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Quantification of functional selectivity at the human α_{1A}-adrenoceptor

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Running Title: Functional selectivity at the α_{1A}-AR

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Abbreviations: α-AR, α-adrenoceptor; cAMP, 3',5'-cyclic adenosine monophosphate; CHO-KI, Chinese hamster ovary; ECAR, extracellular acidification rate; GPCR, G protein-coupled receptor; IP, inositol phosphates; PTX, Pertussis toxin

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ABSTRACT

Although G protein-coupled receptors (GPCRs) are often categorized in terms of their primary coupling to a given type of Gα protein subunit, it is now well established that many show promiscuous coupling and activate multiple signaling pathways. Furthermore, some agonists selectively activate signaling pathways by promoting interaction between distinct receptor conformational states and particular Gα subunits or alternative signaling proteins. We have tested the capacity of agonists to stimulate Ca^{2+} release, cAMP accumulation and changes in extracellular acidification rate (ECAR) at the human α_{1A}-adrenoceptor. Signaling bias factors were determined by novel application of an operational model of agonism, and compared to the reference endogenous agonist, norepinephrine; values significantly different from 1.0 indicated an agonist that promoted receptor conformations distinct from that favored by norepinephrine. Oxymetazoline was a full agonist for ECAR and a partial agonist for Ca^{2+} release (bias factor 8.2), but failed to stimulate cAMP production. Phenylephrine showed substantial bias towards ECAR versus Ca^{2+} release or cAMP accumulation (bias factors 21 and 33 respectively), but did not display bias between Ca^{2+} and cAMP pathways. Cirazoline and A61603 displayed bias towards cAMP relative to Ca^{2+} release (bias factors 7.4 and 8.6). Interestingly, epinephrine, a second endogenous adrenoceptor agonist, did not display bias relative to norepinephrine. Our finding that phenylephrine displayed significant signaling bias, despite being highly similar in structure to epinephrine, indicates that subtle differences in agonist-receptor interaction can affect conformational changes in cytoplasmic domains and thereby modulate the repertoire of effector proteins that are activated.

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INTRODUCTION

Although G protein-coupled receptors (GPCRs) are traditionally categorized as Gs-, Gq- or Gi/o-coupled, studies of multiple signaling outputs often indicate that a GPCR has the capacity to interact with more than one G protein subtype as well as alternative signaling or effector proteins such as arrestins. This raises the possibility that ligands may display functional selectivity, promoting differential coupling of receptors to Gα subunits or G protein-independent pathways (reviewed by Galandrin *et al*., 2007; Kenakin, 2007; Audet & Bouvier 2008; Evans *et al*., 2010; Kenakin & Miller, 2010). For example, antipsychotic drugs acting at the dopamine D_2 receptor display functional selectivity with respect to Gi/o-mediated decreases in cyclic AMP (cAMP), compared to receptor recruitment of arrestin-3 (Masri et al., 2008). All clinically effective antipsychotics block arrestin-3 recruitment, despite ranging from partial agonists to inverse agonists when tested for inhibition of cAMP.

In some cases, agonists with only subtle differences in structure still display functional selectivity. Amongst a series of phenethylamines active at the β_2-AR, cyclopentylbutanephrine, α-ethylnoradrenaline and isoetharine are partial agonists for cAMP production relative to isoproterenol, but act as full agonists for arrestin-3 recruitment (Drake *et al*., 2008). These three compounds share an ethyl group on the αC atom, in close proximity to the NH_3^+ group that interacts with Asp113 (3.32). There may be a steric effect of the α-ethyl group that compromises receptor conformational changes linked to G protein activation, without affecting receptor phosphorylation or arrestin binding. In cardiac myocytes, the (S,R) isomer of fenoterol stimulates substantially higher activation of Gαi2 than the (R,R) isomer, whereas (R,R)-fenoterol produces higher activation of Gαs than (S,R)-fenoterol (Woo *et al*., 2009). This differential Gs/Gi coupling is apparent in functional assays including myocyte

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contractility and Erk1/2 phosphorylation, and the authors suggest that agonists selectively able to stimulate β_2-AR Gs coupling, without stimulating β_2-AR Gi coupling or β_1-AR activation, may have considerable therapeutic benefit.

In matched cells expressing a given receptor, functional selectivity can be unambiguously demonstrated when two ligands promote a reversal of efficacy or potency between two pathways, that is, drug A has higher efficacy/potency than drug B for pathway 1, but a lower efficacy/potency than drug B for pathway 2 (Urban *et al*., 2007; Kenakin, 2007; Kenakin & Miller, 2010). However, such observations do not provide capacity for quantification and statistical analysis, and there are reported instances where drugs do not display reversal of potency or efficacy, but clearly promote distinct receptor conformations (for example, Galandrin *et al*., 2008). The operational model of agonism, originally derived by Black & Leff (1983), can provide a means of quantifying signalling bias by comparing τ/K_A ratios between agonists (Figueroa *et al*., 2009; Gregory *et al*., 2010; Kenakin & Miller, 2010; Koole et al., 2010). In this model, the parameter τ encompasses receptor density and the efficacy of the agonist, and K_A is the equilibrium dissociation constant of the agonist-receptor complex. The composite parameter, τ/K_A , is thus a convenient "transduction ratio" (Kenakin & Miller, 2010) that can account for functional selectivity in the activity of drugs with high affinity/low efficacy or low affinity/high efficacy.

In the current study we have used this approach to calculate bias factors for agonists acting at the human α_{1A}-adrenoceptor (AR). The three α_1-ARs preferentially stimulate coupling to Gq, activating phospholipase C, increasing intracellular levels of inositol (1,4,5) triphosphate (IP_3) and intracellular Ca^{2+}, and releasing diacylglycerol that in turn activates protein kinase C isoforms. Studies in tissues that endogenously express α_1-ARs, or in recombinant cells

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expressing each of the α_1-AR subtypes, have also demonstrated activation of phospholipase A_2, phospholipase D, adenylate cyclase and MAP kinases (Graham et al., 1996; Perez et al., 1993; Zhong and Minneman 1999; Piascik and Perez 2001). We have examined the ability of α_{1A}-ARs stably expressed in CHO-K1 cells to stimulate Ca^{2+} release, cAMP accumulation and changes in extracellular acidification rate (ECAR) upon agonist exposure. We compared the endogenous agonist norepinephrine, another two phenethylamines (methoxamine and phenylephrine), and three imidazolines, cirazoline, oxymetazoline and A61603 (Figure 1). All 6 agonists stimulated release of intracellular Ca^{2+} and ECAR measured in a cytosensor microphysiometer. Oxymetazoline was a partial agonist for Ca^{2+} release and a full agonist in the cytosensor, yet failed to stimulate cAMP production even at high concentrations. By calculating bias factors for the agonists relative to norepinephrine, we have found several clear and quantitiative indications of functional selectivity. In contrast to this group of compounds, the two endogenous agonists, norepinephrine and epinephrine, showed no bias relative to each other.

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MATERIALS AND METHODS

Materials. Sources of drugs were as follows: [^{125}I]-HEAT (2-[β-(4-hydroxy-3-[^{125}I]-iodophenyl) ethylamino-methyl]tetralone) (Prosearch); [^{3}H]-prazosin (Amersham Pharmacia Biotech); (-) norepinephrine bitartrate, phenylephrine, phentolamine HCl (Sigma); methoxamine, oxymetazoline, cirazoline (Tocris); A61603 (N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydro-naphthalen-1-yl]methane sulphonamide) (Tocris). DMEM/HAMS F12 50:50 mix, foetal bovine serum, glutamine, penicillin/streptomycin (Thermotrace); Platinum Pfx DNA polymerase, Lipofectamine, phenol red-free Optimem, Fluoro-4 (Invitrogen, Eugene OR, USA), G418, IBMX (Sigma). Cytosensor consumables were from Selby Biolab, except for transwell cups that were from Costar.

Cloning of the human α_{1A}-AR. Plasmid containing the human α_{1A-4}-AR cDNA was a gift from Dr Thomas Chang (Roche Bioscience, Palo Alto, CA). The coding region of this receptor was subcloned into the multiple cloning site of pcDNA3.1. The α_{1A-4}-AR cDNA was converted to the α_{1A-1}-AR as follows. A silent mutation was introduced into the α_{1A-4}-AR construct using the Stratagene QuikChange mutagenesis kit to generate a BsrGI restriction site (TGT ACC→TGT ACA) corresponding to Cys^{419} Thr^{420}, immediately upstream of the point at which the four α_{1A}-AR splice variants differ (Chang et al., 1998). The 3' end of the α_{1A-1}-AR coding region was generated by polymerase chain reaction (PCR) on human genomic DNA, using Platinum Pfx high fidelity DNA polymerase. The forward primer incorporated the BsrG1 site (5' AATCCTCCTGTACAACAGCCCGGGTGAG 3') and the reverse primer included the α_{1A-1}-AR termination codon and a downstream Xba I site (5' CCTCTGCATCTAGACTGTCCTAGACTTCCTC 3'). The α_{1A-1}-AR PCR fragment was digested with BsrG1 and Xba I, then ligated into pcDNA3.1-α_{1A-4}-AR plasmid from which the corresponding cassette had been removed. The complete α_{1A-1}-AR insert and junctions

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with pcDNA3.1 were checked by DNA sequencing on both strands (Micromon, Monash University, Australia)

Cell culture and production of clones stably expressing α_{1A}-ARs. Chinese hamster ovary (CHO-K1) cells were grown in a 50:50 Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 medium supplemented with 10% (v/v) foetal bovine serum (FBS), glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C with 5% CO_2. Transfections were performed using Lipofectamine and the transfected cells selected in media containing 800 μg/ml G418, and maintained in media containing 400 μg/ml G418. Clonal cell lines were isolated by screening G418-resistant colonies and preliminary screening of clones was made using a single point [^{3}H]-prazosin (2000 pM) whole cell binding assay. Suitable clones were amplified and receptor levels determined in saturation binding assays using [^{125}I]-HEAT.

Radioligand Binding Studies. Cells were harvested as previously described by Hutchinson et al., (2002). Briefly, cells from a 75 cm^2 flask were washed twice with HEPES buffered saline and scraped from flasks with lysis buffer (25 mM Tris pH 7.5 room temperature, 1 mM EDTA, 200 μg/ml bacitracin, 10 μg/ml leupeptin, 2.5 μg/ml pepstatin A, 2.5 μg/ml aprotinin). Cells were homogenized with a Dounce homogeniser and centrifuged at low speed (1000 x *g*, 10 min) to remove cell debris. Supernatants were pooled and centrifuged (39,000 x *g*, 15 min, 4°C). The pellet was homogenized in binding buffer (50 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 200 μg/ml bacitracin, 10 μg/ml leupeptin, 2.5 μg/ml pepstatin A, 2.5 μg/ml aprotinin) and frozen at -70°C until required. Membranes (10-20 μg protein) were incubated with [^{125}I]-HEAT (20-1200 pM) in a total volume of 100 μl for 90 mins at room temperature (21°C). Phentolamine (100 μM) was used to define non-specific binding. Competition binding experiments were conducted using a range of concentrations of

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unlabelled ligand and [^{125}I]-HEAT (500 pM), also at room temperature for 90 min (Sharpe et al., 2003). Reactions were terminated by rapid filtration through GF/C filters pre-soaked for 30 mins in 0.5% polyethyleneimine using a Packard Filtermate cell harvester. Filters were washed three times with wash buffer (50 mM Tris pH 7.4, 4°C), dried, and 25 μl Microscint O (Packard) added and radioactivity counted on a Packard Top Count. Protein concentrations were quantitated using the Lowry (1951) assay. All experiments were performed in duplicate using 5 different membrane preparations.

Measurement of intracellular free Ca^{2+} concentration. CHO-K1 cells expressing the α_{1A}-AR were seeded at 5 x 10^4 cells per well in 96-well plates overnight. On the day of the experiment, the media was removed and cells washed three times in a modified Hanks' buffered saline solution (HBSS; composition in mM: NaCl 150, KCl 2.6, $MgCl_2.2H_2O$ 1.18, D-glucose 10, Hepes 10, $CaCl_2.2H_2O$ 2.2, probenecid 2, pH 7.4) containing BSA 0.5% (w/v). In light-diminished conditions cells were treated with fluoro-4 (0.1% v/v in modified HBSS, 1 h, 37 ºC). Excess fluoro-4 not taken up by the cells was removed by washing twice in modified HBSS and then incubated for a further 30 min before the assay plate was transferred to a FlexStation (Molecular Devices, Palo Alto CA, USA). Real-time fluorescence measurements were recorded every 1.7 seconds over 200 seconds, with drug additions occurring after 17 seconds, using an excitation wavelength of 485 nm and reading emission wavelength of 520 nm. All experiments were performed in duplicate. Agonist responses represent the difference between basal fluorescence and peak $[Ca^{2+}]i$ measurements expressed as a percentage of the response to A23187 (1 μM) in each experiment.

Cyclic AMP accumulation studies. Cells were seeded into 96 well plates at 10^4 cells/well in media with 0.5% v/v FBS, the night before the experiment. On the day of the experiment the media was replaced with stimulation buffer (1 mg/ml BSA, 0.5 mM IBMX, and 5 mM HEPES, pH 7.4 in Hanks' balanced salt solution, 50 μl/well). Cells were exposed to agonists

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(diluted to 2x final concentration in stimulation buffer, 50 µl/well) for 30 min at 37°C, then reactions were terminated by addition of 100 µl of lysis buffer (1 mg/ml BSA, 0.3% (v/v) Tween 20, 5 mM HEPES, and 1 mM IBMX, pH 7.4). cAMP was assayed using αScreen in an EnVision plate reader (PerkinElmer, VIC, Australia). Responses to forskolin (10^{-4} M) were determined in parallel with agonist-stimulated cAMP accumulation for each batch of cells, and results are expressed as a percentage of the response to forskolin. All experiments were performed in duplicate and n is the number of independent experiments.

Cytosensor microphysiometer studies. The cytosensor microphysiometer (Molecular Devices Corp., Sunnyvale, CA, USA) is a light-addressable silicon sensor-based device that measures increases in the metabolic activity of isolated cells as increases in extracellular acidification rate (ECAR; McConnell et al., 1992). Cells were seeded into 12 mm transwell cups (3 µm pore size; Costar) at $5x10^5$ cells per cup in media with 0.5% FBS, and allowed to grow overnight. The next day, the assembled cups were placed in the sensor chambers in the cytosensor and perfused with modified RPMI 1640 medium (Molecular Devices) at 100 µl/min at 37°C. After initial set-up, cells were superfused with media for 2 hr to stabilize baseline extracellular acidification rates, then cumulative concentration-response curves were constructed for each agonist. Each concentration was perfused for 14 min, consisting of 7 two -min pump cycles during which the flow was stopped for the last 40 sec, and the acidification rate measured for 30 sec. The maximum reading from the 7-cycle recording corresponding to each drug concentration was expressed as a % of the basal acidification rate measured over a period of 10 min before the addition of agonist. All drugs were diluted in medium and concentration-response experiments were performed in the presence of (-) propranolol (10^{-6}M) to block any contributions from endogenous β_2-AR. Results were expressed as % basal ECAR and n values represent cells grown in different flasks before plating into the transwell cups.

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Data analysis. All values are expressed as mean ± s.e. mean of n. Data was analysed using non-linear curve fitting (Graph Pad PRISM v5.02) to obtain pEC_{50} values for the cytosensor microphysiometer, cAMP accumulation, and $[Ca^{2+}]i$ assays, or pK_i, pK_D and B_{max} values from radioligand binding assays. To quantify signaling bias, agonist concentration response curves were analysed by non-linear regression using an operational model of agonism (Black and Leff, 1983; Gregory et al, 2010) in a method similar to that described by Figueroa *et al.* (2009), to define τ/K_A ratios for each agonist, for each pathway:

$$Y = Basal + \frac{(E_m - Basal)\cdot TR^n \cdot [A]^n}{[A]^n \cdot TR^n + \left(1 + \frac{[A]}{K_A}\right)^n} Basal \qquad (1)$$

where E_m is the maximal possible response of the system (not the agonist), Basal is the basal level of response in the absence of agonist, K_A denotes the functional equilibrium dissociation constant of the agonist for the ground (G protein-uncoupled) state of the receptor, n is the slope of the transducer function that links occupancy to response, and TR (transduction ratio) is the ratio of τ/K_A, where τ is an index of the coupling efficiency (or efficacy) of the agonist – this parameter incorporates the affinity of the agonist for the active state of the receptor that triggers signaling, as well as the efficiency of coupling of the receptor to its cognate G protein(s) and subsequent cellular stimulus-response transduction mechanisms (Leff and Harper, 1989). Note that K_A values are derived directly from the non-linear regression analysis of agonist concentration-response curves. The estimated TR values were used in the comparison of functional selectivity mediated by each agonist across the various pathways, as described in the Results. A detailed exploration of this method for determining agonist signaling bias will be presented separately.[1]

[1] Kenakin, Novick, Watson, Muniz-Medina and Christopoulos, manuscript in preparation

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RESULTS

Expression of the α_{1A}-AR in CHO-KI cells

Based on saturation binding with [^{125}I]-HEAT, the α_{1A}-AR clone chosen for these studies displayed a B_{max} value of 531±94 fmol/mg protein, and a pK_D of 9.2±0.09. Further characterisation of the α_{1A}-AR was carried out with competition binding experiments using the agonists norepinephrine, methoxamine, phenylephrine, oxymetazoline, cirazoline and A61603. All of the agonists competed for [^{125}I] HEAT binding with binding curves fitting a single site isotherm. The rank order of binding affinities for the α_{1A}-AR agonists was oxymetazoline (pK_i 7.5±0.2) = A61603 (7.3±0.1) > cirazoline (6.7±0.2) >> norepinephrine (5.3±0.1) > phenylephrine (4.9±0.1) = methoxamine (4.8±0.1) (n=3-7). Note that imidazoline agonists have substantially higher affinity for the α_{1A}-AR than the phenethylamines.

Effects of α_{1A}-AR agonists on intracellular Ca^{2+} release

In CHO-K1 cells expressing the α_{1A}-AR, all the agonists stimulated Ca^{2+} release (Figure 2, Table 1). E_{max} values ranged from 97.4% for A61603 (relative to 1 μM A23187) down to 78.4% for oxymetazoline. It is noteworthy that norepinephrine and phenylephrine behaved as high efficacy, low affinity agonists, as the ratio of the apparent binding affinity (Ki) to EC_{50} is approximately 1700. Such a high "amplification" ratio (Strange, 2008) reflects substantially higher agonist affinity for the active state(s) than for the inactive state of the receptor and/or high efficiency of signal transduction for the measured pathway, in this case Ca^{2+} release. In contrast to norepinephrine and phenylephrine, oxymetazoline and cirazoline display only 36- and 47-fold amplification factors. A61603 and methoxamine differ in pK_i for the α_{1A}-AR by over two log units, but display similar amplification of the response relative to their binding affinities (250- and 320-fold respectively).

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Effects of α_{1A}-AR agonists on cAMP accumulation

All three α_1-AR subtypes promote agonist-stimulated Gq activation and Ca^{2+} release, but only the α_{1A}- and α_{1B}-AR stimulate adenylate cyclase and cAMP production (Shibata et al., 2003). This suggests that the receptor coupling determinants for the Ca^{2+} and cAMP pathways differ, and thus that the immediate post-receptor step for each of these pathways is distinct. We found that incubation of CHO-α_{1A}-AR cells with norepinephrine, phenylephrine, methoxamine, cirazoline or A61603 stimulated concentration-dependent increases in cAMP accumulation as shown in Figure 3. Concentration-response curves were also performed in the presence of inhibiting concentrations of propranolol (10^{-6} M) to block any endogenous β-ARs. This did not affect cAMP responses, demonstrating that endogenous β-ARs were not responsible for the α_{1A}-AR agonist-stimulated cAMP accumulation (data not shown). In addition, no response was seen in non-transfected CHO-K1 cells stimulated with norepinephrine (data not shown). In contrast to the other agonists, oxymetazoline failed to increase cAMP production above basal levels at concentrations up to 10^{-4} M. To test for possible physiological antagonism of responses to oxymetazoline mediated by endogenous α_2-AR in CHO-K1 cells, experiments were performed in the presence of the α_2-AR antagonist rauwolscine (10^{-7} M). These experiments failed to reveal cAMP accumulation in response to oxymetazoline. To test whether oxymetazoline was directing strong coupling of the α_{1A}-AR to Gi, thereby cancelling out any stimulatory effect on cAMP accumulation, cells were pre-treated with PTX 16 hours prior to agonist stimulation. This likewise failed to reveal any cAMP accumulation in response to oxymetazoline (data not shown). cAMP responses are expressed as a percentage of the control forskolin (10^{-4} M) response, and E_{max} and potency values for each agonist are summarized in Table 1. E_{max} values were similar for the three phenethylamines and A61603, whereas cirazoline was a partial agonist. It is striking

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that all of the drugs able to activate cAMP accumulation do so with pEC_{50} values very close to their affinity values.

Effects of α_1-AR agonists on ECAR

To examine additional pathways that may be activated by the α_{1A}-AR, the cytosensor microphysiometer was utilized as a measure of total cellular activity stimulated by agonists. Incubation of cells stably expressing the α_{1A}-AR with agonists stimulated concentration-dependent increases in extracellular acidification rate (ECAR, Figure 4). E_{max} values ranged from increases of 146.5 to 152.2 % basal (Table 1). In essence all of the compounds tested were full agonists relative to norepinephrine, representing a substantial change in the behaviour of oxymetazoline and cirazoline compared to Ca^{2+} release or cAMP responses. As seen in the Ca^{2+} release assay, there were substantial differences in the degree of amplification displayed by each agonist, varying from 2350-fold for phenylephrine down to 9-fold for oxymetazoline. Unlike Ca^{2+} release, however, norepinephrine promoted only 105-fold amplification, approximately 20-fold lower than that seen with phenylephrine.

Analysis of functional selectivity at the α_{1A}-AR

Our data indicated that there are interesting differences in agonist activity between the three measured signaling outputs. We thus carried out a further analysis of this data to test the hypothesis that at least some agonists display functional selectivity, that is, they preferentially induce or stabilize different receptor conformations that in turn couple to two or more signaling pathways (Urban *et al*., 2007; Kenakin, 2007; Evans *et al*., 2010; Kenakin & Miller, 2010). One hallmark of functional selectivity is that two agonists show a reversal in efficacy between two signaling assays, though a reversal in potency between two agonists can also be accommodated only by the involvement of distinct receptor conformations

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(Kenakin, 2007). Amongst the data presented in Table 1, there were no reversals in E_{max} between agonists, however there were several instances of a reversal of potency. As shown in Figure 2, oxymetazoline stimulated a small cAMP response at 1 mM, and thus exhibited very low potency even though no pEC_{50} value could be derived. Oxymetazoline had higher potency for Ca^{2+} release than phenylephrine, but clearly lower potency for cAMP. Similarly, oxymetazoline had higher potency for both Ca^{2+} release and ECAR than methoxamine, but lower potency for cAMP accumulation. Interestingly, phenylephrine had higher potency for ECAR than norepinephrine, but lower potency for Ca^{2+} release.

While these reversals in potency indicate functional selectivity, there are two major limitations. First, a subjective analysis of the data does not allow us to ascribe statistical significance to bias in drug activity across the three signaling endpoints. Second, while a reversal in efficacy or potency between drugs may be a sufficient condition for demonstrating functional selectivity, there is evidence that such reversals are not a necessary condition (Evans *et al*., 2010). Thus, we applied an operational model of agonism (equation 1) to derive quantitative measures of functional selectivity between agonists for the different signaling assays. To exclude possible bias introduced by the cellular host system (as evidenced by the substantial differences in response amplification displayed by norepinephrine across the three assays), the transduction ratios derived from application of the operational model of agonism were normalized to those of a reference agonist, in this case the endogenous agonist, norepinephrine. Under these conditions, if the test agonist and the reference agonist activate the two pathways via a common receptor conformation, the bias factor should be 1.0, irrespective of differences in response amplification between pathways. In contrast, significant deviation of bias factors from 1.0 indicates the involvement of distinct conformations for the different agonists. Table 2 shows the logarithms of the operational model τ/K_A ratios for each pathway (logTR), the logTR values normalised to that of

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norepinephrine ($logTR_n$), and the final bias factor calculations for signaling by the α_{1A}-AR (difference between $LogTR_n$ values for a given agonist across different pathways; see Supplemental Table 1 for statistical analysis of data).

Phenylephrine and oxymetazoline showed significant bias towards ECAR compared to Ca^{2+} release. In the case of phenylephrine, the bias factor of 21 reflects a high τ/K_A ratio for ECAR relative to norepinephrine, despite the lower τ/K_A ratio for Ca^{2+} release. A high bias factor of 8.2 was observed for oxymetazoline because unlike norepinephrine, this drug displayed the same τ/K_A ratio for ECAR and Ca^{2+} release (log τ/K_A values of 8.46 and 8.52 respectively). Phenylephrine also displayed a high bias factor of 33 between ECAR and cAMP accumulation. Again this reflects the fact that the cAMP τ/K_A ratio was substantially lower for phenylephrine than for norepinephrine while the ECAR ratio is higher. The same is true to a lesser extent for methoxamine (bias factor 4.0), but was not seen in the case of the two imidazolines. A different pattern emerged between Ca^{2+} release and cAMP accumulation. Here, the two phenethylamines (methoxamine and phenylephrine) both showed no significant bias relative to norepinephrine, whereas the two imidazolines (cirazoline and A61603) showed a clear 7.4- and 8.6-fold bias respectively towards cAMP signaling. As there was no reversal of potency between norepinephrine and cirazoline or A61603 for cAMP accumulation versus Ca^{2+} release, this result illustrates the power of being able to quantify functional selectivity using the operational model.

In order to check that these findings of signaling bias were not artefacts of the particular clonal cell line used or receptor abundance, we repeated the analysis using CHO-K1 cells expressing the α_{1A}-AR with a B_{max} of 204 fmol/mg protein. The key finding was still evident in these cells, namely that the bias factors for phenylephrine relative to norepinephrine were

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37 for ECAR compared to Ca^{2+} release and 34 for ECAR versus cAMP, whereas there was no bias between the Ca^{2+} and cAMP pathways (bias factor 0.9).

Endogenous agonists for the α_{1A}-AR do not display functional selectivity

Phenylephrine displayed a substantially higher capacity than norepinephrine to activate signaling pathways leading to changes in ECAR, suggesting that it preferentially induces or stabilizes an α_{1A}-AR conformation that couples strongly to one or more of these pathways. In contrast, the lack of bias between cAMP and Ca^{2+} indicates that the conformation associated with each of phenylephrine and norepinephrine is coupled equally well to pathways mediating Ca^{2+} release and cAMP accumulation. As shown in Figure 1, norepinephrine and phenylephrine share the meta-hydroxyl group on the ring structure, and the chiral β-hydroxyl, but there are two key differences in their chemical structure. Norepinephrine has a catechol ring with both the meta- and a para-hydroxyl group whereas phenylephrine lacks the para-hydroxyl. On the other hand, phenylephrine has a methyl substituent on the active amine group that is not present in norepinephrine. We analysed signaling bias between norepinephrine and epinephrine because the latter has both the catechol ring and the N-methyl group (Figure 5A). As shown in Figure 5B-D, norepinephrine and epinephrine are full agonists for each of the signaling pathways. Epinephrine always has somewhat higher potency than norepinephrine (Table 3), but there is no signaling bias between these compounds based on τ/K_A ratio calculations (Table 4).

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DISCUSSION

We show in this study that phenethylamine and imidazoline agonists display functional selectivity at the human α_{1A}-AR. In particular, phenylephrine shows substantial bias towards ECAR versus Ca^{2+} release or cAMP accumulation compared to norepinephrine, but does not display any bias between the Ca^{2+} and cAMP pathways. The additional endogenous agonist, epinephrine, does not show bias relative to norepinephrine in any of the signaling outputs measured. Our findings do not indicate any overall class effect between the phenethylamines and imidazolines, as oxymetazoline is unable to stimulate cAMP accumulation whereas it shows significant bias towards ECAR relative to Ca^{2+} release. The other two imidazolines, however, both display bias towards cAMP relative to Ca^{2+} release but no bias towards ECAR. Importantly, we can now draw conclusions about the functional selectivity of α_{1A}-AR agonists because we have an analytical method that provides a numerical measure of bias.

While many α_{1A}-AR responses, including intracellular Ca^{2+} release, are produced via the classical Gq pathway, coupling of α_1-ARs to additional signaling pathways has been observed in recombinant systems and tissues expressing endogenous receptors (Morgan et al., 1983; Johnson and Minnemann, 1986). Although early studies suggested that α_1-AR mediated cAMP accumulation was secondary to Gq coupling (Perez *et al*., 1993), other studies favor α_1-AR-Gs coupling. Horie *et al.* (1995) showed that in CHO-α_{1B}-AR cells, norepinephrine-stimulated cAMP accumulation was inhibited by an antibody against the Gαs C-terminal. The phospholipase C inhibitor U73122 abolished increases in intracellular Ca^{2+}, but had no effect on cAMP accumulation. In CHO-K1 cells expressing each of the α_1-ARs, phenylephrine stimulation of the α_{1A}- and α_{1B}-AR increased inositol phosphate (IP) and cAMP accumulation, whereas the α_{1D}-AR activated only IP production (Shibata et al., 2003). Thus in CHO-α_{1D}-AR cells, activation of the Gq pathway was not sufficient for stimulation

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of cAMP. Our data on the α_{1A}-AR also support a dissociation between the Gq and cAMP pathways, as cirazoline and A61603 promoted significant bias relative to norepinephrine between Ca^{2+} release and cAMP accumulation. In addition, oxymetazoline was able to stimulate Ca^{2+} release but failed to increase cAMP accumulation. It is noteworthy that although oxymetazoline, cirazoline and A61603 share some common structural elements (Figure 1), they clearly produce distinct α_{1A}-AR conformations that display varying efficiency for cAMP accumulation.

There is increasing understanding of the way in which drugs activate GPCRs, based on recent crystal structures of the β_1-AR, β_2-AR, and adenosine A_{2A} receptors (Rosenbaum *et al*., 2007; Jaakola *et al*., 2008; Warne *et al*., 2008), biophysical and structure-function studies (Swaminath *et al*., 2004; Yao *et al*., 2006, Yao *et al*., 2009), computer modelling and virtual ligand screening (Katritch *et al*., 2009; Reynolds *et al*., 2009). Furthermore, it has been possible to compare the structures of inactive rhodopsin and its active opsin counterpart (Scheerer *et al*., 2008). Binding of an agonist occurs as a multistage process in which successive conformational changes increase the stability of the drug-receptor interaction (Liapakis *et al*., 2004; Swaminath *et al*., 2005). The conformational changes at the extracellular ends of helices III, IV, V and VII are transmitted via changes in the interactions between key amino acid side chains and water molecules in a hydrogen-bonded network that extends toward the cytoplasmic domain of the receptor (Rosenbaum *et al*., 2007). The active opsin structure indicates that upon receptor activation, the C-terminus of Gα subunits binds within a cavity created by outward tilting of helix VI, altered positioning of helix V, and restructuring of the link between helices VII and VIII (Scheerer *et al.*, 2008).

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It is reasonable to suppose that each distinct agonist produces at least subtle differences in the conformation of the ligand binding pocket, however the key question posed by the demonstration of functional selectivity is whether each agonist also produces a unique conformation at the cytoplasmic face of the receptor. Rosenbaum and co-workers (2007) suggest that the core hydrogen-bonded network allows structural flexibility to the receptor, potentially dampening the capacity of different ligands to induce distinct active conformations. This may be the case, for example, between norepinephrine and epinephrine that show no bias at the α_{1A}-AR. However our demonstration of functional selectivity between norepinephrine and the other agonists tested is consistent with the idea that the receptor conformational changes induced by distinct compounds can be transmitted to unique active conformations. We suggest that any demonstration of bias for one pair of signaling outputs must indicate the presence of distinct active conformations. For example, there is clear bias between phenylephrine and norepinephrine when we compare ECAR with either Ca^{2+} release or cAMP accumulation, but not when we compare Ca^{2+} release and cAMP accumulation with each other. This cannot imply that the active conformations promoted by phenylephrine and norepinephrine are the same, but rather that effector proteins for Ca^{2+} release and cAMP signaling are not sensitive to the conformational difference. In contrast, there is clear bias between two of the imidazolines (cirazoline and A61603) and norepinephrine when we compare Ca^{2+} release with cAMP accumulation. In this case the particular receptor conformations induced by cirazoline and A61603 must favour coupling to effectors for the cAMP pathway over $G\alpha q$ coupling.

Our findings concerning the activity of norepinephrine, epinephrine, and phenylephrine at the α_{1A}-AR are of considerable interest from a structural viewpoint. Phenylephrine but not epinephrine showed substantial bias towards ECAR compared to the other two pathways.

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Whereas the structure of phenylephrine differs from norepinephrine by two elements, the only difference between epinephrine and phenylephrine is the para-hydroxyl on the catechol ring. Modelling and structure-function studies of the β_2-AR have shown that each element within the catecholamine structure interacts with particular amino acid side chains in the ligand-binding pocket (Liapakis *et al.*, 2004; Swaminath *et al.*, 2004). The hydroxyl groups on the catechol ring undergo hydrogen bonding with Ser203 (5.42), 204 (5.43) and 207 (5.46), the chiral β-hydroxyl interacts with Asn293 (6.55), the aromatic ring undergoes hydrophobic interaction with Phe290 (6.52), and the bioamine -NH_3^+ group interacts with Asp113 (3.32). In addition, the amine substituent group present in full agonists such as epinephrine and isoproterenol may interact with unidentified residues in TM6 and/or TM7 (Liapakis *et al*., 2004; Swaminath *et al*., 2004). The α_{1A}-AR has only two serine residues in TM5, Ser 188(5.42) and Ser 192(5.46), whereas the α_{1B}-AR and α_{1D}-AR, like the β_2-AR, have all three serines. Our data suggest that the absence of the para-hydroxyl in phenylephrine is associated with a key conformational difference in TM5 compared to epinephrine, which has an otherwise identical structure. While this difference does not affect the capacity of phenylephrine to promote Gαq coupling, it does increase coupling to effectors for ECAR. It would be interesting to test whether a ligand that lacks both the para-hydroxyl and the N-methyl group (norphenylephrine) also shows bias towards ECAR, and to compare the behaviour of all these compounds at the α_{1A}-AR and α_{1B}-AR that differ in the number of TM5 serine residues. We also intend to use high-throughput signaling assays to identify the nature of effectors that promote changes in ECAR and are strongly coupled to the α_{1A}-AR in the presence of phenylephrine.

Another major finding from our study is that norepinephrine and epinephrine display no bias in signaling by the α_{1A}-AR for the three pathways studied. This raises the question of

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whether multiple endogenous agonists have in other cases evolved due to their capacity for differential signal transduction at particular receptors. Indeed, we have recently demonstrated signaling bias for multiple endogenous agonists of the GLP1 receptor (Koole et al., 2010), and this may be relevant to other GPCRs that possess multiple endogenous ligands, including the chemokine CXCR2 and CCL7 receptors, cholecystokinin CCK1(A), cholecystokinin CCK2(B), endothelin ET_A and ET_B, melanocortin MC4, purinergic $P2Y_2$, GLP2, VPAC1, VPAC2 receptors, and calcitonin and related receptors (list derived from http://www.iuphar-db.org/DATABASE/GPCRListForward). The case of adrenoceptors is somewhat different, as both norepinephrine and epinephrine act at 9 different receptor subtypes, and may display functional selectivity at subtypes other than the α_{1A}-AR. A major functional difference between the two endogenous agonists is that norepinephrine is a neurotransmitter that is released into synapses and neuromuscular junctions, whereas epinephrine is a circulating hormone that acts on the adrenoceptors present in a wide range of peripheral cell types. Selective pressure to maintain the interaction between adrenoceptor subtypes and two distinct agonists may have arisen primarily because of differences in the cell distribution of the target receptors rather than due to functional selectivity.

In conclusion, we have shown that a series of agonists at the human α_{1A}-AR display functional selectivity across three signaling pathways. The analytical method used has allowed us to derive numerical values for the degree of signaling bias, and to test for statistical significance. As shown here, a rigorous description of functional selectivity facilitates correlations between ligand structure and the ability to promote distinct active receptor conformations. More generally, this type of analysis will provide a platform for elucidating the mechanistic basis of functional selectivity at GPCRs by suggesting suitable ligands for receptor crystallisation, or classes of ligands for modelling studies.

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Authorship Contribution

Participated in research design: Evans, Broxton, Hutchinson, Summers.

Conducted experiments: Broxton, Merlin, Sato, Hutchinson.

Contributed new reagents or analytic tools: Christopoulos.

Performed data analysis: Evans, Christopoulos.

Wrote or contributed to the writing of the manuscript: Evans, Broxton, Hutchinson, Christopoulos, Summers.

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Footnotes

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Legends for Figures

FIGURE 1. Chemical structures of α_{1A}-AR agonists used in the study. (A) phenethylamines, (B) imidazolines.

FIGURE 2. Concentration-response curves for stimulation of Ca^{2+} release by the α_{1A}-AR. CHO-KI cells stably expressing the human α_{1A}-AR were treated 0.1% fluoro-4 at 37 ºC for 1h then washed and incubated for a further 30 min. Real-time fluorescence measurements were recorded every 1.7 sec, with drug additions occurring after 17 sec. Agonist responses represent the difference between basal fluorescence and the peak $[Ca^{2+}]i$ (reached within 20 sec of agonist addition), expressed as a percentage of the response to the Ca^{2+} ionophore A23187 (1 μM). Dose-dependent Ca^{2+} release was stimulated by (A) phenethylamine and (B) imidazoline agonists. Values are means ± SEM of 6 independent experiments. In (B), the norepinephrine curve is shown as a dashed line for comparison.

FIGURE 3. Concentration-response curves for stimulation of cAMP accumulation by the α_{1A}-AR. CHO-K1 cells stably expressing the α_{1A}-AR were exposed to agonists for 30 min in stimulation buffer containing 0.5 mM IBMX to inhibit phosphodiesterases. Responses to forskolin (10^{-4} M) were determined in parallel with agonist-stimulated cAMP accumulation for each batch of cells, and results are expressed as a percentage of the response to forskolin. A dose-dependent cAMP response was stimulated by (A) norepinephrine, phenylephrine, methoxamine, and (B) cirazoline and A61603, but not oxymetazoline. Values are means ± SEM of 4-5 independent experiments. In (B), the norepinephrine curve is shown as a dashed line for comparison.

FIGURE 4. Concentration-response curves for changes in extracellular acidification rate measured in the cytosensor microphysiometer. Cells stably expressing the α_{1A}-AR were grown overnight in transwell cups then placed in cytosensor chambers and perfused with modified low-buffered RPMI 1640 medium. After 2 h pre-equilibration, the basal acidification rate was measured over a period of 10 mins, then successive agonist concentrations were added at 14 min intervals. The maximum reading from the 7- cycle recording corresponding to each drug concentration was expressed as a % of the basal acidification rate. Changes in ECAR were stimulated by (A) phenethylamine and (B) imidazoline agonists in a dose-dependent manner. Values are means ± SEM of 4 independent experiments. In (B), the norepinephrine curve is shown as a dashed line for comparison.

FIGURE 5. Comparison of the activity of the endogenous agonists norepinephrine and epinephrine at the human α_{1A}-AR. (A) Structure of epinephrine. (B-D) Concentration-response curves for Ca^{2+} release, cAMP and changes in ECAR were determined as described in Figures 2-4. Values are means ± SEM of 7-9 independent experiments.

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TABLE 1: Potency (pEC_{50}), maximum response (E_{max}), and α value (E_{max} relative to norepinephrine) of agonists at the human α_{1A}-AR.

	Ca^{2+} release	cAMP	ECAR
Norepinephrine			
pEC_{50}	8.58 ± 0.07	5.55 ± 0.05	7.36 ± 0.04
E_{max}	92.8 ± 1.7	27.3 ± 0.5	152.2 ± 1.1
α	1.00 (6)	1.00 (5)	1.00 (4)
Methoxamine			
pEC_{50}	7.44 ± 0.05	4.39 ± 0.06	6.95 ± 0.04
E_{max}	87.7 ± 1.6	25.7 ± 0.8	146.5 ± 0.9
α	0.95 (6)	0.94 (5)	0.96 (4)
Phenylephrine			
pEC_{50}	8.10 ± 0.06	4.93 ± 0.06	8.29 ± 0.07
E_{max}	91.7 ± 1.6	23.4 ± 0.6	148.3 ± 1.6
α	0.99 (6)	0.86 (5)	0.97 (4)
Oxymetazoline			
pEC_{50}	8.86 ± 0.10	nd	8.55 ± 0.05
E_{max}	78.4 ± 2.4	nd	146.6 ± 1.0
α	0.84 (6)	nd	0.96 (4)
Cirazoline			
pEC_{50}	8.54 ± 0.12	6.89 ± 0.08	8.13 ± 0.03
E_{max}	97.0 ± 3.5	14.8 ± 0.5	148.5 ± 0.6
α	1.05 (6)	0.54 (5)	0.98 (4)
A61603			
pEC_{50}	9.63 ± 0.06	7.59 ± 0.05	9.09 ± 0.05
E_{max}	97.4 ± 2.0	28.6 ± 0.5	147.6 ± 1.1
α	1.05 (6)	1.05 (5)	0.97 (4)

TABLE 2.

Calculation of bias factors for phenethylamine and imidazoline agonists at the α_{1A}-AR.

AGONIST	Ca^{2+} release (n=6)		cAMP (n=5)		ECAR (n=4)		$logTR_n$ [cAMP-Ca^{2+}] (Bias factor)	$logTR_n$ [ECAR-cAMP] (Bias factor)	$logTR_n$ [ECAR-Ca^{2+}] (Bias factor)
	logTR	$logTR_n$	logTR	$logTR_n$	logTR	$logTR_n$			
Norepinephrine	8.46 ± 0.14	0.00 ± 0.19	5.48 ± 0.11	0.00 ± 0.16	7.48 ± 0.11	0.00 ± 0.18	0.00 ± 0.25 (1.0)	0.00 ± 0.24 (1.0)	0.00 ± 0.26 (1.0)
Methoxamine	7.24 ± 0.14	-1.22 ± 0.20	4.27 ± 0.13	-1.21 ± 0.18	6.87 ± 0.11	-0.61 ± 0.18	0.01 ± 0.26 (1.0)	0.60 ± 0.25* (**4.0**)	0.61 ± 0.26 (4.0)
Phenylephrine	7.94 ± 0.14	-0.52 ± 0.20	4.76 ± 0.14	-0.72 ± 0.18	8.27 ± 0.12	0.79 ± 0.18	-0.20 ± 0.27 (0.63)	1.52 ± 0.26*** (**33**)	1.31 ± 0.27** (**21**)
Oxymetazoline	8.52 ± 0.15	0.06 ± 0.21	na	na	8.46 ± 0.12	0.98 ± 0.19	na	na	0.91 ± 0.28* (**8.2**)
Cirazoline	8.57 ± 0.14	0.11 ± 0.20	6.46 ± 0.22	0.98 ± 0.25	8.10 ± 0.11	0.62 ± 0.18	0.87 ± 0.32* (**7.4**)	-0.36 ± 0.31 (0.44)	0.51 ± 0.27 (3.2)
A61603	9.58 ± 0.15	1.12 ± 0.20	7.54 ± 0.12	2.06 ± 0.16	9.03 ± 0.11	1.55 ± 0.19	0.94 ± 0.26** (**8.6**)	-0.51 ± 0.25 (0.31)	0.43 ± 0.28 (2.7)

LogTR is the log of the transduction ratio (τ/K_A), and $logTR_n$ values are normalized to the logTR for norepinephrine. Bias factors in bold are significantly different from 1.0. To avoid propagation of error due to multiple data manipulation steps, Student's t-tests were carried out on the raw logTR data rather than data normalized to norepinephrine (see Supplemental Table 1), *P<0.05, **P<0.01, ***P<0.001. na, not applicable.

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TABLE 3. Potency (pEC_{50}), maximum response (E_{max}), and α value (E_{max} relative to norepinephrine) of the endogenous agonists norepinephrine and epinephrine at the α_{1A}-AR.

	Ca^{2+} release	cAMP	ECAR
Norepinephrine			
pEC_{50}	8.83 ± 0.06	5.55 ± 0.07	7.49 ± 0.09
Emax	84.4 ± 1.9	32.8 ± 0.9	159.3 ± 2.7
α	1.00 (9)	1.00 (7)	1.00 (8)
Epinephrine			
pEC_{50}	9.32 ± 0.08	5.83 ± 0.08	7.93 ± 0.06
Emax	86.6 ± 2.3	31.4 ± 0.8	159.9 ± 1.6
α	1.03 (9)	0.96 (7)	1.00 (8)

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TABLE 4.

Calculation of bias factors for norepinephrine and epinephrine at the α_{1A}-AR.

AGONIST	Ca^{2+} release (n=9)		cAMP (n=7)		ECAR (n=8)	
	logTR	$logTR_n$	logTR	$logTR_n$	logTR	$logTR_n$
Norepinephrine	8.78 ± 0.09	0.00 ± 0.16	5.59 ± 0.08	0.00 ± 0.12	7.49 ± 0.09	0.00 ± 0.13
Epinephrine	9.33 ± 0.08	0.55 ± 0.16	5.78 ± 0.08	0.19 ± 0.12	7.94 ± 0.09	0.45 ± 0.12

	$logTR_n$ [cAMP–Ca^{2+}] (Bias factor)	$logTR_n$ [ECAR–cAMP] (Bias factor)	$logTR_n$ [ECAR–Ca^{2+}] (Bias factor)
Norepinephrine	0.00 ± 0.20 (1.0)	0.00 ± 0.18 (1.0)	0.00 ± 0.21 (1.0)
Epinephrine	-0.36 ± 0.20 (0.43)	0.26 ± 0.17 (1.8)	-0.10 ± 0.20 (0.79)

LogTR is the log of the transduction ratio (τ/K_A), and $logTR_n$ values are normalized to the logTR for norepinephrine. To avoid propagation of error due to multiple data manipulation steps, Student's t-tests were carried out on the raw logTR data rather than data normalized to norepinephrine (see Supplemental Table 1), $P<0.05$ was considered significant.

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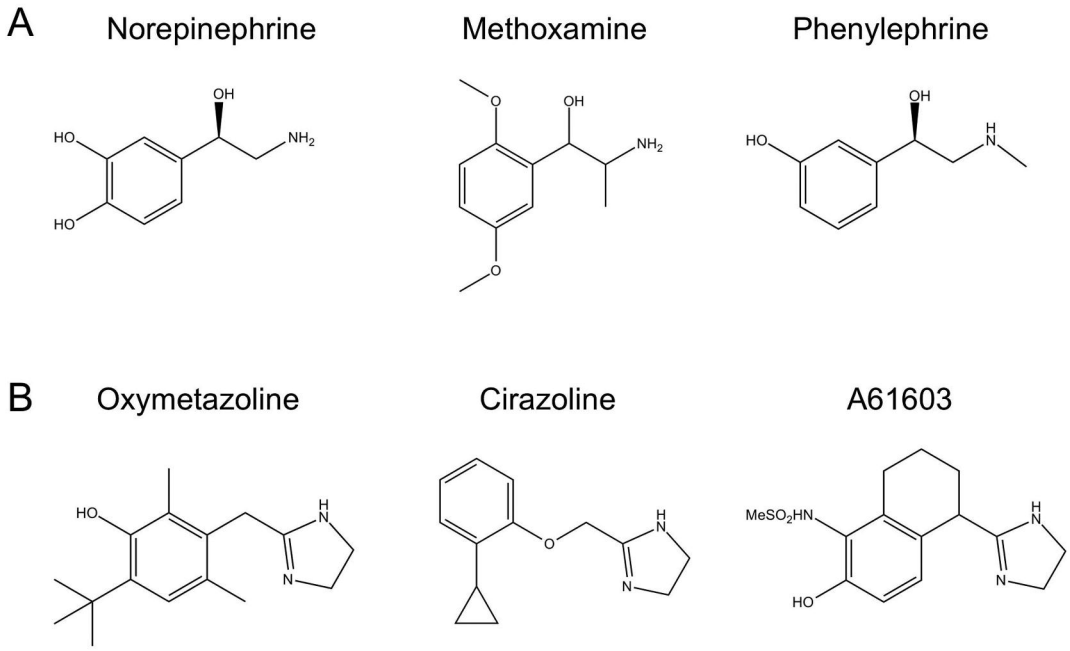

Fig 1

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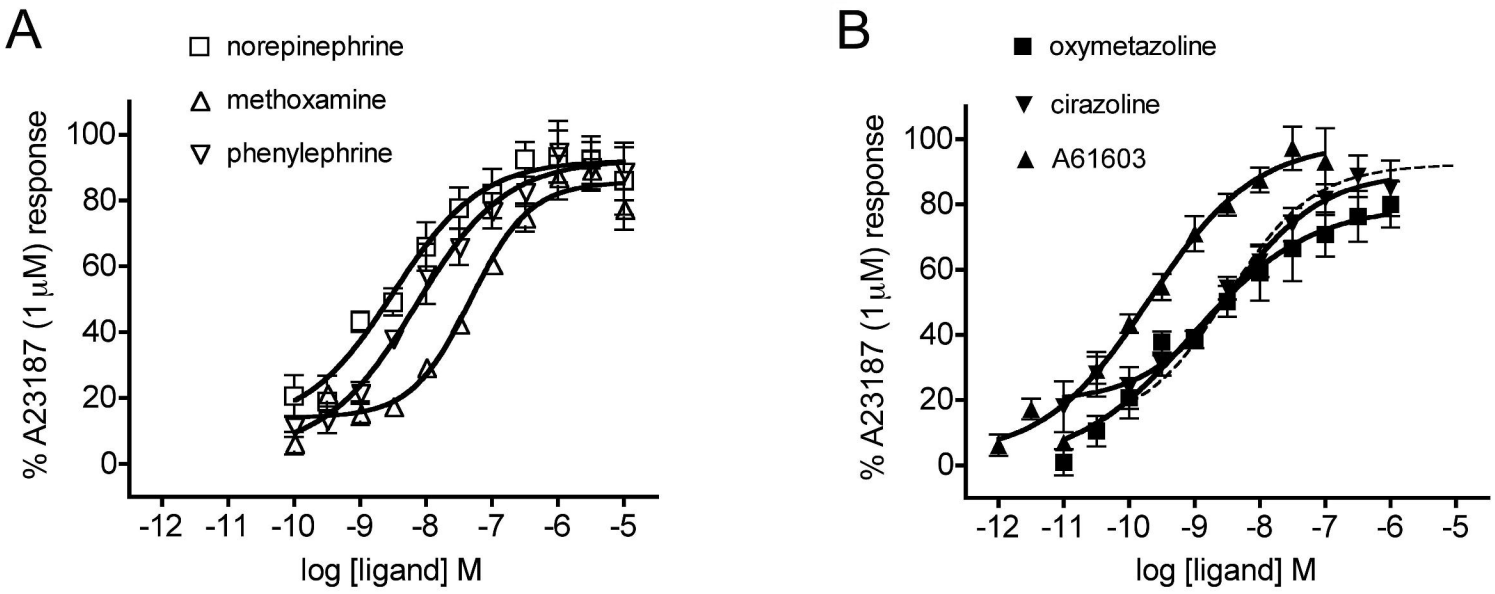

Fig 2

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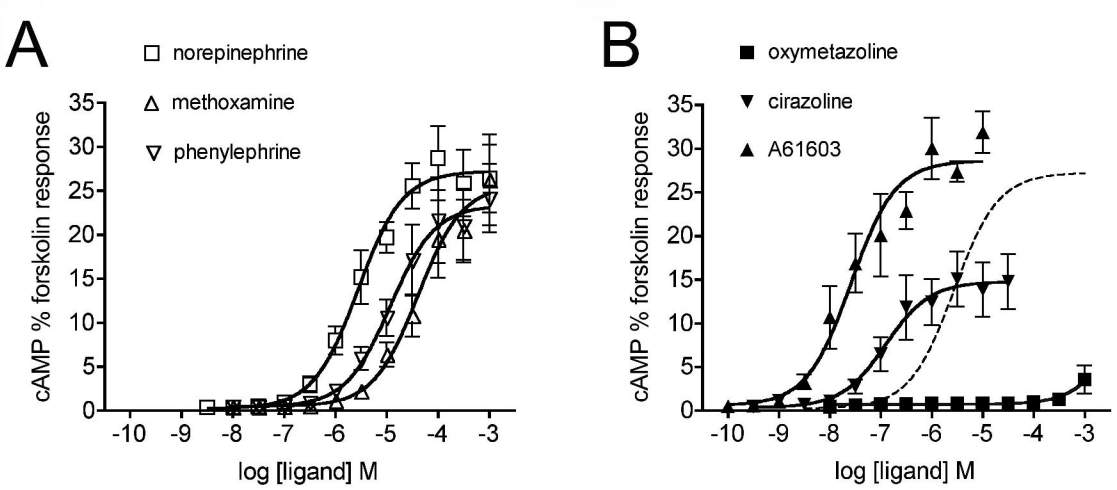

Fig 3

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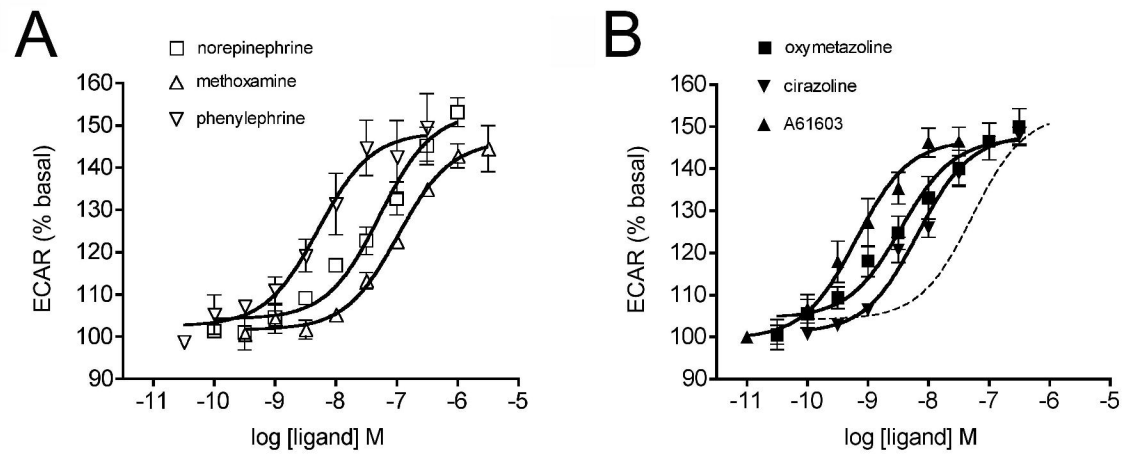

Fig 4

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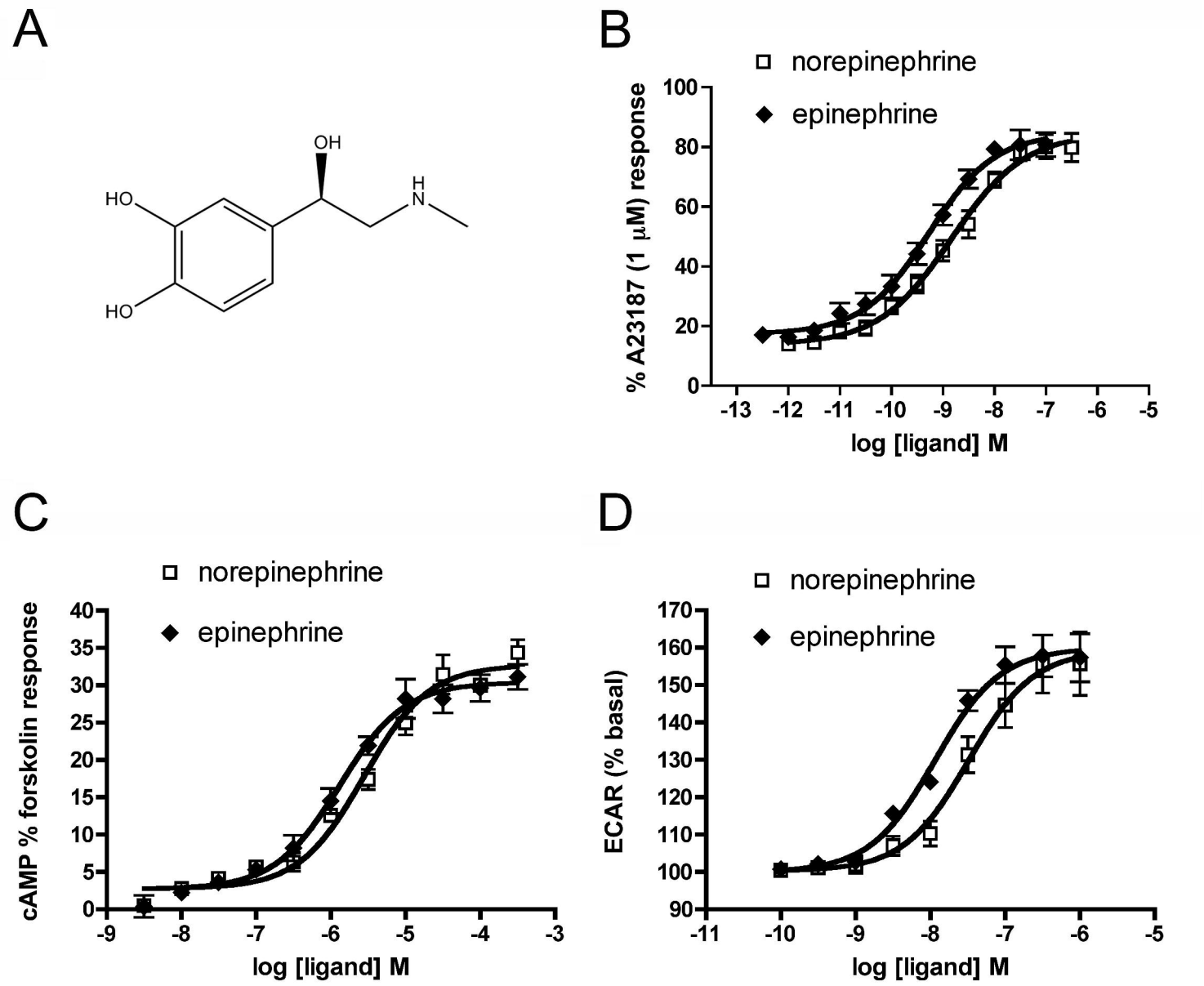

Fig 5