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Distinct Interactions of Human β_1- and β_2-Adrenoceptors with Isoproterenol, Epinephrine, Norepinephrine and Dopamine

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Abbreviations: AC, adenylyl cyclase; βAR, non-specified human β-adrenoceptor; β_1AR, β_1-adrenoceptor; β_1AR-$G_{s\alpha L}$, fusion protein consisting of the human β_1-adrenoceptor and the long splice variant of $G_{s\alpha}$; β_1AR-$G_{s\alpha S}$, fusion protein consisting of the human β_1-adrenoceptor and the short splice variant of $G_{s\alpha}$; β_2AR, human β_2-adrenoceptor; β_2AR_{CAM}, constitutively active mutant of the β_2AR; β_2AR-$G_{s\alpha L}$, fusion protein consisting of the human β_2-adrenoceptor and the long splice variant of $G_{s\alpha}$; β_2AR-$G_{s\alpha S}$, fusion protein consisting of the human β_2-adrenoceptor and the short splice variant of $G_{s\alpha}$; DHA, dihydroalprenolol; DOP, dopamine; EPI, (-)-epinephrine; G_α, 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 β_2-adrenoceptor (β_2AR) 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 β_1-adrenoceptor (β_1AR) and β_2AR 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 βAR agonist, (-)-isoproterenol (ISO), showed marked differences in their efficacies at stabilizing the high-affinity ternary complex at β_1AR-$G_{s\alpha}$ and β_2AR-$G_{s\alpha}$ fusion proteins. Ternary complex formation was more sensitive to disruption by GTP with the β_2AR than with the β_1AR. Generally, in steady-state GTPase assays, ISO, EPI and NE were full agonists, and DOP was a partial agonist. Exceptionally, at β_1AR-$G_{s\alpha L}$, 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 β_2AR-$G_{s\alpha L}$, 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 βAR-$G_{s\alpha L}$- versus βAR-$G_{s\alpha S}$ fusion proteins. In conclusion, our data with βAR-$G_{s\alpha}$ fusion proteins show that endogenous catecholamines and ISO stabilize distinct conformations in the β_1AR and β_2AR.

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Introduction

The β_1AR and β_2AR 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 βARs, serving as a reference compound for the analysis of agonist potencies and efficacies (Rousseau et al., 1996; Hoffmann et al., 2004; Kobilka, 2007). βARs couple to the G-protein G_s (Gilman, 1987; Birnbaumer et al., 1990). G_s consists of a $G_{s\alpha}$-subunit and a Gβγ-complex. In the resting state, the $G_{s\alpha}$-subunit is bound to GDP. Binding of an agonist to a βAR 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, βAR 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 βAR-affinity for agonist and dissociation of G_s into $G_{s\alpha}$ and the Gβγ-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

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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 β_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 β_2AR with a constitutively active β_2AR mutant (β_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 β_2AR depends on the specific G-protein coupling partner (Wenzel-Seifert and Seifert, 2000). These data suggest that ligands stabilize unique β_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 β_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 βAR 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 β_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

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and β_2AR internalization. Computational analysis of the β_2AR confirms the existence of NE- and DOP-specific conformations (Bhattacharya et al., 2008). However, the functional consequences of the distinct β_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 β_1AR and β_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 β_1AR-$G_{s\alpha L}$, β_1AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$ and β_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 β_1AR. A previous study revealed no differences between β_1AR-Gly389-$G_{s\alpha}$- and the corresponding β_1AR-Arg389-$G_{s\alpha}$ fusion proteins (Wenzel-Seifert and Seifert, 2003). [^{32}P]P_i (8,500-9,100 Ci/mmol), [α-^{32}P]ATP (3,000 Ci/mmol) and [^{3}H]DHA (85-90 Ci/mmol) were from Perkin Elmer (Boston, MA). [γ-^{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).

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Cell culture and membrane preparation. βAR-$G_{s\alpha}$ fusion proteins were expressed in Sf9 inset cells. Sf9 cells were cultured in 250 ml disposable Erlenmeyer flasks at 28°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 βAR-$G_{s\alpha}$ 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 μg/ml benzamidine and 10 μg/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°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°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 μg protein/tube) in the presence of 10 nM [^{3}H]DHA. The total volume of the binding reaction was 500 μl. Incubations were performed for 90 min at 25°C and shaking at 250 rpm. Non-specific [^{3}H]DHA binding was determined in the presence of 10 μM (±)-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 βAR-$G_{s\alpha}$ at levels between 3.8-5.2 pmol/mg of membrane protein were used. Tubes contained Sf9 membranes (20-25 μg protein/tube), 1 nM [^{3}H]DHA and agonists at increasing concentrations. Reaction mixtures additionally contained solvent

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(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_2PO_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-

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^{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°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°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°C and terminated by the addition of 20 μl 2.2 N HCl. Denatured protein was sedimented by a 3 min centrifugation at 25°C and 15,000 *x* g. Sixyfive 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.

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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_α 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_α 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

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levels should be avoided (Seifert et al., 1998b). βAR-$G_{s\alpha}$ 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 βAR-$G_{s\alpha}$ 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, βAR-$G_{s\alpha}$ 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 βAR 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 β_1AR-$G_{s\alpha}$- and β_2AR-$G_{s\alpha}$ fusion proteins as assessed by [^{35}S]guanosine 5'-[γ-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_{α} 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 βAR-$G_{s\alpha}$ fusion proteins: effect of GTP. At β_1AR-$G_{s\alpha L}$, 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 β_1AR-$G_{s\alpha L}$. At β_1AR-$G_{s\alpha S}$, like at β_1AR-$G_{s\alpha L}$, DOP was the least effective catecholamine at stabilizing the ternary complex (Fig. 2 and Table 1). At β_1AR-$G_{s\alpha S}$, ISO was a less effective stabilizer of the ternary complex than at β_1AR-$G_{s\alpha L}$. Like at β_1AR-$G_{s\alpha L}$, GTP did not convert the biphasic competition isotherms at β_1AR-$G_{s\alpha S}$ into monophasic isotherms. At both β_1AR-$G_{s\alpha}$ fusion proteins, the order of affinity (K_{ih} and K_{il}) of catecholamines was ISO ~ NE > EPI >> DOP. At the β_1AR 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 β_2AR-$G_{s\alpha L}$, 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 β_2AR-$G_{s\alpha L}$, EPI was the most effective ternary complex stabilizer at β_2AR-$G_{s\alpha S}$, and DOP was the least effective agonist in this regard (Fig. 4). GTP converted the biphasic competition isotherms for EPI, NE and DOP at β_2AR-$G_{s\alpha S}$ into monophasic isotherms, whereas the competition isotherm for ISO remained biphasic. At both β_2AR-$G_{s\alpha}$ fusion proteins, the order

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of affinity (K_{ih} and K_{il}) of catecholamines was ISO ~ EPI > NE > DOP. At the β_2AR 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 βAR-$G_{s\alpha}$ fusion proteins in the GTPase assay. At β_1AR-$G_{s\alpha L}$ and β_1AR-$G_{s\alpha S}$, the order of potency of catecholamines in the GTPase assay was ISO ~ NE > EPI >> DOP (Figs. 5 and 6 and Table 2). At β_1AR-$G_{s\alpha L}$, ISO and EPI were full agonists, whereas NE and DOP were only partial agonists. At β_1AR-$G_{s\alpha S}$, ISO, EPI and NE were all full agonists, whereas DOP was only a partial agonist.

At β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{s\alpha S}$, 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 β_2AR-$G_{s\alpha}$ fusion proteins, but the difference did not reach significance.

Agonist potencies and efficacies at βAR-$G_{s\alpha}$ fusion proteins in the AC assay. At β_1AR-$G_{s\alpha L}$ and β_1AR-$G_{s\alpha S}$, 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 β_1AR-$G_{s\alpha}$ fusion proteins, but the difference did not reach significance.

At β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{s\alpha S}$, 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 β_2AR-$G_{s\alpha L}$,

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ISO and EPI were full agonists, and NE and DOP were partial agonists. At β_2AR-$G_{s\alpha S}$, 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 β_1AR-$G_{s\alpha L}$, 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 β_2AR-$G_{s\alpha S}$ 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 β_1AR and β_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 βAR-$G_{s\alpha}$ fusion proteins, we found a match between the two values only for DOP at β_2AR-$G_{s\alpha S}$ (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 β_1AR and β_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,

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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 β_2AR-$G_{s\alpha S}$, the two EC_{50} values matched, but at β_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 βAR and at the β_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 β_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 β_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 β_1AR-$G_{s\alpha}$- and β_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 β_1AR-$G_{s\alpha S}$, β_2AR-$G_{s\alpha L}$ and β_2AR-$G_{s\alpha S}$ 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 β_1AR-$G_{s\alpha}$ fusion proteins was less sensitive to disruption by GTP than ternary complex formation with the corresponding β_2AR-$G_{s\alpha}$ fusion proteins. These data indicate that the β_1AR couples to $G_{s\alpha}$ more tightly than the β_2AR, 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 β_2AR-$G_{s\alpha L}$, 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 β_2AR 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 β_2AR-$G_{i\alpha}$ 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 βAR conformations.

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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 β_2AR and β_2AR_{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 βAR-$G_{s\alpha S}$- and βAR-$G_{s\alpha L}$ 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 β_1AR and β_2AR 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 βAR-$G_{s\alpha L}$ fusion protein compared to the corresponding βAR-$G_{s\alpha S}$ fusion protein. However, with respect to efficacy, no significant differences emerged for DOP at βAR-$G_{s\alpha S}$- versus βAR-$G_{s\alpha L}$ 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

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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 β_1AR-$G_{s\alpha}$- and β_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 β_2AR and β_2AR internalization studies in HEK293 cells (Swaminath et al., 2004). In contrast, studying AC activation by β_1AR and β_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

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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 β_1AR (Granneman, 2001) and the catechol- and non-catechol binding site in the β_2AR (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 β_2AR conformations. Moreover, previous pharmacological studies revealed that various synthetic ligands stabilize distinct β_1AR conformations (Granemann, 2001; Galandrin et al.,

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2008). Our present study extends the concept of ligand-specific β_1AR conformations to endogenous catecholamines.

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Footnotes

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Legends for Figures

Fig. 1. Competition by ISO, EPI, NE and DOP of [^{3}H]DHA binding in Sf9 membranes expressing β_1AR-$G_{s\alpha L}$: effect of GTP. [^{3}H]DHA binding was determined as described in *Methods*. Reaction mixtures contained Sf9 membranes expressing β_1AR-$G_{s\alpha L}$, 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 β_1AR-$G_{s\alpha S}$: effect of GTP. [^{3}H]DHA binding was determined as described in *Methods*. Reaction mixtures contained Sf9 membranes expressing β_1AR-$G_{s\alpha S}$, 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 [^{3}H]DHA binding in Sf9 membranes expressing β_2AR-$G_{s\alpha L}$: effect of GTP. [^{3}H]DHA binding was determined as described in *Methods*. Reaction mixtures contained Sf9 membranes expressing β_2AR-$G_{s\alpha L}$, 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. 4. Competition by ISO, EPI, NE and DOP of [^{3}H]DHA binding in Sf9 membranes expressing β_2AR-$G_{s\alpha S}$: effect of GTP. [^{3}H]DHA binding was determined as described in *Methods*. Reaction mixtures contained Sf9 membranes expressing β_2AR-$G_{s\alpha S}$, 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. 5. Agonist potencies and efficacies at β_1AR-$G_{s\alpha}$ fusion proteins in the GTPase- and AC assay. The GTPase- and AC assays were performed as described in *Methods*. Reaction mixtures contained the various β_1AR-$G_{s\alpha}$ 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 β_2AR-$G_{s\alpha}$ fusion proteins in the GTPase- and AC assay. The GTPase- and AC assays were performed as described in *Methods*. Reaction mixtures contained the various β_2AR-$G_{s\alpha}$ 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)
β_1AR-$G_{s\alpha L}$					
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)
β_1AR-$G_{s\alpha S}$					
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)
β_2AR-$G_{s\alpha L}$					
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)
β_2AR-$G_{s\alpha S}$					
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 β_1AR-$G_{s\alpha}$- and β_2AR-$G_{s\alpha}$ fusion proteins. Agonist competition binding in Sf9 membranes expressing βAR-$G_{s\alpha}$ 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 βARs, 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 βAR-$G_{s\alpha}$ fusion proteins in the presence of GTP (1 mM).

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Ligand	β_1AR-$G_{s\alpha L}$	β_1AR-$G_{s\alpha S}$	β_2AR-$G_{s\alpha L}$	β_2AR-$G_{s\alpha S}$
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 β_1AR-$G_{s\alpha}$- and β_2AR-$G_{s\alpha}$ 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. *, $p < 0.01$.

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Ligand	β_1AR-$G_{s\alpha L}$	β_1AR-$G_{s\alpha S}$	β_2AR-$G_{s\alpha L}$	β_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 β_1AR-$G_{s\alpha}$- and β_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. *, $p < 0.01$.

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		K_{ih}/EC_{50} GTPase		
Ligand	**β_1AR-$G_{s\alpha L}$**	**β_1AR-$G_{s\alpha S}$**	**β_2AR-$G_{s\alpha L}$**	**β_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

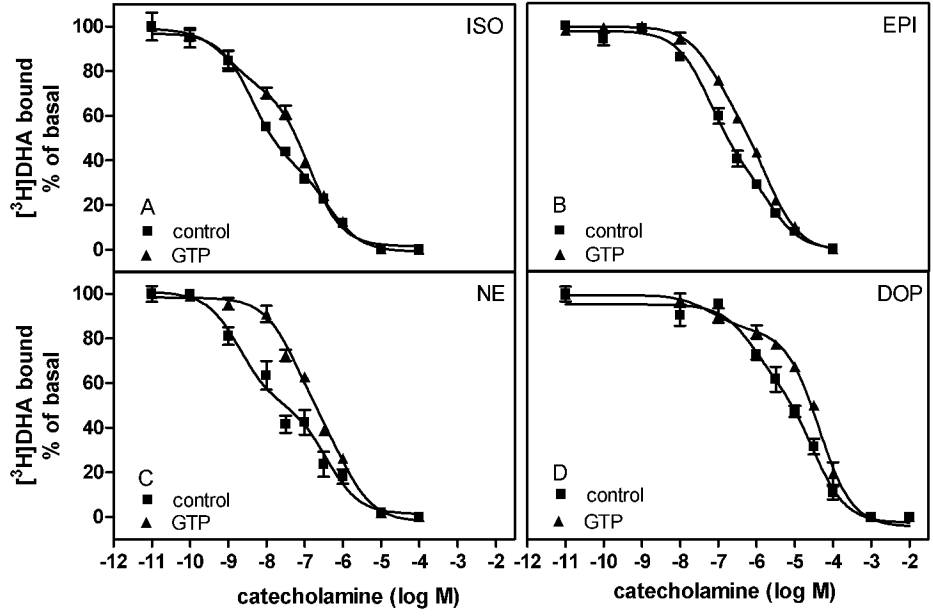
ISO
EPI
NE
DOP
A
B
C
D
control
GTP
[3H]DHA bound
% of basal
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2
catecholamine (log M)

Figure 2

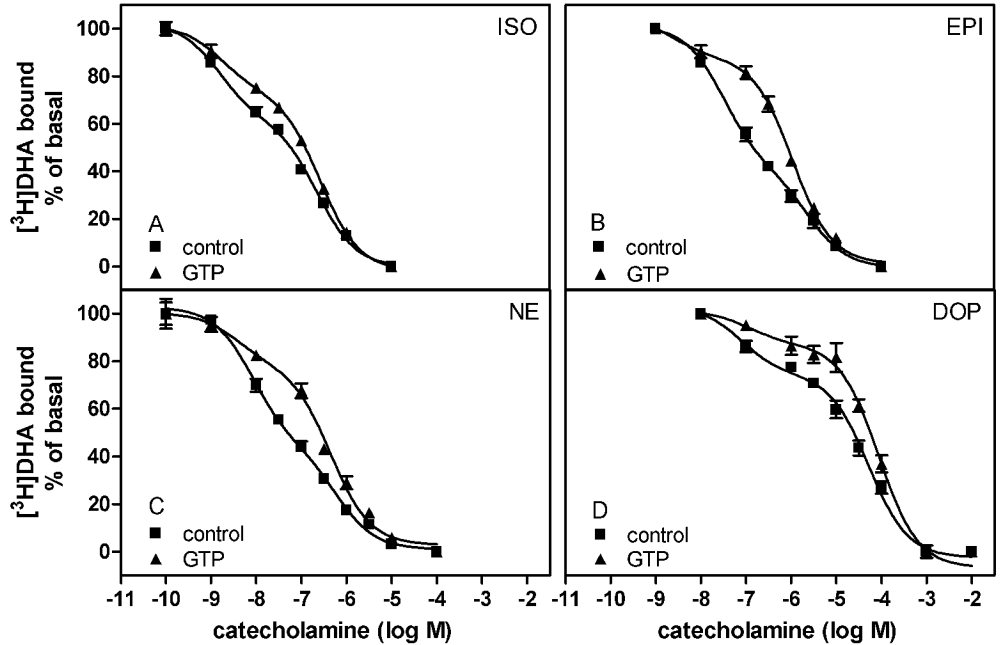
ISO
EPI
NE
DOP
A
B
C
D
control
GTP
[3H]DHA bound
% of basal
catecholamine (log M)
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
-3
-2

Figure 3

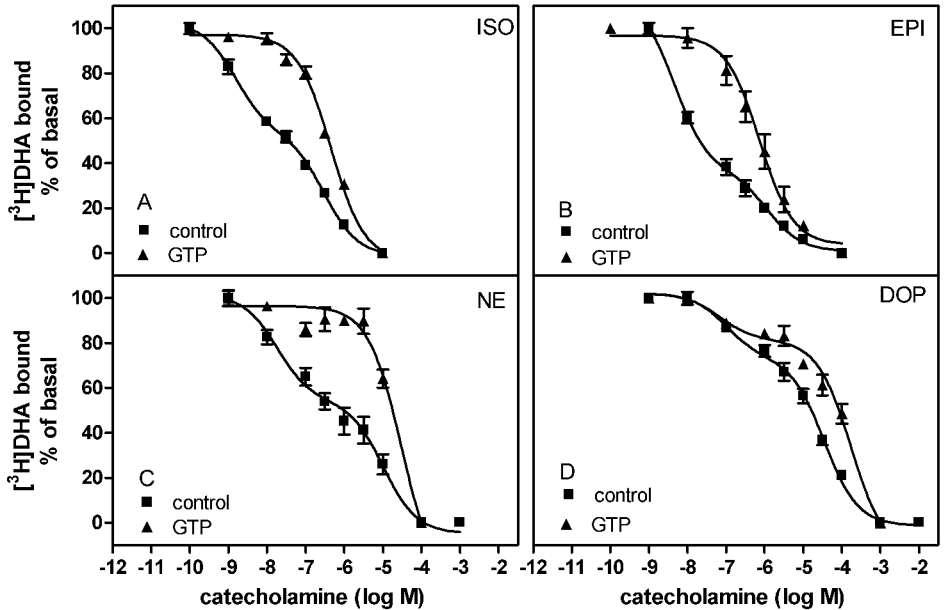
ISO
EPI
NE
DOP
A
B
C
D
control
GTP
[3H]DHA bound
% of basal
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2
catecholamine (log M)

Figure 4

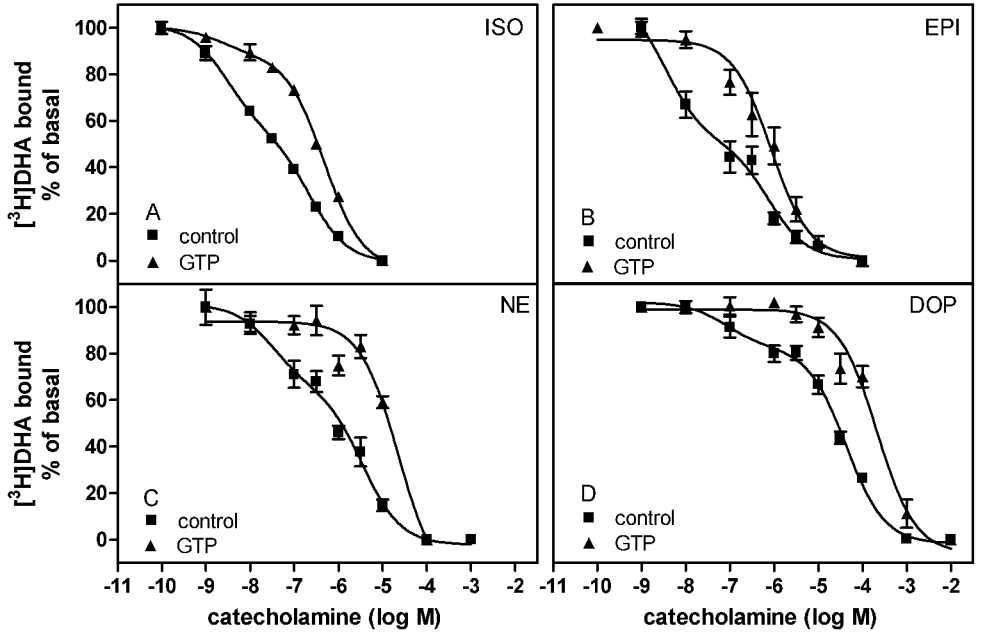
[3H]DHA bound
% of basal
ISO
A
control
GTP
EPI
B
control
GTP
NE
C
control
GTP
DOP
D
control
GTP
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
-3
-2
catecholamine (log M)

Figure 5

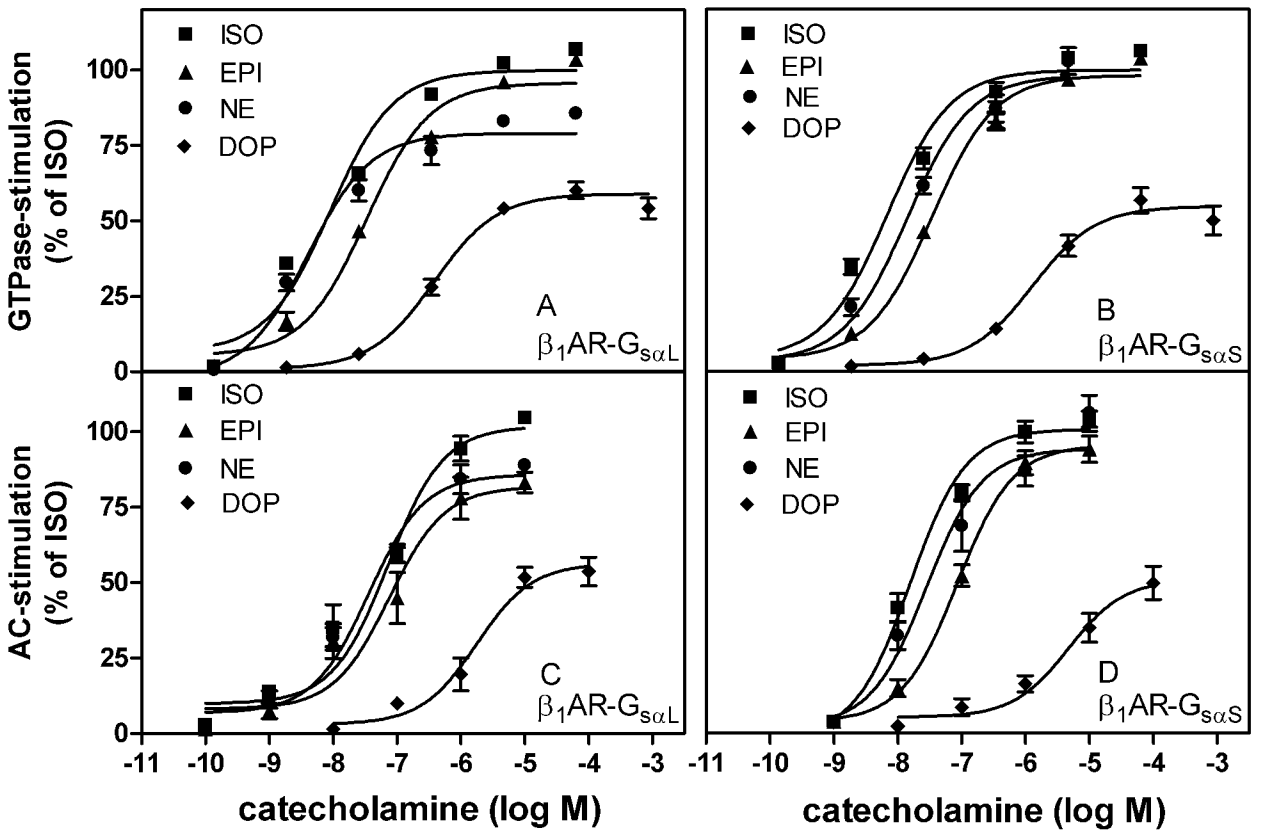
GTPase-stimulation (% of ISO)
AC-stimulation (% of ISO)
ISO
EPI
NE
DOP
A
β_1AR-$G_{s\alpha L}$
B
β_1AR-$G_{s\alpha S}$
C
β_1AR-$G_{s\alpha L}$
D
β_1AR-$G_{s\alpha S}$
0
25
50
75
100
-11
-10
-9
-8
-7
-6
-5
-4
-3
catecholamine (log M)

Figure 6