# Distinct Interactions of Human β₁- and β₂-Adrenoceptors with Isoproterenol, Epinephrine, Norepinephrine and Dopamine

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**Running title:** β-Adrenoceptors and Catecholamines

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**Abbreviations:** AC, adenylyl cyclase; βAR, non-specified human β-adrenoceptor; $\beta_1$AR, $\beta_1$-adrenoceptor; $\beta_1$AR-$G_{s\alpha L}$, fusion protein consisting of the human $\beta_1$-adrenoceptor and the long splice variant of $G_{s\alpha}$; $\beta_1$AR-$G_{s\alpha S}$, fusion protein consisting of the human $\beta_1$-adrenoceptor and the short splice variant of $G_{s\alpha}$; $\beta_2$AR, human $\beta_2$-adrenoceptor; $\beta_2$AR$_{CAM}$, constitutively active mutant of the $\beta_2$AR; $\beta_2$AR-$G_{s\alpha L}$, fusion protein consisting of the human $\beta_2$-adrenoceptor and the long splice variant of $G_{s\alpha}$; $\beta_2$AR-$G_{s\alpha S}$, fusion protein consisting of the human $\beta_2$-adrenoceptor and the short splice variant of $G_{s\alpha}$; DHA, dihydroalprenolol; DOP, dopamine; EPI, (-)-epinephrine; $G_\alpha$, non-specified G-protein α-subunit; GPCR, G-protein-coupled receptor; NE, (-)-norepinephrine; ISO, (-)-isoproterenol

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# Abstract

Fluorescence studies with purified human $\beta_2$-adrenoceptor ($\beta_2$AR) revealed that the endogenous catecholamines, (-)-epinephrine (EPI), (-)-norepinephrine (NE), and dopamine (DOP), stabilize distinct active receptor conformations. However, the functional relevance of these ligand-specific conformations is as yet poorly understood. We addressed this question by studying fusion proteins of the $\beta_1$-adrenoceptor ($\beta_1$AR) and $\beta_2$AR with the short and long splice variants of $G_{s\alpha}$ ($G_{s\alpha S}$ and $G_{s\alpha L}$), respectively. Fusion proteins ensure efficient receptor/G-protein coupling and defined stoichiometry of the coupling partners. EPI, NE, DOP and the prototypical synthetic $\beta$AR agonist, (-)-isoproterenol (ISO), showed marked differences in their efficacies at stabilizing the high-affinity ternary complex at $\beta_1$AR-$G_{s\alpha}$ and $\beta_2$AR-$G_{s\alpha}$ fusion proteins. Ternary complex formation was more sensitive to disruption by GTP with the $\beta_2$AR than with the $\beta_1$AR. Generally, in steady-state GTPase assays, ISO, EPI and NE were full agonists, and DOP was a partial agonist. Exceptionally, at $\beta_1$AR-$G_{s\alpha L}$, NE was only a partial agonist. Generally, in adenylyl cyclase assays, ISO, EPI and NE were full agonists, and DOP was a partial agonist. Exceptionally, at $\beta_2$AR-$G_{s\alpha L}$, NE was only a partial agonist. There was no correlation between efficacy at stabilizing the ternary complex and activating GTPase, and there were also dissociations between $K_i$ values for high-affinity agonist binding and $EC_{50}$ values for GTPase activation. In contrast to synthetic partial agonists, DOP did not exhibit increased efficacy at $\beta$AR-$G_{s\alpha L}$- versus $\beta$AR-$G_{s\alpha S}$ fusion proteins. In conclusion, our data with $\beta$AR-$G_{s\alpha}$ fusion proteins show that endogenous catecholamines and ISO stabilize distinct conformations in the $\beta_1$AR and $\beta_2$AR.

## Introduction

The $\beta_1$AR and $\beta_2$AR are GPCRs, play crucial roles in the regulation of

cardiovascular functions and are activated by the catecholamines EPI, NE, DOP and ISO

(Rohrer and Kobilka, 1998; Rockman et al., 2002). ISO is a prototypical synthetic agonist at

$\beta$ARs, serving as a reference compound for the analysis of agonist potencies and efficacies

(Rousseau et al., 1996; Hoffmann et al., 2004; Kobilka, 2007). $\beta$ARs couple to the G-protein

$G_s$ (Gilman, 1987; Birnbaumer et al., 1990). $G_s$ consists of a $G_{s\alpha}$-subunit and a $G\beta\gamma$-complex.

In the resting state, the $G_{s\alpha}$-subunit is bound to GDP. Binding of an agonist to a $\beta$AR

stabilizes a GPCR conformation that allows it to promote the dissociation of GDP from $G_{s\alpha}$,

the rate-limiting step of the G-protein cycle (Gilman, 1987; Kobilka, 2007). $G_{s\alpha}$ exists as a

short splice variant ($G_{s\alpha S}$) and a long splice variant ($G_{s\alpha L}$), $G_{s\alpha S}$ possessing a higher GDP-

affinity than $G_{s\alpha L}$ (Seifert et al., 1998a). Subsequently to GDP-dissociation from $G_{s\alpha}$, a

ternary complex consisting of the agonist, $\beta$AR and nucleotide-free $G_{s\alpha}G_{\beta\gamma}$ forms (DeLean et

al., 1980; Kent et al., 1980; Seifert et al., 1998a). This complex possesses high agonist-

affinity. Upon binding of GTP to $G_{s\alpha}$, the ternary complex is disrupted, resulting in a

decrease of the $\beta$AR-affinity for agonist and dissociation of $G_s$ into $G_{s\alpha}$ and the $G\beta\gamma$-

complex. The ternary complex model of GPCR activation assumes that there is a correlation

between the efficacy of an agonist at promoting high-affinity agonist binding and its efficacy

at promoting GDP/GTP exchange and down-stream effector activation (DeLean et al., 1980;

Kent et al., 1980). GTP-bound $G_{s\alpha}$ activates the effector AC that catalyzes the conversion of

ATP into the second messenger cAMP (Sunahara et al., 1996). cAMP, through interaction

with specific protein kinases, ion channels and nucleotide exchange factors, changes cell

functions (Rehmann et al., 2007). Termination of G-protein activation is achieved by the

high-affinity GTPase activity of $G_{s\alpha}$, cleaving GTP to GDP and $P_i$ (Gilman, 1987).

Subsequently, GDP-bound $G_{s\alpha}$ and $G\beta\gamma$ reassociate, closing the G-protein cycle.

While the ternary complex model is capable of describing basic aspects of GPCR/G-protein/effector interactions, the model is not sufficient at fully explaining GPCR-mediated signal transduction (DeLean et al., 1980; Kent et al., 1980; Kobilka, 2007). For example, by studying a panel of synthetic agonists with different efficacies at $\beta_2AR$ in the presence of inosine 5'-triphosphate, we observed dissociations in the efficacies of agonists at inosine 5'-triphosphate hydrolysis and AC activation (Seifert et al., 1999b). Furthermore, by comparing $\beta_2AR$ with a constitutively active $\beta_2AR$ mutant ($\beta_2AR_{CAM}$), we noticed dissociations in the efficacies of agonists at stabilizing the ternary complex and promoting GTP hydrolysis (Seifert et al., 2001). Moreover, the pharmacological profile of the $\beta_2AR$ depends on the specific G-protein coupling partner (Wenzel-Seifert and Seifert, 2000). These data suggest that ligands stabilize unique $\beta_2AR$ conformations that differ from each other in their efficacy at promoting ternary complex formation on one hand and the overall G-protein cycle on the other hand. Studies with other GPCRs including the $\beta_1AR$ further corroborate the concept of ligand-specific GPCR conformations (Granneman, 2001; Kenakin, 2007; Galandrin et al., 2008).

So far, most studies concerning ligand-specific $\beta AR$ conformations were conducted with synthetic ligands, while relatively little attention has been paid to the endogenous catecholamines EPI, NE and DOP. Intriguingly, fluorescence studies with purified $\beta_2AR$ revealed that endogenous catecholamines stabilize two kinetically distinct active conformational states (Swaminath et al., 2004). Specifically, DOP induces only the rapid conformational change, whereas ISO, EPI and NE induce both the rapid and slow conformational change. ISO and EPI are more efficient than NE at promoting fluorescence changes, whereas ISO, EPI and NE are similarly efficient at activating cAMP accumulation

and $\beta_2AR$ internalization. Computational analysis of the $\beta_2AR$ confirms the existence of NE- and DOP-specific conformations (Bhattacharya et al., 2008). However, the functional consequences of the distinct $\beta_2AR$ conformations stabilized by endogenous catecholamines and ISO are still poorly understood. Therefore, the aim of our present study was to comprehensively characterize the interactions of the endogenous catecholamines EPI, NE and DOP in comparison to ISO at $\beta_1AR$ and $\beta_2AR$ fused to either to $G_{s\alpha L}$ or $G_{s\alpha S}$. Specific advantages and disadvantages of the fusion protein approach are outlined in the "Methods" section.

## Methods

**Materials.** The construction of baculoviruses encoding $\beta_1AR$-$G_{s\alpha L}$, $\beta_1AR$-$G_{s\alpha S}$, $\beta_2AR$-$G_{s\alpha L}$ and $\beta_2AR$-$G_{s\alpha S}$ was described earlier (Seifert et al., 1998a,b; Wenzel-Seifert et al., 2002). In our present study, we analyzed the Gly389 isoform of the $\beta_1AR$. A previous study revealed no differences between $\beta_1AR$-Gly389-$G_{s\alpha}$- and the corresponding $\beta_1AR$-Arg389-$G_{s\alpha}$ fusion proteins (Wenzel-Seifert and Seifert, 2003). [$^{32}$P]P$_i$ (8,500-9,100 Ci/mmol), [$\alpha$-$^{32}$P]ATP (3,000 Ci/mmol) and [$^3$H]DHA (85-90 Ci/mmol) were from Perkin Elmer (Boston, MA). [$\gamma$-$^{32}$P]GTP was synthesized enzymatically from GDP and [$^{32}$P]P$_i$ as described (Walseth and Johnson, 1979). Unlabeled nucleotides were from Roche (Mannheim, Germany). ISO, EPI, NE and DOP were from Sigma (St. Louis, MO). Stock solutions of catecholamines (10 mM each) were prepared fresh daily in 1 mM HCl. Catecholamine dilutions were also prepared in 1 mM HCl. All catecholamine solutions were kept at 4°C and under light protection until experiments were performed. Binding experiments, lasting 90 min, were also conducted under light protection. Glass fiber filters (GF/C) were from Schleicher and Schuell (Dassel, Germany).

**Cell culture and membrane preparation.** $\beta$AR-G$_{s\alpha}$ fusion proteins were expressed in Sf9 inset cells. Sf9 cells were cultured in 250 ml disposable Erlenmeyer flasks at 28$^o$C under rotation at 125 rpm in SF 900 II medium (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) fetal calf serum (Cambrex, East Rutherford, NJ) and 0.1 mg/ml gentamicin (Cambrex) (Seifert et al., 1998a,b). Cells were maintained at a density of 1.0-6.0 x 10$^6$ cells/ml. Sf9 cells were seeded at 3.0 x 10$^6$ cells/ml and infected with 1: 100-1 : 1,000 dilutions of high-titer baculovirus stocks encoding $\beta$AR-G$_{s\alpha}$ fusion proteins. Cells were cultured for 48 h before membrane preparation. Sf9 membranes were prepared as described (Seifert et al., 1998a,b), using 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml benzamidine and 10 $\mu$g/ml leupeptin as protease inhibitors. Membranes were suspended in binding buffer (12.5 mM MgCl$_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). Membranes were stored at -80$^o$C for periods of up to 6 months (longer periods of time were not analyzed in this study) without loss of functional activity in the various assays employed.

**[$^3$H]DHA binding assay.** Membranes were thawed and sedimented by a 10 min centrifugation at 4$^o$C and 15,000 x $g$ to remove residual endogenous guanine nucleotides as far as possible and resuspended in binding buffer. Expression levels of fusion proteins were determined by incubating Sf9 membranes (20-25 $\mu$g protein/tube) in the presence of 10 nM [$^3$H]DHA. The total volume of the binding reaction was 500 $\mu$l. Incubations were performed for 90 min at 25$^o$C and shaking at 250 rpm. Non-specific [$^3$H]DHA binding was determined in the presence of 10 $\mu$M ($\pm$)-alprenolol. Non-specific [$^3$H]DHA binding amounted to less than 10-15% of total [$^3$H]DHAbinding. For agonist competition binding experiments, membranes expressing $\beta$AR-G$_{s\alpha}$ at levels between 3.8-5.2 pmol/mg of membrane protein were used. Tubes contained Sf9 membranes (20-25 $\mu$g protein/tube), 1 nM [$^3$H]DHA and agonists at increasing concentrations. Reaction mixtures additionally contained solvent

(control) or GTP (1 mM). Binding experiments were conducted under light protection. Bound

[$^3$H]DHA was separated from free [$^3$H]DHA by filtration through GF/C filters using a 48-

well harvester (Brandel, Gaithersburg, MD), followed by three washes with 2 ml binding

buffer (4$^o$C). Filter-bound radioactivity was determined by liquid scintillation counting using

Rotiszint eco plus cocktail (Roth, Karlsruhe, Germany).

**Steady-state GTPase activity assay.** The GTPase assay was performed as

described previously with minor modifications (Seifert et al., 1998a,b). Briefly, Sf9

membranes were thawed, sedimented by centrifugation at 15,000 x $g$ for 10 min at 4°C, and

resuspended in 10 mM Tris/HCl, pH 7.4. For GTPase assays, membranes expressing βAR-

G$_{s\alpha}$ at levels between 1.7-5.2 pmol/mg of membrane protein were used. Assay tubes

contained Sf9 membranes (10 µg of protein/tube), 1.0 mM MgCl$_2$, 0.1 mM EDTA, 0.1 mM

ATP, 100 nM GTP, 0.1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 µg of

creatine kinase, and 0.2% (w/v) bovine serum albumin in 50 mM Tris/HCl, pH 7.4, and

catecholamines at various concentrations. Reaction mixtures (80 µl) were incubated for 2 min

at 25°C before the addition of 20 µl [γ-$^{32}$P]GTP (0.1 µCi/tube). All stock and work dilutions

of [γ-$^{32}$P]GTP were prepared in 20 mM Tris/HCl, pH 7.4. Reactions were conducted for 20

min at 25°C. Reactions were terminated by the addition of 900 µl slurry consisting of 5%

(w/v) activated charcoal and 50 mM NaH$_2$PO$_4$, pH 2.0. Charcoal absorbs nucleotides but not

P$_i$. Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at

15,000 x g. Six hundred µl of supernatant fluid of reaction mixtures were removed, and $^{32}$P$_i$

was determined by Čerenkov radiation in 3 ml water. Enzyme activities were corrected for

spontaneous degradation of [γ-$^{32}$P]GTP. Spontaneous [γ-$^{32}$P]GTP degradation was

determined in tubes containing all of the above described components plus a high

concentration of unlabeled GTP (1 mM) that, by competition with [γ-$^{32}$P]GTP, prevents [γ-

$^{32}$P]GTP hydrolysis by enzymatic activities present in Sf9 membranes. Spontaneous [γ-$^{32}$P]GTP degradation amounted to <1% of the total amount of radioactivity. The experimental conditions chosen ensured that not more than 10% of the total amount of [γ-$^{32}$P]GTP added was converted to $^{32}$P$_i$.

**AC activity assay.** The AC assay was performed as described previously with minor modifications (Seifert et al., 1998a,b). Membranes were thawed and sedimented by a 10 min centrifugation at 4$^{o}$C and 15,000 x $g$ to remove residual endogenous guanine nucleotides as far as possible and resuspended in binding buffer. For AC assays, membranes expressing βAR-G$_{s\alpha}$ at levels between 1.1-2.0 pmol/mg of membrane protein were used. Tubes contained Sf9 membranes expressing fusion proteins (30 μg protein/tube), 5 mM MgCl$_2$, 0.4 mM EDTA, 30 mM Tris/HCl, pH 7.4, GTP (10 μM) and catecholamines at various concentrations. Assay tubes containing membranes and additions in a total volume of 30 μl were incubated for 3 min at 37$^{o}$C before starting reactions with 20 μl reaction mixture containing (final) [α-$^{32}$P]ATP (0.5-1.0 μCi/tube) plus 40 μM unlabeled ATP, 0.1 mM cAMP and a regenerating system consisting of 2.7 mM mono(cyclohexyl)ammonium phosphoenolpyruvate, 0.125 IU pyruvate kinase and 1 IU myokinase. Reactions were conducted for 20 min at 37$^{o}$C and terminated by the addition of 20 μl 2.2 N HCl. Denatured protein was sedimented by a 3 min centrifugation at 25$^{o}$C and 15,000 $x$ g. Sixtyfive μl of the supernatant fluid were applied onto disposable columns filled with 1.3 g neutral alumina (MP alumina N-super-I, MP Biomedicals, Eschwege, Germany). [$^{32}$P]cAMP was separated from [α-$^{32}$P]ATP by elution of [$^{32}$P]cAMP with 4 ml 0.1 M ammonium acetate, pH 7.0. Recovery of [$^{32}$P]cAMP was ~80%. Blank values were routinely ~0.01% of the total amount of [α-$^{32}$P]ATP added. [$^{32}$P]cAMP was determined by Čerenkov radiation in 10 ml water.

**Advantages and disadvantages of using βAR-G$_{s\alpha}$ fusion proteins as model systems in the [$^3$H]DHA binding-, GTPase- and AC assay.** GPCR-G$_\alpha$ fusion proteins ensure close proximity between, and defined 1:1 stoichiometry of, the coupling partners (Seifert et al., 1999a; Milligan et al., 2007). The fusion facilitates efficient GPCR/G-protein coupling under defined experimental conditions. This effect of the fusion is most prominent for G$_s$-coupled GPCRs. Specifically, in conventional βAR-G$_{s\alpha}$ co-expression systems, ternary complex formation is less efficient than in fusion proteins, rendering a detailed comparison of various ligands very difficult (Seifert et al., 1998b). Moreover, in fusion proteins, the efficiency of ternary complex formation is independent of the specific expression level of the construct, thereby greatly facilitating comparison of various receptors coupled to different G-proteins (Seifert et al., 1998a; Wenzel-Seifert et al., 1999, 2001, 2002). It has been repeatedly observed that ternary complex formation in βAR-G$_\alpha$ fusion proteins can be (partially) insensitive to disruption by guanine nucleotides (Seifert et al., 1998b, 2001; Wenzel-Seifert and Seifert, 2000). This is not a peculiar property of fusion proteins but also a property of conventional co-expression systems (Seifert et al., 1998b).

With respect to steady-state GTP hydrolysis, it is exceedingly difficult to perform a detailed pharmacological analysis in a conventional βAR-G$_{s\alpha}$ co-expression system because the signal-to noise ratio is very low (Seifert et al., 1998a). However, with fusion proteins, a detailed pharmacological analysis is feasible due to the high signal-to noise ratio (Seifert et al., 1998a, 1999b, 2001). Moreover, ligand potencies and efficacies are independent of the expression level of constructs (Seifert et al., 1998a, 1999b, 2001).

Concerning the analysis of AC activity, such studies can be performed with conventional βAR-G$_{s\alpha}$ co-expression systems since signal amplification at the effector level is sufficiently large (Seifert et al., 1998b). However, one has to keep in mind that the number of available AC molecules is the limiting factor in the system so that high βAR expression

levels should be avoided (Seifert et al., 1998b). $\beta$AR-$G_{s\alpha}$ fusion proteins are also capable of mediating efficient AC activation (Seifert et al., 1998a,b, 1999b). In order to avoid depletion of AC molecules, we only used membranes with relatively low $\beta$AR-$G_{s\alpha}$ expression levels, i.e. in the range between 1.1-2.0 pmol/mg. These conditions ensure good signals while avoiding AC depletion (Seifert et al., 1998a,b, 1999b; Wenzel-Seifert et al., 2002).

Evidently, $\beta$AR-$G_{s\alpha}$ fusion proteins are artificial and do not occur physiologically. However, in previous studies, we had carefully compared the properties of fusion proteins with those of non-fused $\beta$AR and $G_{s\alpha}$ proteins and did not reveal large differences in terms of ligand affinities/potencies and efficacies, validating the approach (Seifert et al., 1999a). Previous studies also revealed that the functional integrity of $\beta_1$AR-$G_{s\alpha}$- and $\beta_2$AR-$G_{s\alpha}$ fusion proteins as assessed by [$^{35}$S]guanosine 5'-[$\gamma$-thio]triphosphate saturation binding is similar (Seifert et al., 1998a,b; Wenzel-Seifert and Seifert, 2000, 2003; Wenzel-Seifert et al., 2002). Thus, it is unlikely that there are large differences in functionally relevant membrane insertion between the various fusion proteins. Based on these considerations, the conclusions obtained with GPCR-$G_{\alpha}$ fusion proteins can be cautiously transferred to non-fused systems.

**Miscellaneous.** Protein concentrations were determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Data shown in Figs. 1-6 were analyzed by non-linear regression (Prism 5.0 software, GraphPad-Prism, San Diego, CA). Statistical comparisons of the efficacies of ISO *versus* endogenous catecholamines in the GTPase assay and AC assay (Tables 2 and 3) were performed using ANOVA followed by Dunnet's multiple comparison post test.

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## Results

**Competition by ISO, EPI, NE and DOP of [$^{3}$H]DHA binding in Sf9 membranes**

**expressing $\beta$AR-G$_{s\alpha}$ fusion proteins: effect of GTP.** At $\beta_1$AR-G$_{s\alpha L}$, all catecholamines

studied (ISO, EPI, NE and DOP) inhibited [$^{3}$H]DHA binding according to biphasic

competition isotherms. DOP was the least effective catecholamine at stabilizing the ternary

complex as reflected by the low R$_h$ (%) value (Fig. 1 and Table 1). GTP shifted the agonist-

competition curves to the right, with the shift being most pronounced for NE. However, with

all catecholamines studied, GTP did not achieve a complete conversion into a monophasic

competition isotherm at $\beta_1$AR-G$_{s\alpha L}$. At $\beta_1$AR-G$_{s\alpha S}$, like at $\beta_1$AR-G$_{s\alpha L}$, DOP was the least

effective catecholamine at stabilizing the ternary complex (Fig. 2 and Table 1). At $\beta_1$AR-

G$_{s\alpha S}$, ISO was a less effective stabilizer of the ternary complex than at $\beta_1$AR-G$_{s\alpha L}$. Like at

$\beta_1$AR-G$_{s\alpha L}$, GTP did not convert the biphasic competition isotherms at $\beta_1$AR-G$_{s\alpha S}$ into

monophasic isotherms. At both $\beta_1$AR-G$_{s\alpha}$ fusion proteins, the order of affinity (K$_{ih}$ and K$_{il}$) of

catecholamines was ISO ~ NE > EPI >> DOP. At the $\beta_1$AR expressed in CHO cells, the

order of affinity was ISO > NE ~ EPI (DOP was not studied) (Hoffmann et al., 2004), but in

this system, only K$_{il}$ values in the presence of GTP were determined, annihilating the impact

of G$_{s\alpha}$ on agonist-affinity.

At $\beta_2$AR-G$_{s\alpha L}$, EPI was the most effective ternary complex stabilizer, and DOP was

the least effective agonist in this respect (Fig. 3 and Table 1). Except for DOP, GTP

converted the biphasic agonist competition isotherms into monophasic isotherms. Similar to

$\beta_2$AR-G$_{s\alpha L}$, EPI was the most effective ternary complex stabilizer at $\beta_2$AR-G$_{s\alpha S}$, and DOP

was the least effective agonist in this regard (Fig. 4). GTP converted the biphasic competition

isotherms for EPI, NE and DOP at $\beta_2$AR-G$_{s\alpha S}$ into monophasic isotherms, whereas the

competition isotherm for ISO remained biphasic. At both $\beta_2$AR-G$_{s\alpha}$ fusion proteins, the order

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of affinity ($K_{ih}$ and $K_{il}$) of catecholamines was ISO ~ EPI > NE > DOP. At the $\beta_2$AR

expressed in CHO cells, the order of affinity was ISO ~ EPI >> NE (DOP was not studied)

(Hoffmann et al., 2004), but in this system, only $K_{il}$ values in the presence of GTP were

determined, annihilating the impact of $G_{s\alpha}$ on agonist-affinity.

**Agonist potencies and efficacies at $\beta$AR-$G_{s\alpha}$ fusion proteins in the GTPase**

**assay.** At $\beta_1$AR-$G_{s\alpha L}$ and $\beta_1$AR-$G_{s\alpha S}$, the order of potency of catecholamines in the GTPase

assay was ISO ~ NE > EPI >> DOP (Figs. 5 and 6 and Table 2). At $\beta_1$AR-$G_{s\alpha L}$, ISO and EPI

were full agonists, whereas NE and DOP were only partial agonists. At $\beta_1$AR-$G_{s\alpha S}$, ISO, EPI

and NE were all full agonists, whereas DOP was only a partial agonist.

At $\beta_2$AR-$G_{s\alpha L}$ and $\beta_2$AR-$G_{s\alpha S}$, the order of potency of catecholamines in the GTPase

assay was ISO ~ EPI > NE >> DOP. At both fusion proteins, ISO, EPI and NE were full

agonists, whereas DOP was a partial agonist (Figs. 5 and 6 and Table 2). There was a trend

towards strong partial agonism of NE *versus* ISO at both $\beta_2$AR-$G_{s\alpha}$ fusion proteins, but the

difference did not reach significance.

**Agonist potencies and efficacies at $\beta$AR-$G_{s\alpha}$ fusion proteins in the AC assay.** At

$\beta_1$AR-$G_{s\alpha L}$ and $\beta_1$AR-$G_{s\alpha S}$, catecholamines activated AC in the presence of GTP in the order

of potency ISO ~ NE ~ EPI >> DOP (Figs. 5 and 6 and Table 3). At both fusion proteins,

ISO, EPI and NE were full agonists, while DOP was a partial agonist. There was a trend

towards strong partial agonism of EPI and NE *versus* ISO at both $\beta_1$AR-$G_{s\alpha}$ fusion proteins,

but the difference did not reach significance.

At $\beta_2$AR-$G_{s\alpha L}$ and $\beta_2$AR-$G_{s\alpha S}$, catecholamines activated AC in the presence of GTP

in the order of potency ISO ~ EPI > NE >> DOP (Figs. 5 and 6 and Table 3). At $\beta_2$AR-$G_{s\alpha L}$,

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ISO and EPI were full agonists, and NE and DOP were partial agonists. At $\beta_2AR\text{-}G_{s\alpha S}$, ISO and EPI exhibited full agonism, NE exhibited a non-significant trend towards partial agonism, and DOP was a partial agonist.

**Correlations between agonist-affinities in the agonist competition binding assay and potencies for GTPase activation as well as correlation between potencies in the GTPase- and AC assay.** The ternary complex model predicts that here should be a correlation between the $K_{ih}$ values obtained in the agonist competition binding assay and the $EC_{50}$ values for GTPase- and AC activation (Kent et al., 1980; Seifert et al., 2001). Our experimental data do not fulfill the prediction. Specifically, just for one ligand, i.e. DOP at $\beta_1AR\text{-}G_{s\alpha L}$, $K_{ih}$ and $EC_{50}$ for GTPase activation differed by no more than two-fold (Table 4). For the other ligands, $EC_{50}$ values were 4-460-fold higher than $K_{ih}$ values, DOP at $\beta_2AR\text{-}G_{s\alpha S}$ showing the most extreme difference between the two parameters. These differences indicate that efficient stimulation of GDP/GTP exchange by the high-affinity agonist-state of a GPCR is rather an exception than the rule. In accordance with the data on the $G_s$-coupled $\beta_1AR$ and $\beta_2AR$, there is evidence for the $G_i$-coupled formyl peptide receptor that the high-affinity agonist state does not mediate GDP/GTP exchange (Gierschik et al., 1989; Wenzel-Seifert et al., 1999). Rather, in the case of the formyl peptide receptor, the low-affinity agonist state mediates GDP/GTP exchange. However, when we correlated $K_{il}$ values with $EC_{50}$ values for GTPase activation at $\beta AR\text{-}G_{s\alpha}$ fusion proteins, we found a match between the two values only for DOP at $\beta_2AR\text{-}G_{s\alpha S}$ (Table 4). For the other ligands, $K_{il}$ values were about 3-20-fold higher than $EC_{50}$ values for GTPase activity. These data indicate that there is considerable variation in the agonist-affinity of the $\beta_1AR$ and $\beta_2AR$ state that mediates GDP/GTP exchange, depending on the specific ligand studied. In most cases, an intermediate agonist-affinity state that is not easily distinguished in radioligand competition binding studies,

appears to mediate GDP/GTP exchange. Furthermore, it would also have been predicted that the $EC_{50}$ values of agonists for activation of GTPase and AC had been identical. For EPI at $\beta_2AR$-$G_{s\alpha S}$, the two $EC_{50}$ values matched, but at $\beta_2AR$-$G_{s\alpha L}$, EPI was more than three-fold more potent in the GTPase assay than in the AC assay (Table 4). Less pronounced differences between these parameters were also observed for other ligands.

## Discussion

The ternary complex model is of fundamental importance for understanding the mechanisms of GPCR/G-protein/effector interactions (Kent et al., 1980; DeLean et al., 1980; Kobilka, 2007). A central paradigm of this model is a correlation between the efficacy of agonists at stabilizing the ternary complex and the efficacy at activating GDP/GTP exchange and effector activation. At the turkey $\beta AR$ and at the $\beta_2AR$-$G_{s\alpha L}$ fusion protein, using a panel of synthetic ligands, such a correlation was, indeed, observed (Kent et al., 1980; Seifert et al., 2001). In contrast, with $\beta_2AR_{CAM}$, no such correlation was apparent (Seifert et al., 2001), indicating that the ternary complex model is not applicable to all GPCRs. However, so far, little attention has been paid to the analysis of the endogenous catecholamines EPI, NE and DOP. Intriguingly, fluorescence studies with purified $\beta_2AR$ and computational studies indicate that these catecholamines stabilize ligand-specific GPCR conformations (Swaminath et al., 2004; Bhattacharya et al., 2008). Moreover, there is evidence that the conformational state of a GPCR depends on the structure and efficacy of a ligand for a specific G-protein and/or signaling pathway (Wenzel-Seifert and Seifert, 2000; Galandrin et al., 2008; Bhattacharya et al., 2008). Therefore, we comprehensively examined the interactions of ISO, EPI, NE and DOP with $\beta_1AR$-$G_{s\alpha}$- and $\beta_2AR$-$G_{s\alpha}$ fusion proteins. We included the two splice

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variants of $G_{s\alpha}$, $G_{s\alpha S}$ and $G_{s\alpha L}$, in our analysis in order to account for the G-protein-specificity aspect of GPCR conformations.

We made several observations that are not compatible with the ternary complex model. First, at $\beta_1$AR-$G_{s\alpha S}$, $\beta_2$AR-$G_{s\alpha L}$ and $\beta_2$AR-$G_{s\alpha S}$ ISO was a full agonist in the GTPase- and AC assay, but in terms of ternary complex formation, ISO was surpassed by EPI and/or NE. These data can be explained by the existence of "frozen" ternary complexes (Seifert et al., 2001; Kenakin, 2007), i.e. complexes that are inefficient at stimulating GTP binding and subsequent effector activation. Second, as a general rule, ternary complex formation with $\beta_1$AR-$G_{s\alpha}$ fusion proteins was less sensitive to disruption by GTP than ternary complex formation with the corresponding $\beta_2$AR-$G_{s\alpha}$ fusion proteins. These data indicate that the $\beta_1$AR couples to $G_{s\alpha}$ more tightly than the $\beta_2$AR, i.e. even the activated $G_{s\alpha}$ protein is still in physical contact with the GPCR, conferring to it high agonist-affinity. Such observations are also not without precedence in the literature (Seifert et al., 1998b, 2001). Third, at $\beta_2$AR-$G_{s\alpha L}$, NE and DOP were more effective at stimulating steady-state GTPase activity than AC activity. The ternary complex model predicts a correlation between those parameters (De Lean et al., 1980; Kent et al., 1989; Seifert et al., 1999b). Thus, certain ligand-specific $\beta_2$AR conformations couple less efficiently to AC than to GDP/GTP exchange. These observations imply that a quaternary complex consisting of agonist, GPCR, G-protein and effector exists. Differential regulation of AC by formyl peptide-$G_{i\alpha}$- and $\beta_2$AR-$G_{i\alpha}$ fusion proteins supports this interpretation (Seifert et al., 2002). Fourth, the ternary complex model predicts a correlation between $K_{ih}$ values and $EC_{50}$ values for G-protein activation (Kent et al., 1980; Seifert et al., 2002), but we did not observe such a correlation. Moreover, we did not observe the predicted correlation between $EC_{50}$ values for GTPase- and AC activation. All these data support the concept of ligand-specific $\beta$AR conformations.

The ternary complex model was extended to account for the observation of agonist-independent, i.e. constitutive activity (Samama et al., 1993; Chidiac et al., 1994; Kenakin, 1996). Inverse agonists reduce the constitutive activity of GPCRs. Using the wild-type $\beta_2$AR and $\beta_2$AR$_{CAM}$ as model, increased potency and efficacy of synthetic partial agonists and increased efficacy of inverse agonists emerged as hallmark of high constitutive activity (Samama et al., 1993; Seifert et al., 2001). The comparison of $\beta$AR-G$_{s\alpha S}$- and $\beta$AR-G$_{s\alpha L}$ fusion proteins provides another model system for probing the extended ternary complex model. Specifically, when fused to G$_{s\alpha L}$, but not when fused to G$_{s\alpha S}$, both the $\beta_1$AR and $\beta_2$AR exhibit the properties of high constitutive activity as assessed by increased potency and efficacy of a series of synthetic partial agonists including salbutamol, dobutamine, (-)-ephedrine, dichloroisoproterenol and alprenolol, as well as increased efficacy of inverse agonists (Seifert et al., 1998a; Wenzel-Seifert and Seifert, 2000, 2003; Wenzel-Seifert et al., 2002). These data are explained by the fact that G$_{s\alpha L}$ possesses a lower GDP-affinity than G$_{s\alpha S}$ (Seifert et al., 1998a), facilitating GDP dissociation by the agonist-free GPCR or GPCR bound to a partial agonist.

The fact that at all four fusion proteins studied, DOP was a partial agonist both in the GTPase- and AC assay, offered a unique opportunity to probe the validity of the extended ternary complex model using a natural partial agonist. In agreement with the model, both in the GTPase- and in the AC assay, DOP exhibited increased potency at a $\beta$AR-G$_{s\alpha L}$ fusion protein compared to the corresponding $\beta$AR-G$_{s\alpha S}$ fusion protein. However, with respect to efficacy, no significant differences emerged for DOP at $\beta$AR-G$_{s\alpha S}$- versus $\beta$AR-G$_{s\alpha L}$ fusion proteins although for synthetic ligands with comparable efficacy, i.e. salbutamol and dobutamine, such differences were apparent (Seifert et al., 1998a; Wenzel-Seifert and Seifert, 2000; Wenzel-Seifert et al., 2002). Intriguingly, deviations from the predictions of the extended ternary complex model were also observed for certain partial agonists at the human

histamine $H_2$-receptor fused to $G_{s\alpha S}$ and $G_{s\alpha L}$ (Wenzel-Seifert et al., 2001) and the highly

constitutively active canine $H_2$-receptor fused to $G_{s\alpha S}$ (Preuss et al., 2007).

It is a convention to use the synthetic agonist ISO as reference compound for

assessing ligand efficacy at βARs (Rousseau et al., 1996; Hoffmann et al., 2004; Kobilka,

2007). Our data on four $\beta_1AR$-$G_{s\alpha}$- and $\beta_2AR$-$G_{s\alpha}$ fusion proteins in two different assays

(GTPase and AC) corroborate the validity of this convention, i.e. in none of the systems

studied did EPI or NE surpass ISO in terms of efficacy. Rather, there was a trend, more

pronounced for NE than for EPI, towards reduced efficacy of endogenous catecholamines.

DOP was a partial agonist under all conditions studied. Our data on ISO, EPI, NE and DOP

data fit to fluorescence studies with purified $\beta_2AR$ and $\beta_2AR$ internalization studies in

HEK293 cells (Swaminath et al., 2004). In contrast, studying AC activation by $\beta_1AR$ and

$\beta_2AR$ expressed in CHO cells, there was a trend of reduced efficacy of ISO compared to EPI

and NE at most GPCR expression levels studied (Hoffmann et al., 2004). The high efficacy

of NE in the latter study contrasts with fluorescence and GPCR internalization data

(Swaminath et al., 2004) and our data. The molecular basis for these differences in efficacy is

unknown, but the aggregate data show that the efficacy of ISO, EPI and NE is sensitive to the

specific experimental conditions. Previous studies revealed that the efficacy of ligands at

βARs depends on the specific G-protein and the specific signal transduction pathway studied

(Wenzel-Seifert and Seifert, 2000; Kenakin, 2007; Galandrin et al., 2008; Bhattacharya et al.,

2008). Thus, any given ligand actually possesses multiple efficacies.

In the present study, we only considered coupling of βARs to $G_s$-proteins. However,

given the fact that various synthetic βAR ligands show distinct pharmacological properties

when studying different G-protein families such as $G_i$ and $G_q$ and signaling pathways such as

the mitogen-activated protein kinase pathway (Wenzel-Seifert and Seifert, 2000; Galandrin et

al., 2008), it is possible that those ligand-specific signaling differences extend to endogenous

catecholamines. Previous studies reported intriguing differences between endogenous

catecholamines in organ systems and the intact organism that could not be explained

satisfactorily (McNay and Goldberg, 1966; Mueller, 1978). Our data suggest that differential

activation of signaling pathways by various catecholamines contribute to their complex in

vivo effects. An in depth-analysis of the unexpectedly complex effects of endogenous

catecholamines on βARs will also help us understand why the clinical effects of

catecholamines in septic shock and renal failure are so controversial and identify

subpopulations of patients that may benefit from therapy with defined catecholamines (Beale

et al., 2004; Myburgh, 2007). By analogy to the developments in the nuclear receptor field

(Regitz-Zagrosek et al., 2007), catecholamines may exhibit organ-and/or cell-type-specific

effects that would facilitate a more specific treatment of disease states while reducing

toxicity. The development of organ- and/or cell-type-specific catecholamine therapy is

feasible in view of the fact that different agonist-binding sites can be exploited in βARs, i. e.

the aryloxypropanolamine binding site in the $\beta_1$AR (Granneman, 2001) and the catechol- and

non-catechol binding site in the $\beta_2$AR (Swaminath et al., 2005). Thus, when studying βARs

at the molecular, cellular, tissue, organ or intact organism level, one should keep in mind that

there is no "standard" catecholamine but that the analysis of multiple agonists from different

chemical classes is required.

Collectively, by applying classic pharmacological methods, i.e. ternary complex

formation-, GTPase- and AC activity assays, we corroborate the concept developed with

sophisticated methods, i.e. fluorescence spectroscopy and molecular modeling (Swaminath et

al., 2004; Bhattacharya et al., 2008), that endogenous catecholamines stabilize distinct active

$\beta_2$AR conformations. Moreover, previous pharmacological studies revealed that various

synthetic ligands stabilize distinct $\beta_1$AR conformations (Granemann, 2001; Galandrin et al.,

2008). Our present study extends the concept of ligand-specific $\beta_1$AR conformations to

endogenous catecholamines.

## Acknowledgements

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## Footnotes

## Legends for Figures

**Fig. 1. Competition by ISO, EPI, NE and DOP of [$^3$H]DHA binding in Sf9 membranes expressing $\beta_1$AR-G$_{s\alpha L}$: effect of GTP.** [$^3$H]DHA binding was determined as described in *Methods*. Reaction mixtures contained Sf9 membranes expressing $\beta_1$AR-G$_{s\alpha L}$, 1 nM [$^3$H]DHA and agonists at increasing concentrations. On the abscissa, $10^{-12}$ indicates the absence of agonist. Reaction mixtures additionally contained distilled water (control) or 1 mM GTP. Data points shown are the means ± SD of 3-4 experiments performed in triplicates. Data were analyzed by non-linear regression for best fit to monophasic or biphasic competition curves. The results of this analysis are summarized in Table 1.

**Fig. 2. Competition by ISO, EPI, NE and DOP of [$^3$H]DHA binding in Sf9 membranes expressing $\beta_1$AR-G$_{s\alpha S}$: effect of GTP.** [$^3$H]DHA binding was determined as described in *Methods*. Reaction mixtures contained Sf9 membranes expressing $\beta_1$AR-G$_{s\alpha S}$, 1 nM [$^3$H]DHA and agonists at increasing concentrations. On the abscissa, $10^{-11}$ indicates the absence of agonist. Reaction mixtures additionally contained distilled water (control) or 1 mM GTP. Data points shown are the means ± SD of 3-4 experiments performed in triplicates. Data were analyzed by non-linear regression for best fit to monophasic or biphasic competition curves. The results of this analysis are summarized in Table 1.

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**Fig. 3. Competition by ISO, EPI, NE and DOP of [³H]DHA binding in Sf9 membranes expressing β₂AR-G$_{s\alpha L}$: effect of GTP.** [³H]DHA binding was determined as described in *Methods*. Reaction mixtures contained Sf9 membranes expressing β₂AR-G$_{s\alpha L}$, 1 nM [³H]DHA and agonists at increasing concentrations. On the abscissa, $10^{-12}$ indicates the absence of agonist. Reaction mixtures additionally contained distilled water (control) or 1 mM GTP. Data points shown are the means ± SD of 3-4 experiments performed in triplicates. Data were analyzed by non-linear regression for best fit to monophasic or biphasic competition curves. The results of this analysis are summarized in Table 1.

**Fig. 4. Competition by ISO, EPI, NE and DOP of [³H]DHA binding in Sf9 membranes expressing β₂AR-G$_{s\alpha S}$: effect of GTP.** [³H]DHA binding was determined as described in *Methods*. Reaction mixtures contained Sf9 membranes expressing β₂AR-G$_{s\alpha S}$, 1 nM [³H]DHA and agonists at increasing concentrations. On the abscissa, $10^{-11}$ indicates the absence of agonist. Reaction mixtures additionally contained distilled water (control) or 1 mM GTP. Data points shown are the means ± SD of 3-4 experiments performed in triplicates. Data were analyzed by non-linear regression for best fit to monophasic or biphasic competition curves. The results of this analysis are summarized in Table 1.

**Fig. 5. Agonist potencies and efficacies at β₁AR-G$_{s\alpha}$ fusion proteins in the GTPase- and AC assay.** The GTPase- and AC assays were performed as described in *Methods*. Reaction mixtures contained the various β₁AR-G$_{s\alpha}$ fusion protein membranes and the catecholamines ISO, EPI, NE and DOP at the concentrations indicated on the abscissa. The stimulatory effects of EPI, NE and DOP were referred to the maximum stimulatory effect of ISO, serving as reference compound. Panels A and B show GTPase data and panels C and D show AC data. Data points are the means of 5-6 experiments performed in duplicates. Data shown were analyzed by non-linear regression and were best fit to monophasic saturation curves.

**Fig. 6. Agonist potencies and efficacies at β₂AR-G$_{s\alpha}$ fusion proteins in the GTPase- and AC assay.** The GTPase- and AC assays were performed as described in *Methods*. Reaction mixtures contained the various β₂AR-G$_{s\alpha}$ fusion protein membranes and the catecholamines ISO, EPI, NE and DOP at the concentrations indicated on the abscissa. The stimulatory effects of EPI, NE and DOP were referred to the maximum stimulatory effect of ISO, serving as reference compound. Panels A and B show GTPase data and panels C and D show AC data. Data points are the means of 5-6 experiments performed in duplicates. Data shown were analyzed by non-linear regression and were best fit to monophasic saturation curves.

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| Agonist | $K_{ih}$ (nM) | $K_{il}$ (nM) | $R_h$ (%) | $K_{ihGTP}$ (nM) | $K_{ilGTP}$ (nM) |
|---|---|---|---|---|---|
| **$\beta_1$AR-G$_{s\alpha L}$** | | | | | |
| ISO | 2.5 (1.3-4.8) | 260 (120-580) | 60 (51-69) | 0.6 (0.2-1.8) | 79 (57-110) |
| EPI | 35 (18-68) | 1,400 (520-3,500) | 61 (47-75) | 53 (25-110) | 1,200 (760-2,000) |
| NE | 1.2 (0.4-3.7) | 230 (88-590) | 51 (39-62) | 33 (13-86) | 630 (200-2,000) |
| DOP | 490 (100-2,300) | 19,000 (8,000-46,000) | 36 (16-56) | 40 (3.4-470) | 26,000 (18,000-38,000) |
| **$\beta_1$AR-G$_{s\alpha S}$** | | | | | |
| ISO | 1.0 (0.5-1.8) | 150 (110-200) | 40 (35-41) | 1.0 (0.5-2.5) | 170 (140-220) |
| EPI | 21 (11-39) | 1,600 (810-3,200) | 59 (50-67) | 1.5 (0.1-640) | 710 (550-920) |
| NE | 5.3 (3.2-8.8) | 390 (230-690) | 57 (49-64) | 2.4 (0.2-25) | 290 (190-460) |
| DOP | 52 (16-160) | 35,000 (25,000-48,000) | 27 (21-34) | 77 (3.9-1,500) | 59,000 (41,000-85,000) |
| **$\beta_2$AR-G$_{s\alpha L}$** | | | | | |
| ISO | 0.4 (0.2-0.7) | 82 (58-120) | 48 (43-52) | - | 120 (100-140) |
| EPI | 1.2 (0.7-2.1) | 320 (150-680) | 69 (63-74) | - | 190 (130-300) |
| NE | 4.9 (1.6-15) | 2,800 (1,300-6,300) | 44 (36-52) | - | 8,200 (5,100-13,000) |
| DOP | 25 (7.8-78) | 9,300 (6,500-13,000) | 28 (22-34) | 29 (5.7-150) | 54,000 (34,000-86,000) |
| **$\beta_2$AR-G$_{s\alpha S}$** | | | | | |
| ISO | 0.8 (0.4-1.5) | 60 (41-87) | 45 (39-51) | 0.6 (0.1-8.4) | 130 (97-160) |
| EPI | 0.9 (0.2-4.9) | 200 (69-570) | 55 (44-67) | - | 270 (170-410) |
| NE | 8.6 (1.3-57) | 960 (440-2,100) | 34 (22-47) | - | 6,000 (3,500-10,000) |
| DOP | 21 (2.8-160) | 11,000 (7,900-16,000) | 19 (11-27) | - | 61,000 (34,000-110,000) |

**Table 1. Agonist binding properties of $\beta_1$AR-G$_{s\alpha}$- and $\beta_2$AR-G$_{s\alpha}$ fusion proteins.** Agonist

competition binding in Sf9 membranes expressing $\beta$AR-G$_{s\alpha}$ fusion proteins was performed as

described in *Methods*. The data shown in Figs. 1-4 were analyzed by non-linear regression for best fit

to monophasic or biphasic competition curves. Data shown are the means of 3-4 experiments

performed in triplicates. Numbers in parentheses represent the 95% confidence intervals. $K_h$ and $K_l$

designate the dissociation constants for the high- and low-affinity state of $\beta$ARs, respectively. $R_h$ (%)

indicates the percentage of high-affinity agonist binding sites. $K_{hGTP}$ and $K_{lGTP}$ designate the

dissociation constants for the high- and low-affinity state of $\beta$AR-G$_{s\alpha}$ fusion proteins in the presence

of GTP (1 mM).

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| Ligand | $\beta_1AR\text{-}G_{s\alpha L}$ | $\beta_1AR\text{-}G_{s\alpha S}$ | $\beta_2AR\text{-}G_{s\alpha L}$ | $\beta_2AR\text{-}G_{s\alpha S}$ |
|---|---|---|---|---|
| **$EC_{50}$ (nM)** | | | | |
| ISO | 40 (30-54) | 32 (23-45) | 8.7 (6.4-12) | 14 (11-18) |
| EPI | 130 (100-170) | 130 (100-170) | 16 (12-22) | 43 (29-62) |
| NE | 20 (13-30) | 60 (40-88) | 130 (93-190) | 290 (200-430) |
| DOP | 1,100 (760-1,600) | 3.200 (1,800-5,800) | 1,600 (1,000-2,400) | 9,700 (6,300-15,000) |
| **$E_{max}$ (%)** | | | | |
| ISO | 100 (97-103) | 100 (96-104) | 100 (97-103) | 100 (97-103) |
| EPI | 96 (92-99) | 98 (95-101) | 100 (97-103) | 100 (97-107) |
| NE | 79* (75-83) | 98 (92-104) | 93 (88-98) | 92 (87-98) |
| DOP | 59* (55-63) | 55* (50-59) | 82* (77-88) | 77* (71-83) |

**Table 2. Agonist potencies and efficacies at $\beta_1AR\text{-}G_{s\alpha}$- and $\beta_2AR\text{-}G_{s\alpha}$ fusion proteins in the GTPase assay.** Agonist potencies and efficacies in the GTPase assay were determined as described in *Methods*. The data shown in Figs. 5A, 5B, 6A and 6B were analyzed by non-linear regression and were best fit to monophasic saturation curves. Data shown are the means of 5-6 experiments performed in duplicates. Numbers in parentheses represent the 95% confidence intervals. Statistical significance of differences in $E_{max}$ values of endogenous catecholamines *versus* the reference compound ISO was assessed using ANOVA followed by Dunnet's multiple comparison post test. *, p < 0.01.

| Ligand | $\beta_1AR-G_{s\alpha L}$ | $\beta_1AR-G_{s\alpha S}$ | $\beta_2AR-G_{s\alpha L}$ | $\beta_2AR-G_{s\alpha S}$ |
|---|---|---|---|---|
| **$EC_{50}$ (nM)** | | | | |
| ISO | 72 (47-110) | 16 (11-22) | 13 (7.0-23) | 18 (11-32) |
| EPI | 76 (32-180) | 85 (60-120) | 57 (33-99) | 37 (19-71) |
| NE | 36 (23-56) | 28 (14-57) | 230 (98-540) | 490 (230-1,000) |
| DOP | 1,800 (770-4,000) | 4,700 (1,800-13,000) | 1500 (390-5,700) | 8000 (3,700-17,000) |
| **$E_{max}$ (%)** | | | | |
| ISO | 100 (95-109) | 100 (96-105) | 100 (93-108) | 100 (93-107) |
| EPI | 82 (69-95) | 96 (90-100) | 98 (91-106) | 100 (90-110) |
| NE | 86 (80-92) | 94 (85-104) | 78* (65-91) | 92 (81-103) |
| DOP | 56* (50-63) | 51* (41-61) | 58* (47-70) | 70* (59-79) |

**Table 3. Agonist potencies and efficacies at $\beta_1AR-G_{s\alpha}$- and $\beta_2AR-G_{s\alpha}$ fusion proteins in the AC assay.** Agonist potencies and efficacies in the AC assay were determined as described in *Methods*. The data shown in Figs. 5C, 5D, 6C and 6D were analyzed by non-linear regression and were best fit to monophasic saturation curves. Data shown are the means of 5-6 experiments performed in duplicates. Numbers in parentheses represent the 95% confidence intervals. Statistical significance of differences in $E_{max}$ values of endogenous catecholamines *versus* the reference compound ISO was assessed using ANOVA followed by Dunnet's multiple comparison post test. *, $p < 0.01$.

| Ligand | $\beta_1AR-G_{s\alpha L}$ | $K_{ih}/EC_{50}$ GTPase $\beta_1AR-G_{s\alpha S}$ | $\beta_2AR-G_{s\alpha L}$ | $\beta_2AR-G_{s\alpha S}$ |
|---|---|---|---|---|
| ISO | 0.0625 | 0.0313 | 0.0460 | 0.0571 |
| EPI | 0.2692 | 0.1615 | 0.0750 | 0.0201 |
| NE | 0.0600 | 0.0883 | 0.0377 | 0.0297 |
| DOP | 0.4455 | 0.0163 | 0.0131 | 0.0027 |
|  |  | $K_{il}/EC_{50}$ GTPase |  |  |
| ISO | 6.5 | 4.7 | 9.4 | 4.3 |
| EPI | 10.8 | 12. | 20.0 | 4.7 |
| NE | 11.5 | 6.5 | 21.5 | 3.3 |
| DOP | 17.3 | 10.9 | 5.8 | 1.1 |
|  |  | $EC_{50}$ GTPase/ $EC_{50}$ AC |  |  |
| ISO | 0.55 | 2.00 | 0.67 | 0.78 |
| EPI | 1.71 | 1.53 | 0.28 | 1.16 |
| NE | 0.55 | 2.14 | 0.57 | 0.59 |
| DOP | 0.61 | 0.68 | 1.07 | 1.21 |

**Table 4. Correlations between agonist-affinities in the agonist competition binding assay and potencies for GTPase activation as well as correlation between potencies in the GTPase- and AC assay.** The $K_{ih}$- and $K_{il}$ values were taken from Table 1. The $EC_{50}$ values for GTPase activation were taken from Table 2, and the $EC_{50}$ values for AC activation were taken from Table 3. Coefficients of the various parameters were calculated for the four catecholamines studied.
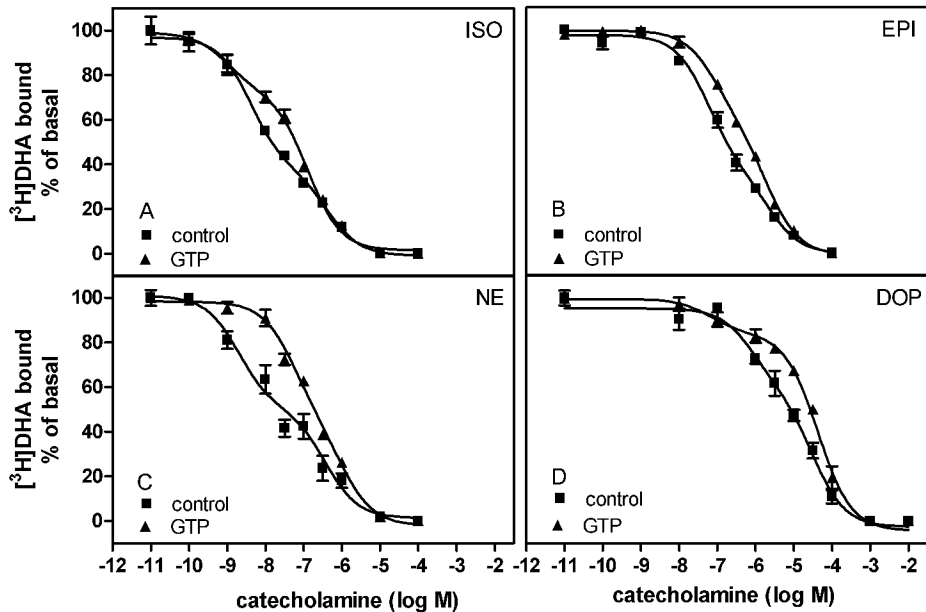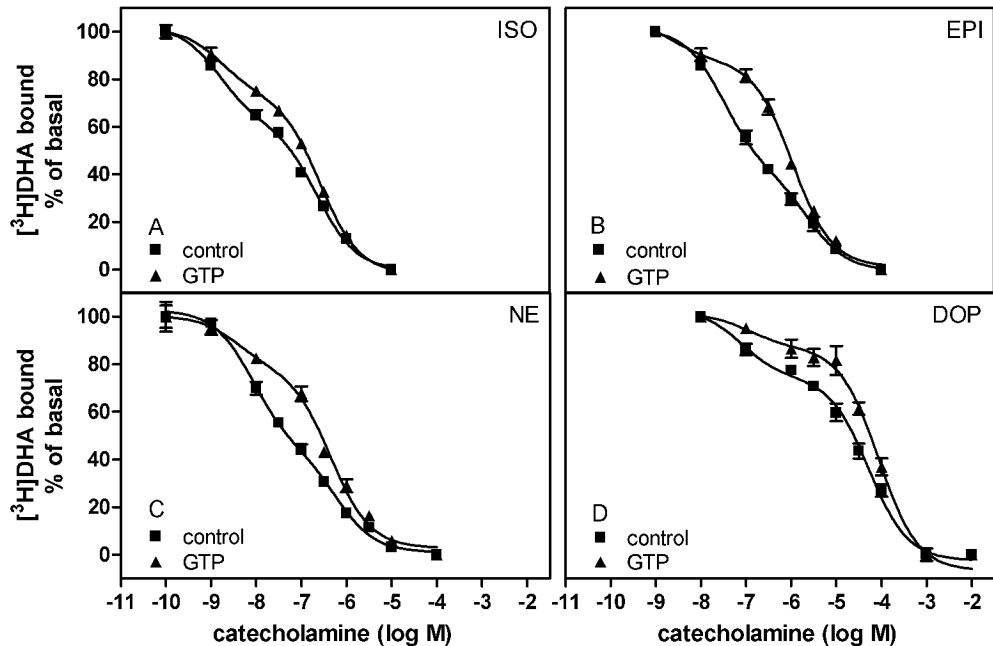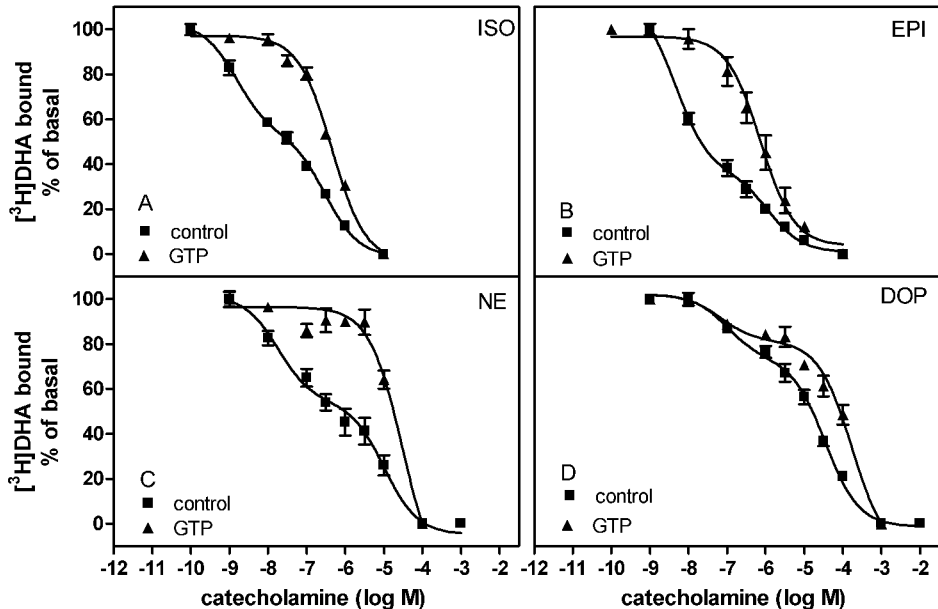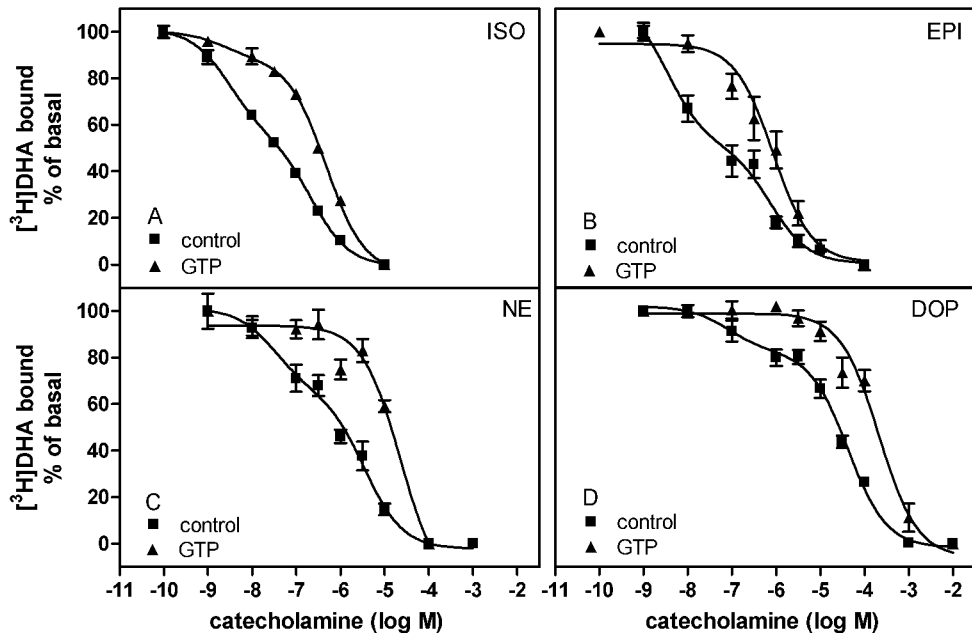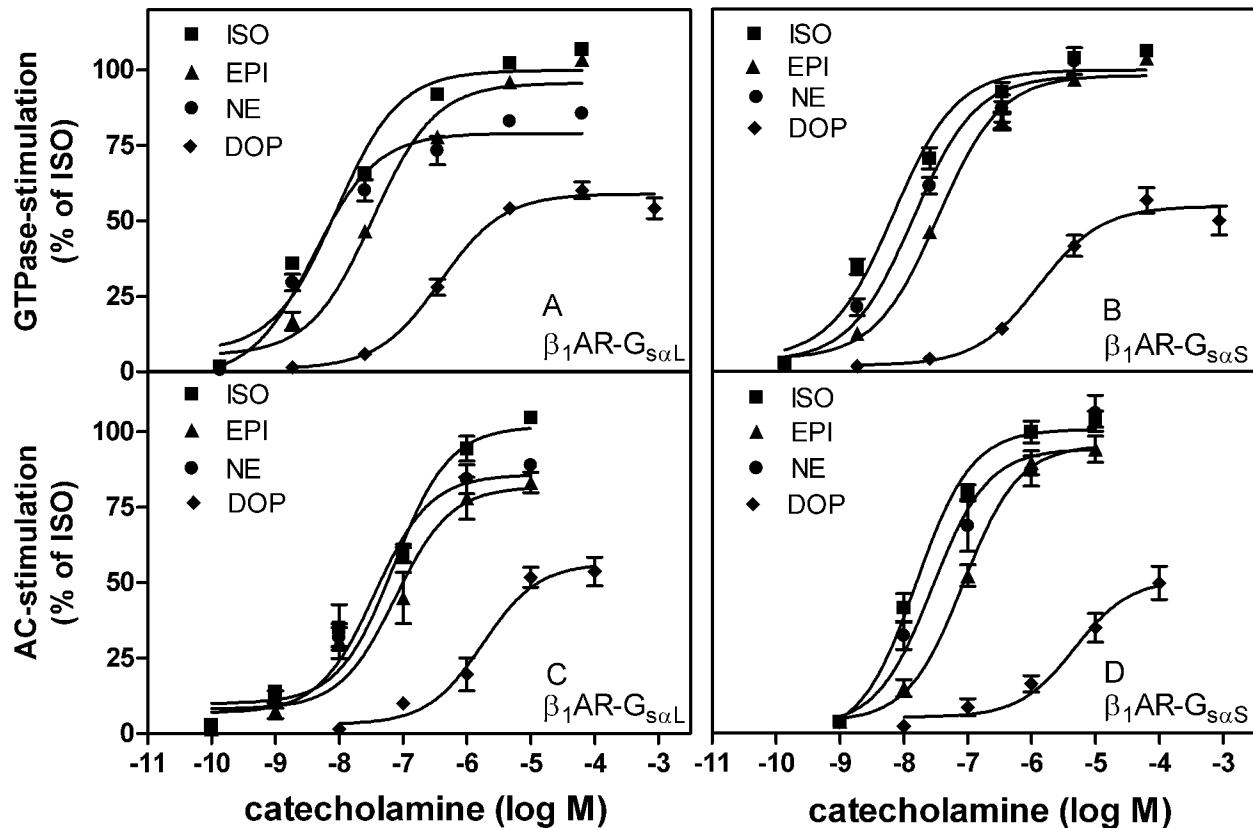
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

# Figure 6