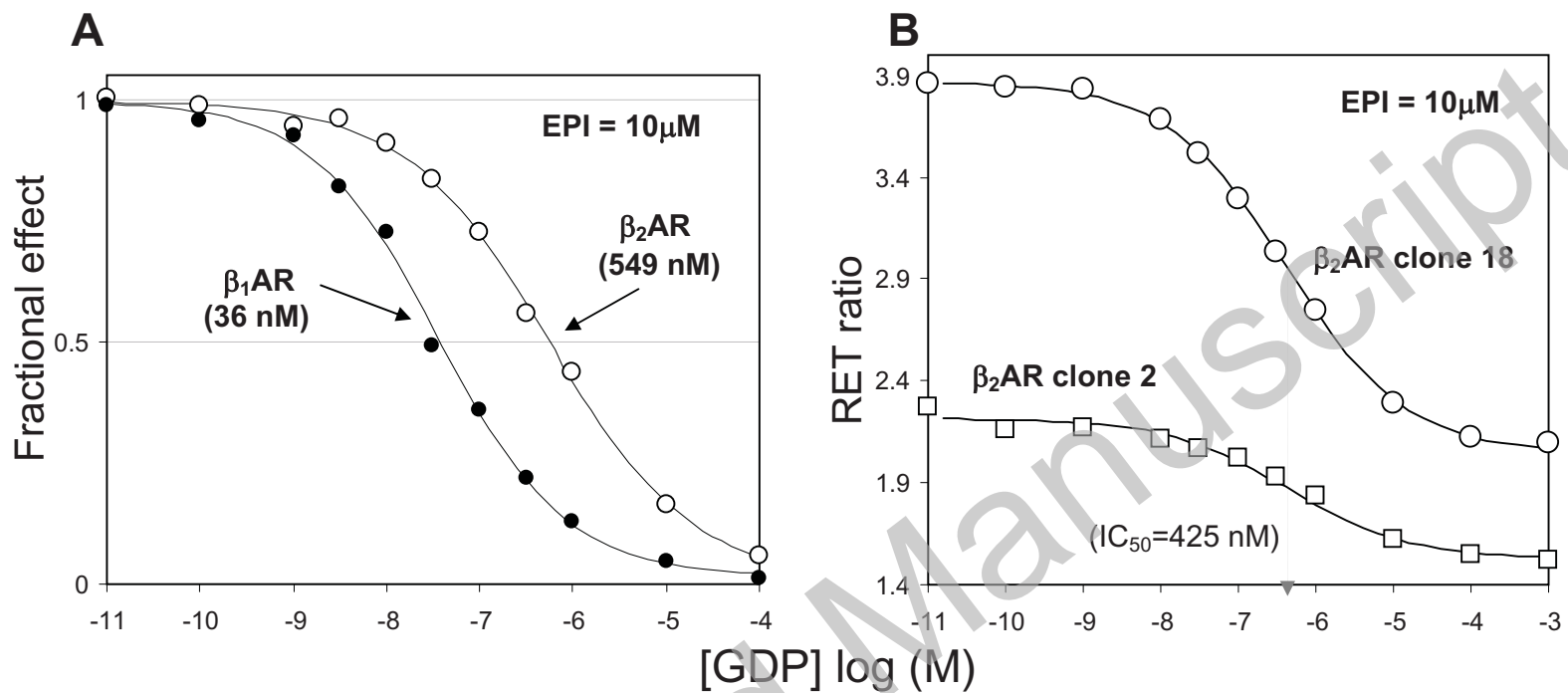


Figure 3

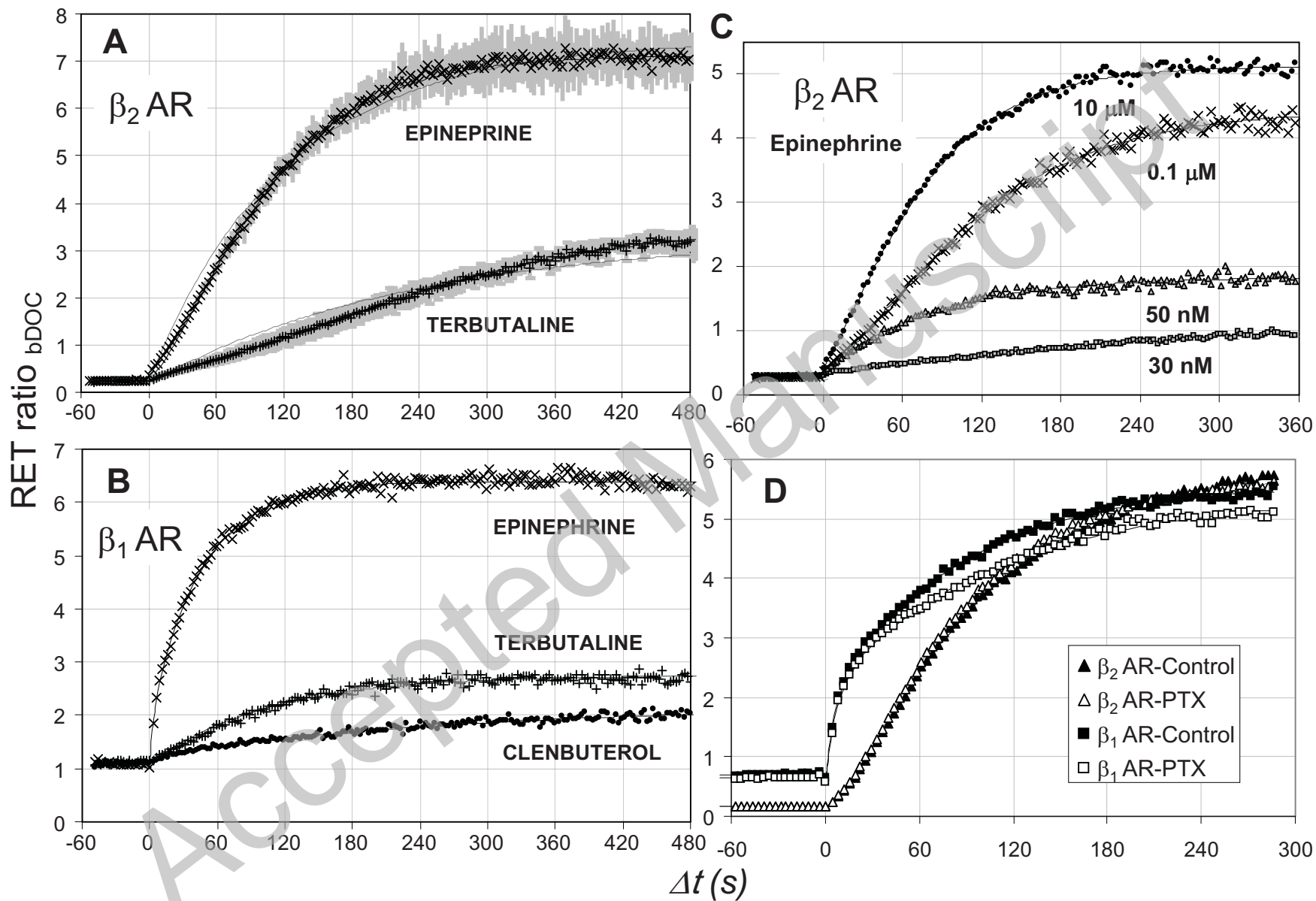


Figure 4

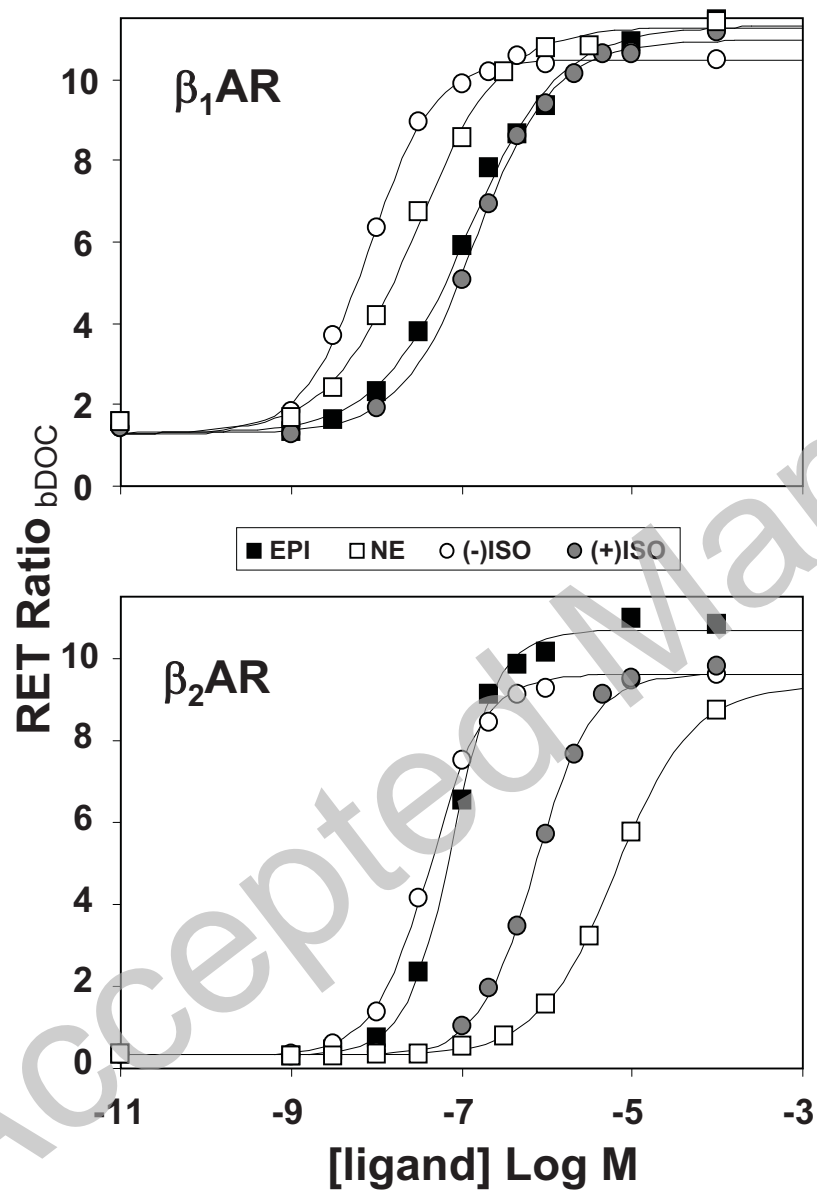


Figure 5

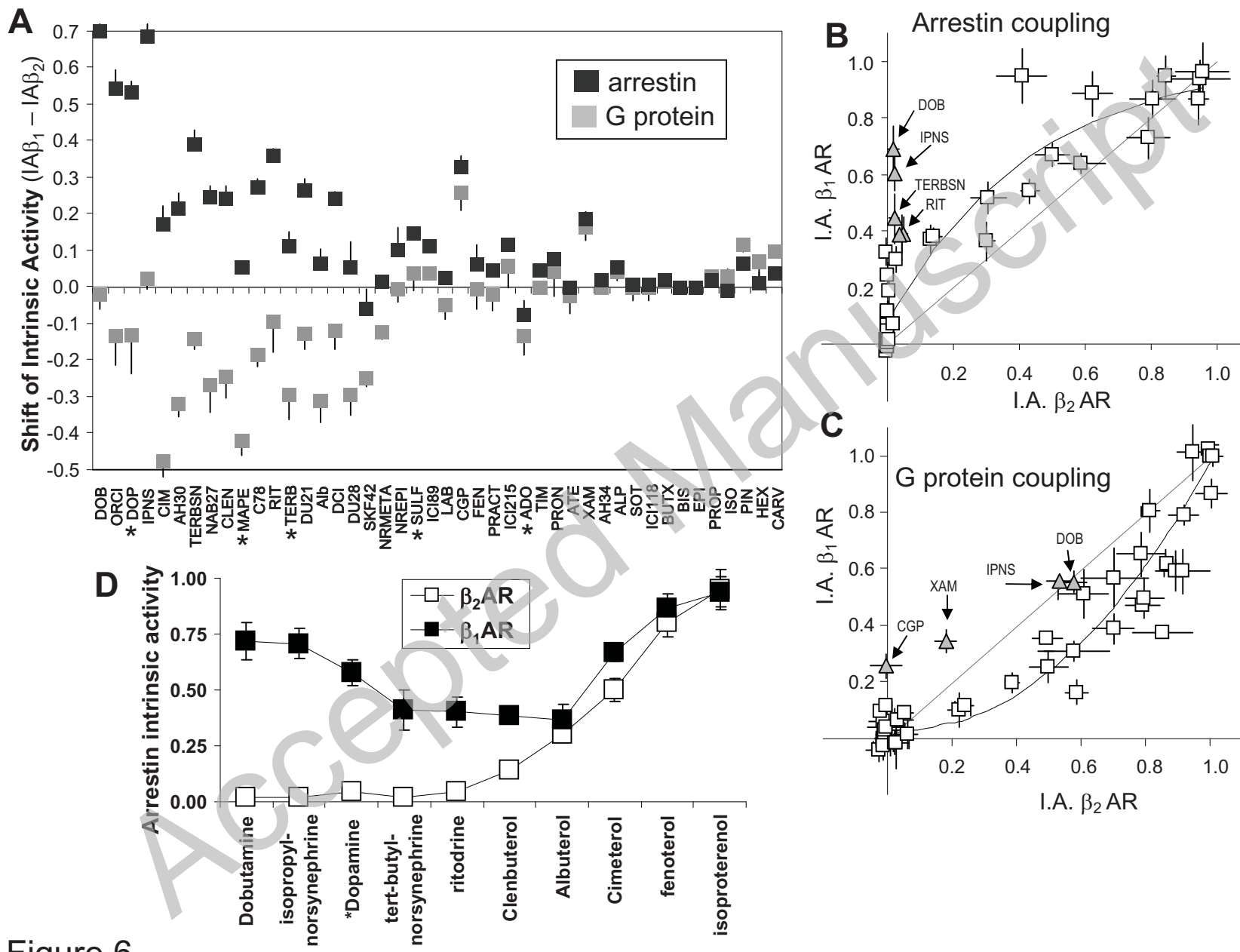


Figure 6

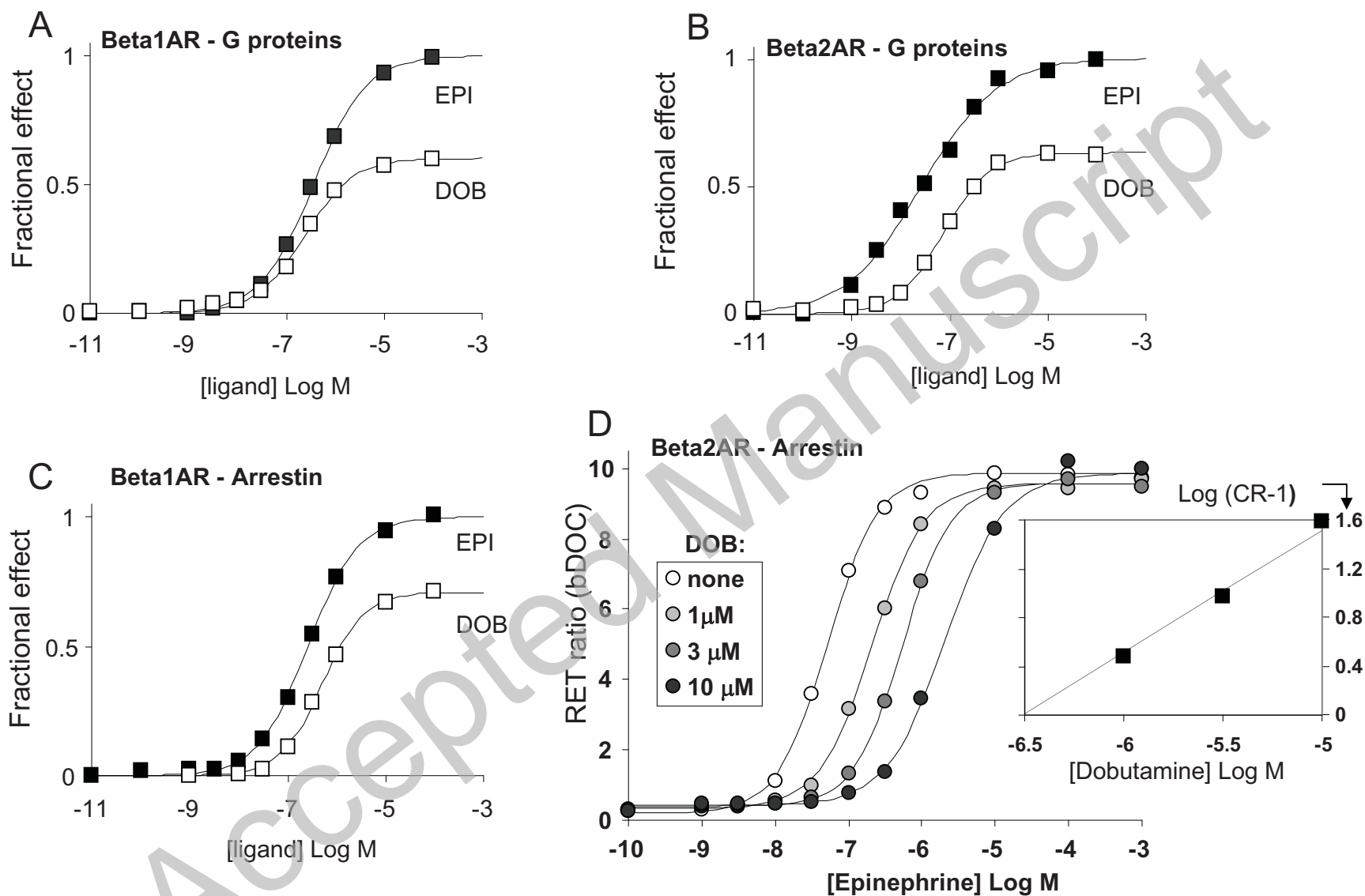


Figure 7