Differential Signaling of the Endogenous Agonists at the β2-Adrenergic Receptor*§

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The concept of “functional selectivity” or “biased signaling” suggests that a ligand can have distinct efficacies with regard to different signaling pathways. We have investigated the question of whether biased signaling may be related to distinct agonist-induced conformational changes in receptors using the β2-adrenergic receptor (β2AR) and its two endogenous ligands epinephrine and norepinephrine as a model system. Agonist-induced conformational changes were determined in a fluorescently tagged β2AR FRET sensor. In this β2AR sensor, norepinephrine caused signals that amounted to only ≈50% of those induced by epinephrine and the standard “full” agonist isoproterenol. Furthermore, norepinephrine-induced changes in the β2AR FRET sensor were slower than those induced by epinephrine (rate constants, 47 versus 128 ms). A similar partial β2AR activation signal was revealed for the synthetic agonists fenoterol and terbutaline. However, norepinephrine was almost as efficient as epinephrine (and isoproterenol) in causing activation of Gs and adenylyl cyclase. In contrast, fenoterol was quite efficient in triggering β-arrestin2 recruitment to the cell surface and its interaction with β2AR, as well as internalization of the receptors, whereas norepinephrine caused partial and slow changes in these assays. We conclude that partial agonism of norepinephrine at the β2AR is related to the induction of a different active conformation and that this conformation is efficient in signaling to Gs and less efficient in signaling to β-arrestin2. These observations extend the concept of biased signaling to the endogenous agonists of the β2AR and link it to distinct conformational changes in the receptor.

G-protein-coupled receptors are members of a highly regulated transduction machinery that generates different signal outputs. Classical receptor theory postulated that receptors switch between “off” and “on” states. Ligands were thought to alter the balance between receptors in the two states and to differ only in their efficacy or potency. In this concept, full agonists should activate all signal transduction pathways of a given receptor to the same extent. However, it has become apparent over recent years that the situation is far more complex and that ligands may cause differential activation of specific signaling pathways (1). Several terms have been coined for this phenomenon, such as “biased agonism,” “functional selectivity,” “signal trafficking,” “agonist-directed trafficking,” or “ligand-induced differential signaling” (2). In particular, it has been shown for several receptor systems that ligands may differentially activate two major pathways: “classical” G-protein-mediated signals versus “nonclassical” activation via β-arrestins and often involving MAPKs. β-Arrestins are recruited to receptors in response to agonist activation and agonist-induced phosphorylation by G-protein-coupled receptor kinases (GRKs)2 (3–5). They were initially thought to only disrupt receptor/G-protein signaling and thereby terminate signaling to G-proteins but have since been recognized to play a role in clathrin-dependent receptor internalization and to trigger several nonclassical signaling pathways such as activation of the MAPK cascade (6, 7). Initial studies comparing for the β2AR agonist-induced activation of G-proteins with agonist-induced receptor phosphorylation by GRKs revealed a close correlation (8). Different compounds displayed the same extent of partial or full activity in both readouts of receptor activation. These data appeared to suggest that the same active conformation(s) of the receptor induce both downstream events.

However, a growing body of experiments provides evidence that this classical view of receptor function is incomplete and that ligands may cause distinct responses for different downstream effects, including most notably G-protein-dependent versus β-arrestin-dependent pathways (9). Such data have been obtained for many receptors, including serotonin, opioid, vasopressin, dopamine, and β-adrenergic receptors (1, 2, 10). These observations include ligands that differentially affect G-protein activation versus receptor internalization (11) as well as compounds that differentially activate the MAPK cascade compared with G-protein-dependent signaling (12–14).

For the β2AR, this topic has been addressed in several studies. Although a detailed earlier study on several synthetic ligands revealed a very good proportionality between various effects (15), later studies revealed several synthetic ligands with differential activation of β-arrestin-dependent versus G-protein-dependent signals (16, 17). However, these studies leave open the question of whether the physiological ligands of these receptors, epinephrine and norepinephrine, also differ in their abilities to trigger downstream responses. The different release

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2 The abbreviations used are: GRK, G-protein-coupled receptor kinase; β2AR, β2-adrenergic receptor; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; GPCR, G-protein-coupled receptor.
mechanisms and functional roles of the two endogenous agonists raise the possibility that they might also display distinct mechanisms of receptor activation and signaling. Such different activities between epinephrine and norepinephrine have recently been reported for cardiomyocyte β2AR, where norepinephrine was observed to induce slower GRK2-mediated phosphorylation, receptor internalization and recycling, and no coupling to Gs when compared with epinephrine (18).

It has been suggested that differential responses to different ligands may be due to distinct active receptor conformations, which may be specifically induced by different ligands (12, 19–23). The existence of such distinct receptor conformations has also been inferred from different fluorescence patterns of labeled purified receptors in response to full and partial agonists (10) as well as different kinetics of these fluorescence changes (24). Similar kinetic differences between conformational changes in response to full, partial, and inverse agonists have also been reported for α1-adrenergic receptors in intact cells (see below) and have been interpreted as evidence for distinct receptor conformations (25).

To probe receptor conformational changes in intact cells, we have developed a FRET-based technology (26). This involves the labeling of receptors with fluorescent proteins or with small tetracysteine-based labels in their third intracellular loop and their C termini (27, 28). The close proximity of the two labels permits the transfer of energy between a donor label (e.g. cyan fluorescent protein (CFP)) and an acceptor label (e.g. yellow fluorescent protein (YFP)), and the binding of agonists to these receptors causes a change in FRET that reports the agonist-induced conformational change (29). Similar technologies based on FRET between interacting proteins have been developed recently for cardiomyocyte β2AR, where norepinephrine was observed to induce slower GRK2-mediated phosphorylation, receptor internalization and recycling, and no coupling to Gs when compared with epinephrine (18).

In the present study we have combined these technologies with classical techniques of monitoring downstream responses to receptor activation to assess potential differences between the two endogenous β2AR ligands epinephrine and norepinephrine in eliciting specific conformational changes and downstream responses. The standard full agonist isoproterenol plus the two partial agonists fenoterol and terbutaline were used as reference compounds. We report that indeed these assays provide evidence for agonist-specific conformations and signaling in response to the two endogenous ligands in intact cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—All of the β2AR agonists were purchased from Sigma-Aldrich, except for (−)-isoproterenol hydrochloride, which was from Tocris/Biozol (Eching, Germany). Unless indicated otherwise, all of the β2AR agonists were used at receptor saturating concentrations, calculated as 10–20-fold Ks (33) as follows: (−)-isoproterenol hydrochloride, 10 μM; (−)-epinephrine (+)-bitartrate, 10 μM; (±)-fenoterol hydrobromide, 10 μM; (−)-norepinephrine (+)-bitartrate, 300 μM; and terbutaline hemisulfate, 150 μM.

Alprenolol hydrochloride was provided by Astra Chemicals (Wedel, Germany). The cell culture reagents were obtained from PAN-Biotec GmbH (Aidenbach, Germany). Penicillin (100 units/ml), streptomycin (100 μg/ml), i-glutamine, and G-418 were purchased from Invitrogen. Effectene was from Qiagen, polyethylenimine was from Sigma-Aldrich, and Lipofectamine2000 and Opti-MEM were from Invitrogen. Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA). Polyclonal primary antibodies to anti-Ser(P)-355/356 and to the C terminus of the β2AR were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Molecular Biology**—The pcDNA3 plasmid encoding for the human β2AR as well as pcDNA3 plasmids containing the cDNA sequence for Gaβγ-YFP, CFP-Gγ2, or β2AR-YFP have been described previously (31, 33). The plasmids encoding bovine β-arrestin2 fused to YFP (5), as well as GRK2 and GRK5 (49) have been described previously.

Constructions of recombinant β2AR FRET sensors were performed by PCR using Pfu DNA polymerase. The influenza-he-magglutinin signal sequence followed by the FLAG epitope was fused to the N terminus to facilitate cell surface expression and detection. The cDNA encoding the enhanced CFP sequence was inserted between Asp-251 and Gly-252 into the third intracellular loop of the β2AR. The cDNA encoding the enhanced YFP sequence was fused to position Arg-343 (β2AR-343), Glu-369 (β2AR-369), or at the very end of the C terminus of the β2AR (β2AR-413). For the β2AR-369 construct, a variant was also constructed with the positions of YFP and CFP inverted. All of the constructs were verified by sequencing and cloned into pcDNA3 for transient expression in eukaryotic cells.

**Cell Culture**—HEK293 cells were cultured in DMEM supplemented with 2 mM glutamine, 10% FCS, 0.1 mg/ml streptomycin, and 100 units/ml penicillin in an atmosphere of 7% CO2 and at 37 °C. CHO cells stably expressing the wild-type β2AR (34) were kept in DMEM/F-12 medium with the same supplements as described for HEK293 cells; the cells were grown in an atmosphere of 5% CO2.

**Stable Transfection of Cells**—For stable expression, HEK293 cells were transfected with plasmid encoding for β2AR-YFP using Effectene as transfection reagent according to the manufacturer’s instructions. Transfected clones were selected with 600 μg/ml of the neomycin analog G-418, single clonal lines were isolated by limiting dilution and were maintained in DMEM supplemented with 200 μg/ml G-418. Expression of the receptor was verified by radioligand binding.

**Transient Expression or Co-expression of G-protein Subunits, β2AR (FRET Sensor) or β-Arrestin2-CFP**—For FRET or confocal experiments, HEK293 (for measuring β-arrestin translocation or conformational changes in the FRET sensor) or CHO cells stably expressing β2AR (G-protein activation experiments) were grown on poly-D-lysine-coated glass coverslips in six-well plates. Transient transfection of HEK293 cells with the β2AR FRET sensors were performed with Lipofectamine 2000 according to the manufacturer’s instructions. Briefly, the transfection mixes were set in Opti-MEM with an optimized 2:1 Lipofectamine 2000:DNA ratio using 0.2 μg of β2AR FRET sensor cDNA/well.

CHO cells were co-transfected with the following amounts of plasmids/well: 2 μg of Gaβγ-YFP, 0.2 μg of Gγ2-CFP, and 0.5 μg of Gβ1 in a mixture of 2.5 μg of polyethylenimine and 100 μl of
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Opti-MEM, whereas for HEK293 cells the amounts were 0.17 μg of β2AR-YFP and 0.15 μg of β-arrestin2-CFP (FRET measurements) or 0.17 μg of β2AR and 0.15 μg of β-arrestin2-YFP (confocal microscopy) per well using Effectene according to the manufacturer’s instructions. These amounts were chosen to give equimolar expression of G-proteins or receptors versus β-arrestin2, respectively, as determined by fluorescence or Western blotting (31).

Radioligand Binding—Membrane preparation and ligand binding of 125I-CYP were performed as described previously (33). Competition binding experiments were performed with 50 pm 125I-CYP and various concentrations of isoproterenol; binding studies were done in the presence of 100 μM GTP to induce a low affinity state for agonists. Nonspecific binding was determined in the presence of 10 μM alprenolol. Binding studies were analyzed with Origin software (OriginLab Corp.) to calculate Kd and Ks values.

Determination of Cell Surface β2ARs with [3H]CGP12177—HEK293 cells stably expressing β2AR-YFP or β2AR were seeded on poly-d-lysine-coated 12-well plates and grown to 80% confluence. The medium was replaced with HBSS (10 mM HEPES, pH 7, 150 mM NaCl, 2.5 mM KCl, 4 mM CaCl2, 10 mM glucose) and incubated for 30 min at room temperature. HBSS was renewed. To initiate receptor internalization, the cells were exposed to agonists (10 μM isoproterenol, 10 μM epinephrine, 300 μM norepinephrine, 10 μM fenoterol, or 150 μM terbutaline; final concentrations) in HBSS (or the same volume of HBSS alone for controls). After incubation for the indicated periods of time at 37 °C, the cells were immediately placed on ice and washed three times with PBS. Surface receptors were detected by incubation with 1 ml of HBSS containing 250 μl (30 nm) [3H]CGP12177 (Amersham Biosciences) at 4 °C for 2 h under gentle shaking. Nonspecific binding was determined with 10 μM alprenolol. The cells were washed three times with ice-cold PBS, lysed by adding 500 μl of 0.5 M NaOH/well, and incubated for 1 h at 37 °C. Bound radioligand was determined by scintillation counting. Radioligand binding was measured in triplicate. [3H]CGP12177 binding, which quantifies cell surface receptors, was plotted over agonist exposure time, and the curves were fitted to a mono-exponential decay using Origin 8.0.

Determination of Adenylyl Cyclase Activity—Membrane preparation and determination of adenylyl cyclase activity were performed as described previously (33). In brief, the membranes were incubated with [α-32P]cAMP for 20 min, and the product [α-32P]cAMP was isolated by chromatography and determined by scintillation counting. Accumulation of [α-32P]cAMP was linear over the entire incubation period under all conditions.

FRET Measurements—FRET experiments were done 48 h after transfection. Fluorescence microscopy was performed with an Axiovert 200 inverted microscope (Zeiss, Jena, Germany) using a 63× oil immersion objective, a dual emission photometric system, and a polychrome IV (both from Till Photonics, Gräfeling, Germany). To minimize photobleaching, the illumination time was set to ≤60 ms with a frequency of 10 Hz (for kinetics 50 Hz). Fluorescence was measured at 535 ± 15 nm (F535) and 480 ± 20 nm (F480) (dichroic long-pass beam splitter, 505 nm). The excitation wavelength was set to 436 ± 10 nm (dichroic long-pass beam splitter, 460 nm; Chroma Technology). Special care was taken to ensure that fluorescence levels and distribution were similar in the examined cells. The emission intensities were corrected for the respective spillover of CFP into the YFP channel and direct YFP excitation to give corrected intensities, FCFP and FFYP. FRET was monitored from the emission ratio of YFP to CFP (FYP/FCFP).

To detect agonist-induced changes in FRET, the cells were continuously superfused with external buffer (137 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 10 mM HEPES, pH 7.3). Agonists were freshly prepared and applied using a rapid superfusion system (ALA Scientific Instruments), permitting solution exchange times of 5–10 ms. Signals detected by avalanche photodiodes were digitized using an analog/digital converter (Digidata 1322A; Axon Instruments, Union City, CA), and fluorescence intensities were acquired using CLAMPEX 8.1 software (Axon Instruments, Foster City, CA). Kinetic data were fitted with a mono-exponential function using Origin 8.0.

Confocal Microscopy—All of the confocal microscopy experiments were performed on a Leica TCP SP2 system. HEK293 cells either stably expressing β2AR-YFP or transiently transfected with β2AR and β-arrestin2-YFP, as described above, were seeded on poly-d-lysine-coated coverslips that were mounted on a custom-built holder. YFP was excited with the 514-nm line of an argon laser, and a dual beam splitter 458/414 nm was used. The images were taken with a 63× objective using the factory settings for YFP. Settings for recording images (laser power, pinhole size, detector gain, amplifier offset, amplifier gain, etc.) were kept constant within and between experiments.

Time series were recorded using the standard Leica software package (version 2.61). After taking a first picture, the images were taken at 30-s intervals. The increase of membrane fluorescence (β-arrestin2 translocation), the loss of membrane fluorescence, and the increase of cytosolic fluorescence (internalization) over time were quantified using the Leica confocal software package (version 2.61) as described by Oakley et al. (35). The resulting fluorescence intensity values were normalized to the initial values and plotted against time to analyze β-arrestin translocation or receptor internalization. The standard error was calculated by error propagation.

Phosphorylation Assays—β2AR Ser-355/356 phosphorylation was measured in whole HEK293 cells seeded on 6-well plates and transiently transfected with 1.2 μg of β2AR cDNA or with a mixture of 1.2 μg of β2AR and 0.8 μg of either GRK2 or GRK5 cDNA. After transfection, the cells were grown in serum-starved medium (0.5% FCS) for 48 h. After 5 min of stimulation with the indicated agonists at 37 °C, the cells were washed once rapidly with ice-cold 20 mM HEPES and 1.0 mM EDTA, pH 7.7, and solubilized by the addition of 250 μl of lysis buffer (20 mM HEPES, pH 7.4, 150 μM NaCl, 0.9% dodecyl-β-maltoside, 20 mM tetrasodium pyrophosphate, 10 mM NaF, 0.1 mM o-acidic acid, 10 μg/ml benzamidine, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin) at 0–4 °C for 30 min. The solubilized cells were transferred to microcentrifuge tubes, sonicated for 10 s, heated at 95 °C for 5 min, and cooled on ice. The solubilized extracts were centrifuged at 14,000 rpm for 5 min at 4 °C. Aliquots of samples were resolved on 12% SDS-PAGE, transferred
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Nitrocellulose membrane, and immunoblotted first with anti-Ser(P)-355/356 antibody at a dilution of 1:500, then stripped, and reprobed with the anti-C terminus antibody 1:2000.

Data Analysis—The values are given as the means ± S.E. of n experiments. Statistical analyses were performed using Statistica version 8. Unless stated otherwise, comparisons between groups were done by analysis of variance followed by Bonferroni’s multiple comparison test.

RESULTS

Expression of the β2AR FRET Sensors in HEK293 Cells—A series of receptor constructs were generated carrying CFP in the third intracellular loop and YFP in various positions of the C terminus as described under “Experimental Procedures.” Similar to analogous constructs of other GPCRs (27, 28, 36, 37), the fluorescent probes were inserted such that the receptor construct would respond to agonist activation with a change in FRET.

Confocal microscopy indicated that 48 h upon transfection into HEK293 cells, the only receptor construct giving clear cell surface expression was the β2AR-369 FRET sensor (data not shown). The fluorescent probes did not affect ligand binding by the receptor, because the radioligand binding experiments with the radioligand 125I-CYP and the agonist isoproterenol were indistinguishable from the wild-type β2AR (Ki values and 95% confidence intervals: 0.34 (0.25–0.45) μM for the FRET sensor and 0.54 (0.38–0.78) μM for the wild-type β2AR). Inverting the positions of CFP and YFP in these constructs changed neither the cell surface expression nor the FRET signals. Therefore, the β2AR-369 FRET sensor (Fig. 1A) was used for further studies.

Receptor Conformational Changes Measured by Intramolecular FRET—FRET signals recorded from single transiently transfected HEK293 cells expressing the β2AR-369 FRET sensor were analyzed under a microscope as described under “Experimental Procedures.” Fig. 1B shows that superfusion with saturating concentrations of epinephrine (10 μM) led to a simultaneous decrease in YFP emission and increase in CFP emission. This resulted in an average decrease of the F_{YFP}/F_{CFP} ratio by ~5%. This loss of FRET indicates the agonist-induced receptor activation, as described previously for various receptor constructs (29). Interestingly, stimulation of the cells with norepinephrine even at high concentrations (300 μM; Fig. 1B) caused only a partial decrease of the F_{YFP}/F_{CFP} ratio compared with epinephrine (~50%). In addition, the effects of norepinephrine were significantly slower (τ = 128 ± 21 ms) than those induced by epinephrine (τ = 47.7 ± 6 ms), even at concentrations causing maximally rapid changes (Fig. 1C). Analogous experiments were performed with saturating concentra-

![FIGURE 1. Ligand-induced conformational changes in a β2AR FRET sensor.](image-url)

- The overall transmembrane topology of the β2AR-369 FRET sensor. CFP was inserted into the third intracellular loop between residues 251 and 252. The C terminus of β2AR was truncated at position 369 and fused to YFP. B–D, effects of different agonists on the β2AR FRET signal. Changes in FRET in response to various agonists were measured at concentrations corresponding to ~10–20-fold their Kᵢ value. B shows the effects of short superfusion with 10 μM epinephrine (E) or 300 μM norepinephrine (NE) recorded in single HEK293 cells expressing the β2AR-369 FRET sensor. The gray curves represent the experimental points, whereas the colored curves represent the five-point sliding averages of the YFP (yellow) and CFP (blue) traces and of the corrected F_{YFP}/F_{CFP} ratio (red). C, maximal rate constants of the signals of epinephrine and norepinephrine recorded as in B but with 1 μM norepinephrine and 0.1 mM epinephrine. The experimental data were fitted to a mono-exponential time course. The data are the means ± S.E., n = 6–9, *p < 0.05; D, amplitudes of agonist-induced intramolecular FRET signals derived from experiments as in B. FRET changes in cells expressing the β2AR-369 FRET sensor after agonist application (10 μM isoproterenol (Iso), 300 μM norepinephrine (NE), 10 μM epinephrine (E), 10 μM fenoterol (Feno), and 150 μM terbutaline (Terb)). The changes were calculated as percentages of the change induced by isoproterenol (10 μM), which was assayed in each individual experiment as a reference. The data are the means ± S.E., n = 3–8, **p < 0.01 versus isoproterenol; ##, p < 0.01 terbutaline versus norepinephrine and fenoterol.
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tions of isoproterenol (10 μM), fenoterol (10 μM), and terbutaline (150 μM). Fig. 1D shows the maximal signals, normalized to the effects of the “standard” β₂AR agonist isoproterenol. Whereas epinephrine caused essentially the same FRET response as isoproterenol (106 ± 5%), responses to norepinephrine and fenoterol were approximately half as big (58 ± 2.6 and 49 ± 7.3%), and the responses to terbutaline were again significantly smaller (21 ± 0.5%). Similar results were obtained when the concentrations of these apparently partial agonists were increased even further, indicating that saturation of the effects had been achieved (norepinephrine, 1 mM; fenoterol, 100 μM; and terbutaline, 1 mM) (data not shown).

Determination of Gₛ Activation—G-protein activation by GPCRs in intact cells can also be investigated with FRET-based approaches, either by studying the interaction between receptors and G-proteins (31, 38) or by measuring changes between the G-protein subunits (25, 30, 39, 40). Because it produces a more robust signal and also because it reflects G-protein activation, we chose the latter technique to compare the effects of different ligands on Gₛ activation by β₂ARs in intact cells. To this end, we co-transfected YFP-tagged Gαₛ together with CFP-tagged Gγ₂, and Gβ₁ in CHO cells stably expressing β₂AR. CHO cells were used in these assays because they produced more robust signals than HEK293 cells (not shown). Fig. 2A shows ligand-induced changes in FRET between the YFP-tagged Gαₛ and the CFP-tagged Gγ₂. These signals amount to up to 15% of the initial Fₚ/YFP/Fₚ/CFP ratio. Compared with the speed of effects measured with the β₂AR-369 FRET sensor, these effects were considerably slower, in line with earlier findings showing that G-protein activation and deactivation are much slower than receptor activation and deactivation (31, 38).

Similar experiments were performed with the same agonists as above, and again their maximum results were normalized to the effects observed with isoproterenol (Fig. 2B). In these experiments, there were no appreciable differences between isoproterenol, epinephrine, and norepinephrine. Responses to fenoterol, however, were reduced by 20–25% compared with isoproterenol, and very little Gₛ stimulation was observed in response to terbutaline.

Adenylyl Cyclase Activation—As a second read-out of Gₛ activation, adenylyl cyclase activation by various ligands was determined in membranes of CHO cells stably expressing β₂AR. Again, the effect obtained with isoproterenol (10 μM) was set to 100%. As shown previously (33), epinephrine (10 μM) as well as norepinephrine (300 μM) caused a similar stimulation of adenylyl cyclase activity, whereas 10 μM fenoterol led to only 76% of the isoproterenol response, and terbutaline (150 μM) showed only 41% of the isoproterenol response. Similar results were obtained using HEK293 cells (supplemental Fig. S1). Generally, the extent of adenylyl cyclase stimulation by the tested ligands correlated well with the extent of Gₛ activation as determined by FRET (Fig. 2C). Notably, in contrast to what was observed for the receptor conformational change, not only epinephrine but also norepinephrine produced full responses in these systems.

β₂AR GRK Site Phosphorylation—To explore whether different agonists may induce different GRK-mediated receptor phosphorylation, we determined the phosphorylation levels in whole HEK293 cells using anti-Ser(P)-355/356-specific antibodies and normalized the agonist-induced responses to those observed with isoproterenol. Fig. 3A shows that in our cell system, endogenously expressed GRKs are sufficient for agonist-induced β₂AR phosphorylation and give rise to similar receptor phosphorylation levels independently of the agonist applied.
The observation that Western blots with anti-GRK-specific antibodies produced a stronger signal for GRK5 than for GRK2 (not shown).

β-Arrestin Translocation—Agonist stimulation of many GPCRs leads to translocation of β-arrestins from the cytosol to the cell surface, and this can be used to monitor activation of the β-arrestin pathway (5, 41). Fig. 4A shows this process in response to several agonists using β-arrestin2-YFP in β2AR-expressing HEK293 cells, visualized by confocal microscopy. Norepinephrine appeared to differ from the other compounds, because only modest β-arrestin2 movement to the cell membrane was visible.

For a quantitative analysis of these experiments, the increase of fluorescence in defined membrane areas was determined over time, and the results were expressed as the percentages of the effect observed with 10 μM isoproterenol (Fig. 4B). These data revealed almost full agonist activity for epinephrine and fenoterol, whereas the effects of terbutaline (~60%) and norepinephrine (~50%) were clearly partial, with norepinephrine being the least efficacious compound in triggering β-arrestin2 translocation.

Because the confocal microscopy technique permits only a semi-quantitative analysis of β-arrestin2 translocation, to investigate β-arrestin2 translocation with a second method, we measured FRET between CFP-labeled β-arrestin2 (donor) and YFP-labeled β2ARs (acceptor). The two constructs were expressed in HEK293 cells, and agonist-induced FRET was measured for the same compounds as before. In these experiments, agonist stimulation causes an approximation of the two labels in the receptor and in β-arrestin2 and hence an increase in FRET (5). Fig. 5A shows the change of FRET ratio in response to three successive stimuli with norepinephrine. Although the amplitudes of the increases in FRET were the same for all three stimuli, the first pulse caused a clearly slower change than the subsequent pulses. We have shown earlier that the kinetics of the first pulse reflect GRK-dependent receptor phosphorylation, whereas those of the subsequent stimuli reflect β-arrestin interaction with already prephosphorylated receptors. The amplitudes of the FRET changes were the same for the first and subsequent stimuli and are depicted in Fig. 5B, again relative to isoproterenol (100%). Overall, the results were similar to those determined in the confocal microscopy β-arrestin2 translocation experiments. Epinephrine behaved as a full agonist, whereas all other compounds caused only partial effects.

A correlation of the results obtained by the two methods used to measure β-arrestin2 translocation is shown in Fig. 5C. Although there was a clear positive correlation between the two methods, this correlation was not perfect; the endogenous ligands epinephrine and norepinephrine caused a relatively larger signal in the FRET assay, whereas the synthetic partial agonists fenoterol and terbutaline elicited relatively larger effects in the confocal microscopy experiments.

The kinetics of the FRET signals of the first stimulus, reflecting GRK-dependent phosphorylation, were essentially the same for all of the agonists tested (Fig. 5D). However, there were ∼2-fold differences for the second stimulus (following a 20-s prestimulus with isoproterenol in all cases; Fig. 5E). Isoproterenol...
enol caused the fastest signals, closely followed by epinephrine and fenoterol, whereas the norepinephrine-induced signals were significantly slower ($p < 0.01$). Terbutaline-induced signals were too small to be kinetically analyzed.

Taken together, these data suggest that the agonist-dependent interaction of prephosphorylated $\beta_2$ARs with $\beta$-arrestins occurs with agonist-dependent kinetics. These kinetics were no different, when a different agonist was used in the first pulse to trigger $\beta_2$AR prephosphorylation (data not shown).

$\beta_2$AR Internalization—Internalization of GPCRs is often dependent on $\beta$-arrestin binding, but $\beta$-arrestin-independent internalization mechanisms have also been reported. Therefore, we determined internalization of $\beta_2$AR as an additional read-out of agonist activation.

To explore this process, we measured $\beta_2$AR internalization as the loss of cell surface receptors after agonist exposure, determined by binding of the hydrophilic radioligand $[^3H]$CGP12177. Fig. 6A shows the decrease of cell surface binding over time after exposure to the usual concentrations of the various agonists. All of the agonists caused a loss of cell surface receptors that occurred with an exponential time course and was essentially complete after 20 min, with a total loss ranging between 20 and 50%. These results were the same for $\beta_2$AR-YFP (Fig. 6A) and unmodified $\beta_2$AR (not shown). The extent of receptor internalization was similar for epinephrine, isoproterenol, and fenoterol (but slower for the latter compound), whereas we observed more modest internalization for norepinephrine and terbutaline (Fig. 6B).

The rates of receptor internalization also appeared to be ligand-dependent (Fig. 6C). Epinephrine and isoproterenol led to a significantly faster receptor internalization compared with the other compounds. Interestingly, fenoterol showed the slowest receptor internalization, even though it eventually caused the same extent of receptor internalization as did isoproterenol. These data indicate that both the extent and the rate of receptor internalization may be agonist-specific.

To confirm these data on $\beta_2$AR internalization with a second assay, we investigated internalization of $\beta_2$AR-YFP stably expressed in HEK293 cells by confocal microscopy. Fig. 6D shows images before and 20 min after exposure to the various agonists. Again, it is clearly visible that full internalization was caused by isoproterenol, epinephrine, and fenoterol, whereas norepinephrine caused only modest and terbutaline very little internalization. These results agree well with those obtained by radioligand binding.

A semi-quantitative analysis of these confocal images as described under “Experimental Procedures” generally gave lower values of internalization compared with those determined with $[^3H]$CGP12177 binding. The most likely explanation is that the confocal microscopy approach is less sensitive...
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than the radioligand-based assay, because it cannot differentiate between cell surface receptors and those that are no longer accessible to hydrophilic ligands but close to the cell membrane. However, we observed the same order of ligand efficacy for β2AR internalization with both the approaches: terbutaline < norepinephrine ≈ fenoterol ≈ epinephrine ≈ isoproterenol (data not shown).

**DISCUSSION**

Several recent studies suggest that GPCR activation is a multistep process and that it may occur via multiple active conformations of the receptors (19, 20, 24). It is very plausible that such distinct conformations underlie the phenomenon of biased signaling, i.e. the ability of agonists to induce different signaling pathways with different efficacies (24). Although evidence for distinct conformations and biased agonism has recently been described for synthetic agonists of several receptors, including the β2AR (16, 17), it remained to be shown whether these phenomena also exist for endogenous agonists.

Our studies revealed a number of interesting observations. First, experiments with a β2AR FRET sensor revealed signals of different amplitudes and kinetics for the two endogenous agonists; in particular, norepinephrine produced only partial signals, which were slower than those caused by epinephrine. Second, Gs and adenylyl cyclase activation by norepinephrine were comparable with those produced by epinephrine and isoproterenol, but this was not the case for terbutaline, another compound producing only partial signals of the β2AR FRET sensor. Third, although there was in general a good correlation between various parameters for β-arrestin2 recruitment and β2AR internalization, there were some exceptions. In particular, confocal microscopy suggested a stronger translocation of β-arrestin2 to the cell surface induced by the partial agonists fenoterol and terbutaline than was evident from β-arrestin/β2AR interaction, and fenoterol appeared to induce a distinct pattern of β2AR internalization, which was slow but of large amplitude.

The β2AR FRET sensor experiments clearly indicate that norepinephrine produced only a partial signal when compared with epinephrine and isoproterenol. Although FRET changes in such sensors do not need to be linearly correlated with the extent of conformational change (25, 26), this suggests that norepinephrine does not induce the same conformational change as epinephrine and isoproterenol. We have observed earlier that partial agonists caused slower FRET signals than full agonists at the α2A-adrenergic receptor (25). In line with these observations, norepinephrine caused slower changes at the β2AR FRET sensor compared with epinephrine.

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**FIGURE 5.** Kinetics of agonist-induced β-arrestin2 β2AR interaction in HEK293 cells. HEK293 cells transiently expressing β2AR-YFP and β-arrestin2-CFP were stimulated with agonists, and the interaction of the labeled proteins was determined by FRET. A, representative FRET experiment showing the normalized FRET ratio (F_{FTP}/F_{CFP}) and repeated stimulation with 300 μM norepinephrine (horizontal bars) in a single HEK293 cell. B, similar experiments were performed for all other agonists at the concentrations used before and normalized to isoproterenol (10 μM). **, p < 0.01 versus isoproterenol; #, p < 0.05 terbutaline versus norepinephrine and fenoterol. C, comparison of β-arrestin2-YFP membrane translocation measured by confocal microscopy (see Fig. 4B) and β-arrestin2/β2AR interactions detected by FRET (see Fig. 5B). Each point represents the mean ± S.E. of more than three experiments. D, kinetics of the agonist-induced β-arrestin2/β2AR interaction. Shown are the time constants of the FRET signals for the first stimulation with the indicated ligands. FRET experiments were performed as described before, and time constants τ (s) were calculated. The data represent the means ± S.E. of more than five experiments. E, kinetics of the agonist-induced β-arrestin2/β2AR interaction. Shown are the time constants τ (s) of the FRET signals for the second stimulation with the indicated ligands, following prestimulation for 20 s with 10 μM isoproterenol, followed by washing. The data represent the means and S.E. of more than five experiments. **, p < 0.01 norepinephrine versus isoproterenol; #, p < 0.05 norepinephrine versus epinephrine; *, p < 0.05 epinephrine versus isoproterenol. Iso, isoproterenol; E, epinephrine; NE, norepinephrine; Feno, fenoterol; Terb, terbutaline.
The modes of binding of epinephrine and norepinephrine to the β₂AR are presumably quite similar and involve the four polar groups: NH₃⁺, β-OH, and the two catechol-OH (42). It has been suggested (43) that interaction of these four groups with the receptor produces full activation.

Full activation in these and similar studies was based on apparently maximal adenylyl cyclase stimulation (43, 44). However, this is not compatible with our apparently maximal adenylyl cyclase stimulation (43, 44). How-with the receptor produces full activation. Interaction of these four groups polar groups: NH₃⁺/H9252,H2O/H9252, β-OH, and the two catechol-OH (42). It has been suggested (43) that interaction of these four groups with the receptor produces full activation.

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B and D). These data reflect a reduced ability of norepinephrine to promote an interaction of β₂AR with β-arrestins and are in agreement with the reduced ability of norepinephrine in triggering the GRK5-dependent β₂AR phosphorylation, which has been suggested to be involved in β-arrestin-dependent pathways independent of Gₛ coupling (49). Thus, this ligand appears to be rather Gₛ-biased when compared with the other compounds. Such a Gₛ bias is also compatible with the observation that norepinephrine is able to recruit a β₂AR reserve at very low expression levels (33).

The determination of receptor internalization confirms the contention that norepinephrine is less efficient in triggering β-arrestin-dependent processes; it induced less β₂AR internalization in both types of assays, and in addition, internalization was slower than with the other agonists, including terbutaline, which again produced only very partial effects. Fenoterol, which has been suggested to be β-arrestin-biased, induced internalization that was also slow but eventually reached the same extent as that seen with epinephrine and isoproterenol.

Taken together, our data indicate that different agonist-induced conformations of the β₂AR are revealed with a receptor FRET sensor and that norepinephrine clearly behaves as a partial agonist in this respect. However, the norepinephrine-activated receptor is quite efficient in recruiting a receptor reserve and the receptor FRET sensor and that norepinephrine clearly behaves as a par-

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REFERENCES

1. Kenakin, T. (2005) Nat. Rev. Drug Discov. 4, 919–927
2. Urban, J. D., Clarke, W. P., von Zastrow, M., Nichols, D. E., Kobilka, B., Weinstein, H., Javitch, J. A., Roth, B. L., Christophoulos, A., Sexton, P. M., Miller, K. J., Spedding, M., and Mailman, R. B. (2007) J. Pharmacol. Exp. Ther. 320, 1–13
3. Benovic, J. L., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 2797–2801
4. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) Science 248, 1547–1550
5. Krasel, C., Bünemann, M., Lorenz, K., and Lohse, M. J. (2005) J. Biol. Chem. 280, 9528–9535
6. Ferguson, S. S., Downey, W. E., 3rd, Colapietro, A. M., Barak, L. S., Ménard, L., and Caron, M. G. (1996) Science 271, 363–366
7. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 383, 447–450
8. Benovic, J. L., Staniszewski, C., Mayor, F., Jr., Caron, M. G., and Lefkowitz, R. J. (1988) J. Biol. Chem. 263, 3893–3897
9. Violin, J. D., and Lefkowitz, R. J. (2007) Trends Pharmacol. Sci. 28, 416–422
10. Swaminath, G., Yang, Y., Lee, T. W., Steenhuis, J., Parnot, C., and Kobilka, B. K. (2004) J. Biol. Chem. 279, 686–691
11. Whistler, J. L., Chuan, H. H., Chu, P., Jan, L. Y., and von Zastrow, M. (1999) Neuron 23, 737–746
12. Kohout, T. A., Nicholas, S. L., Perry, S. J., Reinhart, G., Junger, S., and Struthers, R. S. (2004) J. Biol. Chem. 279, 23214–23222
13. Lewis, M. M., Watts, V. J., Lawler, C. P., Nichols, D. E., and Mailman, R. B. (1998) J. Pharmacol. Exp. Ther. 286, 345–353
14. Urban, J. D., Vargas, G. A., von Zastrow, M., and Mailman, R. B. (2007) Neuropharmacology 32, 67–77
15. January, B., Seibold, A., Whaley, B., Hipkin, R. W., Lin, D., Schonbrunn, A., Barber, R., and Clark, R. B. (1997) J. Biol. Chem. 272, 23871–23879
16. Galandrin, S., and Bouvier, M. (2006) Mol. Pharmacol. 70, 1575–1584
17. Drake, M. T., Violin, J. D., Whalen, E. J., Wisler, J. W., Shenoy, S. K., and Lefkowitz, R. J. (2008) J. Biol. Chem. 283, 5669–5676
18. Wang, Y., DuArcangelis, V., Gao, X., Ramani, B., Jung, Y. S., and Xiang, Y. (2008) J. Biol. Chem. 283, 1799–1807
19. Kobilka, B. K., and Deupi, X. (2007) Trends Pharmacol. Sci. 28, 397–406
20. Hoffmann, C., Zürn, A., Bünemann, M., and Lohse, M. J. (2008) Br. J. Pharmacol. 153, Suppl. 1, S358–S366
21. Arzi, M., Charest, P. G., Angers, S., Rousseau, G., Kohout, T., Bouvier, M., and Pinheiro, G. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 11406–11411
22. Blanpain, C., Vanderwende, J. M., Cihak, J., Wittamer, V., Le Poul, E., Issafra, H., Stangassinger, M., Vassart, G., Marullo, S., Schlöndorff, D., Parmentier, M., and Mack, M. (2002) Mol. Biol. Cell 13, 723–737
23. Whistler, J. L., and von Zastrow, M. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 9914–9919
24. Swaminath, G., Deupi, X., Lee, T. W., Zhu, W., Thian, F. S., Kobilka, T. S., and Kobilka, B. (2005) J. Biol. Chem. 280, 22165–22171
25. Nikolaev, V. O., Hoffmann, C., Bünemann, M., Lohse, M. J., and Vilaradaga, J. P. (2006) J. Biol. Chem. 281, 24506–24511
26. Lohse, M. J., Nikolaev, V. O., Hein, P., Hoffmann, C., Vilaradaga, J. P., and Bünemann, M. (2008) Trends Pharmacol. Sci. 29, 159–165
27. Vilaradaga, J. P., Bünemann, M., Krasel, C., Castro, M., and Lohse, M. J. (2003) Nat. Biotechnol. 21, 807–812
28. Hoffmann, C., Gaietta, G., Bünemann, M., Adams, S. R., Oberdorff-Maass, S., Behr, B., Vilaradaga, J. P., Tsien, R. Y., Ellisman, M. H., and Lohse, M. J. (2005) Nat. Methods 2, 171–176
29. Lohse, M. J., Bünemann, M., Hoffmann, C., Vilaradaga, J. P., and Nikolaev, V. O. (2007) Curr. Opin. Pharmacol. 7, 547–553
30. Bünemann, M., Frank, M., and Lohse, M. J. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 16077–16082
31. Hein, P., Rochais, F., Hoffmann, C., Dorsch, S., Nikolaev, V. O., Engelhardt, S., Berlot, C. H., Lohse, M. J., and Bünemann, M. (2006) J. Biol. Chem. 281, 33345–33351
32. Nikolaev, V. O., Bünemann, M., Hein, L., Hannawacker, A., and Lohse, M. J. (2004) J. Biol. Chem. 279, 37215–37218
33. Hoffmann, C., Leitz, M. R., Oberdorff-Maass, S., Lohse, M. J., and Klotz, K. N. (2004) Naunyn Schmiedebers Arch. Pharmacol. 369, 151–159
34. Lohse, M. J. (1992) Naunyn Schmiedebers Arch. Pharmacol. 345, 444–451
35. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (2001) J. Biol. Chem. 276, 19452–19460
36. Rochais, F., Vilaradaga, J. P., Nikolaev, V. O., Bünemann, M., Lohse, M. J., and Engelhardt, S. (2007) J. Clin. Invest. 117, 229–235
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37. Chachisvilis, M., Zhang, Y. L., and Frangos, J. A. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 15463–15468
38. Hein, P., Frank, M., Hoffmann, C., Lohse, M. J., and Bünemann, M. (2005) EMBO J. 24, 4106–4114
39. Janetopoulos, C., Jin, T., and Devreotes, P. (2001) Science 291, 2408–2411
40. Nikolaev, V. O., Boettcher, C., Dees, C., Bünemann, M., Lohse, M. J., and Zenk, M. H. (2007) J. Biol. Chem. 282, 27126–27132
41. Barak, L. S., Ferguson, S. S., Zhang, J., and Caron, M. G. (1997) J. Biol. Chem. 272, 27497–27500
42. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. (1994) Annu. Rev. Biochem. 63, 101–132
43. Liapakis, G., Chan, W. C., Papadokostaki, M., and Javitch, J. A. (2004) Mol. Pharmacol. 65, 1181–1190
44. Wieland, K., Zuurmond, H. M., Krasel, C., Ijzerman, A. P., and Lohse, M. J. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 9276–9281
45. Levitzki, A., Marbach, I., and Bar-Sinai, A. (1993) Life Sci. 52, 2093–2100
46. Galés, C., Rebois, R. V., Hogue, M., Trieu, P., Breit, A., Hébert, T. E., and Bouvier, M. (2005) Nat. Methods 2, 177–184
47. Hoffmann, C., Ziegler, N., Reiner, S., Krasel, C., and Lohse, M. J. (2008) J. Biol. Chem. 283, 30933–30941
48. Reiner, S., Ziegler, N., Leon, C., Lorenz, K., von Hayn, K., Gachet, C., Lohse, M. J., and Hoffmann, C. (2009) Mol. Pharmacol. 76, 1162–1171
49. Shenoy, S. K., Drake, M. T., Nelson, C. D., Houtz, D. A., Xiao, K., Madabushi, S., Reiter, E., Premont, R. T., Lichtarge, O., and Lefkowitz, R. J. (2006) J. Biol. Chem. 281, 1261–1273