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Fluorescence Resonance Energy Transfer Analysis of α_{2a}-Adrenergic Receptor Activation Reveals Distinct Agonist-Specific Conformational Changes

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ABSTRACT

Several lines of evidence suggest that G-protein-coupled receptors can adopt different active conformations, but their direct demonstration in intact cells is still missing. Using a fluorescence resonance energy transfer (FRET)-based approach we studied conformational changes in α_{2A}-adrenergic receptors in intact cells. The receptors were C-terminally labeled with cyan fluorescent protein and with fluorescein arsenical hairpin binder at different sites in the third intracellular loop: N-terminally close to transmembrane domain V (I3-N), in the middle of the loop (I3-M), or C-terminally close to transmembrane domain VI (I3-C). All constructs retained normal ligand binding and signaling properties. Changes in FRET between the labels were determined in intact cells in response to different agonists. The full agonist norepinephrine evoked similar FRET changes for all three constructs. The strong partial agonists clonidine and dopamine induced partial FRET changes for all constructs. However, the weak partial agonists octopamine and norphenephrine only induced detectable changes in the construct I3-C but no change in I3-M and I3-N. Dopamine-induced FRET-signals were ≈1.5-fold slower than those for norepinephrine in I3-C and I3-M but >3-fold slower in I3-N. Our data indicate that the different ligands induced conformational changes in the receptor that were sensed differently in different positions of the third intracellular loop. This agrees with X-ray receptor structures indicating larger agonist-induced movements at the cytoplasmic ends of transmembrane domain VI than V and suggests that partial agonism is linked to distinct conformational changes within a G-protein-coupled receptor.

Stimulation of G-protein-coupled receptors (GPCRs) by an agonist leads to a conformational change and to a transition of the receptor into an active conformation, which can then couple to its G-protein. Conformational changes have been well established to occur within the transmembrane domains (TMs) III and VI (Gether, 2000; Hubbell et al., 2003). These changes are believed to be transmitted into the third intracellular loop. This loop seems to contain the key domains for coupling to G-proteins, particularly in its C terminus (adjacent to TMVI) but also in its N terminus (adjacent to TMV) regions (Wess, 1998).

Whereas classic theory assumed that receptors simply switch between "off" and "on" states, more recent data indicate that agonists of different efficacy might induce different changes in receptor conformations (Kobilka and Deupi, 2007). To accommodate the growing body of evidence for multiple conformational states into theoretical considerations, different models have been proposed. These models propose either that each agonist might promote its own specific active receptor conformation, thus leading to an almost unlimited number of receptor conformations R_n^*, or suggest that there might be a limited number of active conformations into which different agonists might switch a receptor (Kenakin, 1995). The accumulating evidence for multiple signaling states of GPCRs has been reviewed recently (Perez and Karnik, 2005; Kenakin, 2007; Kobilka and Deupi, 2007; Hoffmann et al., 2008).

With respect to ligand-induced conformational changes, the GPCR that has been best investigated in vitro so far is the purified, fluorescently labeled and reconstituted β_2-adrenergic receptor. In this system, it seems that partial and

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; CFP, cyan fluorescent protein; CL, clonidine; DA, dopamine; EDT, 1,2-ethane dithiol; FlAsH, 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein; FRET, fluorescence resonance energy transfer; HBSS, Hanks' balanced salt solution; NE, norepinephrine; NF, norphenephrine; OC, octopamine; TM, transmembrane domain; [^{3}H]RX821002, 3H-2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline; HEK, human embryonic kidney; PDB, Protein Data Bank; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate.

full agonists lead to distinct active conformations and that partial agonists are able to induce only the first steps of a sequential series of conformational changes, whereas full agonists promote further changes, resulting ultimately in an active conformation that is capable of interacting with all downstream proteins (Swaminath et al., 2004; Yao et al., 2006; Kobilka and Deupi, 2007). This situation is reminiscent of the multiple states that the "light receptor" rhodopsin adopts when its covalently bound ligand retinal is isomerized by light; in this case, a series of conformational changes, visible by changes in the absorption properties of the protein and occurring over approximately 1 ms, ultimately leads to the formation of the fully active metarhodopsin II state (Arshavsky et al., 2002; Hubbell et al., 2003). However, in contrast to rhodopsin, it has not been shown that distinct conformations exist for other G-protein-coupled receptors in intact cells, and the link between the activity of a ligand and a distinct receptor conformation is not clear.

Structural studies of G-protein-coupled receptors have made very significant progress recently (Kobilka and Schertler, 2008). This is because not only structures of inactive forms of rhodopsin and the β_2-adrenergic receptor but also of the partially active opsin structure have been obtained (Palczewski et al., 2000; Li et al., 2004; Rasmussen et al., 2007; Rosenbaum et al., 2007; Park et al., 2008). These studies provide a framework to interpret kinetic experiments on receptor activation that can be carried out in vitro or in living cells.

To study the kinetics and characteristics of receptor activation in living cells, we have developed recently techniques based on fluorescence resonance energy transfer (FRET) to monitor conformational changes in G-protein-coupled receptors. In its initial form, this technique measures FRET between the cyan (CFP) and the yellow variant of the green fluorescent protein and records the changes that result from the addition of ligands (Vilardaga et al., 2003, 2005). To work with smaller labels that can be positioned more accurately, we later used the much smaller fluorescein arsenical hairpin binder (FlAsH) as an alternative label to yellow fluorescent protein (Hoffmann et al., 2005; Nikolaev et al., 2006). FlAsH binds with relatively high specificity to specific tetracysteine motifs that may be as small as six amino acids (Martin et al., 2005).

The small size of these motifs and the rather large third intracellular loop of the α_{2A}-adrenergic receptor (158 amino acids) permit a flexible positioning of the label at different sites within the loop. This led us to explore the possibility that this region of the receptor might change its conformation differently in response to different ligands and, thus, document the existence of different active states of a G-protein-coupled receptor in living cells.

Materials and Methods

Materials. The ligands norepinephrine (NE), dopamine (DA), octopamine (OC), and clonidine (CL) were all obtained from Sigma (St. Louis, MO). Norphenephrine (NF) was obtained from Kraemer& Martin Pharma Handels GmbH (Krefeld, Germany). FlAsH is commercially available from Invitrogen (Carlsbad, CA) as TC-FlAsH. All other chemicals were from standard sources and of the highest purity available.

Molecular Biology. Site-directed mutagenesis was performed on the mouse α_{2A}-adrenergic receptor. The cDNA encoding the enhanced CFP was fused to position Arg449 of the C terminus of the receptor. In addition, the motif FLNCCPGCCMEP was substituted for the sequence from Ala246 to Arg257 (TMV), Ser297 to Arg308 (middle), or Gly350 to Arg361 (TMVI) in the third intracellular loop of the receptor. Constructions were performed using standard polymerase chain reaction mutagenesis techniques and were verified by sequencing. Receptor cDNAs were cloned into pcDNA3 (Invitrogen) for transient expression in HEK-293 cells.

Cell Culture. HEK-293 cells were transfected using Effectene (QIAGEN, Hilden, Germany). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100,000 U/liter penicillin and 100 mg/liter streptomycin at 37°C in 7% CO_2. For fluorescence measurements, cells were seeded on round polylysine-coated coverslips that were placed in six-well plates and transfected 6 h later. Cells were kept in culture for an additional 48 h.

FlAsH-labeling. The labeling was done as described previously (Hoffmann et al., 2005). Transfected cells grown on coverslips were washed twice with Phenol Red-free Hanks' balanced salt solution containing 1 g/liter glucose (HBSS; Invitrogen) and then incubated at 37°C for 1 h with HBSS with 500 nM FlAsH and 12.5 μM 1,2-ethane dithiol (EDT). After FlAsH-EDT incubation, to reduce nonspecific labeling, cells were washed twice with HBSS, incubated for 10 min with HBSS/250 μM EDT, and again washed twice with HBSS before being used for fluorescence measurements.

Fluorescence Measurements. Fluorescence microscopy was performed as described previously (Vilardaga et al., 2003, 2005; Hoffmann et al., 2005; Nikolaev et al., 2006). In brief, cells labeled as described above were washed with HBSS and maintained in buffer A (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.3) at room temperature. Coverslips were mounted on an Attofluor holder (Invitrogen) and placed on a Zeiss inverted microscope (Axiovert 135; Carl Zeiss Inc., Thornwood, NY) equipped with an oil immersion 100× objective and a dual-emission photometric system (Till Photonics, Gräfelfing, Germany). Samples were excited with light from a polychrome IV (Till Photonics). To minimize photobleaching, the illumination time was set to 10 ms, applied with a frequency between 10 and 50 Hz, depending on agonist concentration. The fluorescence signal was recorded from the whole cell. FRET was monitored as the emission ratio of FlAsH to CFP, F_{535}/F_{480}, where F_{535} and F_{480} are the emission intensities at 535 ± 15 and 480 ± 20 nm (beam splitter DCLP 505 nm) upon excitation at 436 ± 10 nm (beam splitter DCLP 460 nm). The emission ratio was corrected by the respective spillover of CFP into the 535-nm channel (spillover of FlAsH into the 480-nm channel was negligible) to give a corrected ratio F^*_{535}/F^*_{480}. The FlAsH emission upon excitation at 480 nm was recorded at the beginning of each experiment to subtract direct excitation of FlAsH (FlAsH emission at 436 nm excitation/FlAsH emission at 480 nm excitation was 0.06).

To determine agonist-induced changes in FRET, cells were continuously superfused with buffer A, and agonists were applied using a computer-assisted solenoid valve-controlled rapid superfusion device ALA-VM8 (ALA Scientific Instruments, Westbury, NY; solution exchange, 5–10 ms). Signals detected by avalanche photodiodes were digitized using an AD converter (Digidata 1322A; Molecular Devices, Sunnyvale, CA) and stored on a personal computer using Clampex 8.1 software (Molecular Devices). The agonist-induced decrease in FRET ratio was fitted to the equation: $A(t) = A \times e^{-t/\tau}$, where τ is the time constant(s), and A is the magnitude of the signal. When necessary for calculating τ, agonist-independent changes in FRET due to photobleaching were subtracted.

Ligand Binding. Membrane preparation and ligand binding were performed as described previously (Bünemann et al., 2001). For saturation binding, cell membranes were incubated for 90 min with 1 to 110 nM [^{3}H]RX821002 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) in binding buffer (5 mM Tris-HCl and 2 mM EDTA, pH 7.4). Competition binding was performed with 4 nM [^{3}H]-RX821002 and increasing concentrations of each agonist. Nonspecific binding was determined in the presence of 1 μM atipamezole

(Thermo Fisher Scientific, Waltham, MA). Saturation and competition binding studies were analyzed with Origin software (OriginLab Corp., Northampton, MA) to calculate K_D and K_i values.

[^{35}S]GTPγS Binding. [^{35}S]GTPγS binding was measured essentially as described previously (Lohse et al., 1992). In brief, 10 μg of membrane protein in 200 μl of binding buffer (20 mM HEPES, 10 mM $MgCl_2$, and 100 mM NaCl, pH 7.4) containing 3 mM guanosine diphosphate and 100 pM [^{35}S]GTPγS (PerkinElmer Life and Analytical Sciences Waltham, MA) were preincubated on ice with or without the indicated agonists. After different times of incubation ranging from 15 s to 10 min at 20°C, the incubation was stopped by filtering the samples through GF/F membrane filters (Whatman, Clifton, NJ) and three washes with ice-cold binding buffer, and bound radioactivity was quantified by liquid scintillation counting.

Rhodopsin Structures. The structures of rhodopsin (Li et al., 2004; PDB entry 1GZM) and opsin (Park et al., 2008; PDB entry 3CAP) were overlaid by least-squares fitting using amino acids 1 to 68, 73 to 221, 262 to 305, and 309 to 322 for superposition giving a root mean square deviation of 1.42 Å and were rendered using the program LSQKAB CCP4 "Program for protein crystallography" (http://www.ccp4.ac.uk/html/lsqkab.html).

Results

To monitor the movements of the α_{2A}-adrenergic receptor during agonist-induced activation, we used a FRET approach using FlAsH/tetra-cysteine tags in the third intracellular loop in combination with CFP at the receptor's C terminus. Figure 1 schematically illustrates the three receptor constructs generated for this study. In each case, CFP was fused to the very C terminus of the α_{2A}-adrenergic receptor, and the tetracysteine motif replaced a stretch of identical length at different positions in the third intracellular loop of the receptor. The three resulting constructs were named according to the position of the tetracysteine motif in the loop. The N-terminal position was termed "I3-N," the position in the center of the loop "I3-M," and the C-terminal position "I3-C."

Upon transfection into HEK-293 cells, all receptor constructs were expressed at the cell surface (data not shown). Radioligand binding experiments in cell membranes (in the presence of GTP to uncouple the receptors from G-proteins and thereby induce a low-affinity state) showed that all three constructs were virtually indistinguishable from wild-type α_{2A}-adrenergic receptors regarding saturation binding with the antagonist [^{3}H]RX821002 and competition with the various full and partial agonists used in this study (Table 1). The obtained K_D and K_i values were similar to published data (Peltonen et al., 2003; Nikolaev et al., 2006). These data indicate that the tetracysteine motifs did not affect ligand binding by the receptors. We have made similar observations for other receptors labeled with FlAsH and CFP, including the adenosine A_{2A}-receptor (Hoffmann et al., 2005), α_{2A}-adrenergic receptor (Nikolaev et al., 2006), and β_1-adrenergic receptor (Rochais et al., 2007).

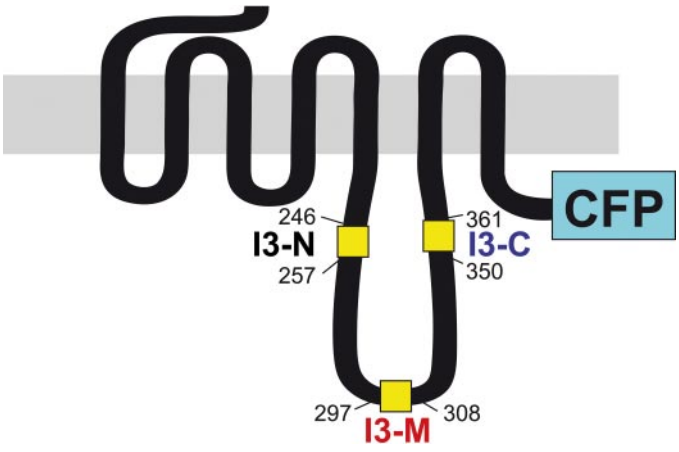

Fig. 1. FRET constructs of the α_{2A}-adrenergic receptor. Schematic representation of the constructs. For all constructs, the donor fluorophore CFP was positioned at the very C terminus of the amino acid sequence. The positions of the different FlAsH-binding sites in the third intracellular loop are marked in yellow. The numbers denote the amino acid segments that were replaced by the binding motif "FLNCCPGMEP." Positions 246 to 257 represent construct I3-N, positions 297–308 the I3 to M construct, and positions 350 to 361 the I3-C construct.

To study whether the receptor constructs were functional with respect to G-protein coupling, we determined [^{35}S]GTPγS binding in cell membranes as a measure for G-protein activation. Figure 2 shows that all constructs induced [^{35}S]GTPγS binding well above the level of nontransfected HEK cells. These assays were done with the full agonist norepinephrine, the strong partial agonists dopamine and clonidine (a structurally independent compound), and two weak partial agonists, norphenephrine and octopamine (Audinot et al., 2002; Peltonen et al., 2003). With all five agonists, wild-type and mutant receptors were indistinguishable in the rates and in the amplitudes of stimulated [^{35}S]GTPγS binding. The effects were quite similar for full and partial agonists (i.e., the partial agonists produced almost full responses), compatible with a significant receptor reserve in this system as observed earlier (Nikolaev et al., 2006). In summary, all three receptor constructs retained the ligand binding and G-protein-signaling properties of the wild-type α_{2A}-adrenergic receptor.

Next, we studied the agonist-induced changes in FRET of the constructs expressed in HEK-293 cells. Cells expressing the respective receptor constructs were labeled with FlAsH, and single cells were monitored under a microscope for CFP and FlAsH fluorescence as described under *Materials and Methods*. Upon superfusion with saturating concentrations of norepinephrine, all three constructs showed a rapid decrease in FRET (Fig. 3). The observed amplitude of the decrease was quite similar for all three constructs, ranging from an average change of 10.0 ± 1.5% for construct I3-N to 14.9 ± 2.2% for construct I3-C (data not shown). For all constructs, the ligand-induced change in the conformation occurred on a millisecond time scale (Fig. 3, A and C). This is in good agreement with data we have obtained earlier for earlier FRET constructs of the α_{2A}-adrenergic receptor (Vilardaga et al., 2003, 2005; Nikolaev et al., 2006) and other similar receptor constructs (Hoffmann et al., 2005; Lohse et al., 2008).

To see whether partial agonists might induce specific changes at the α_{2A}-adrenergic receptor, we used the same set of ligands as in Fig. 2. Most of these ligands are chemically derived from norepinephrine by the deletion of individual hydroxyl groups, whereas clonidine is structurally not related (Fig. 4). Because the activation kinetics depend on ligand concentration and reach a maximum at high concentrations (Vilardaga et al., 2003; Hoffmann et al., 2005; Nikolaev et al., 2006), we used saturating ligand concentrations in each experiment to ensure full and rapid occupancy and activation of the receptors. For each experiment, the subsequent response to norepinephrine was set to 100% as a reference for full receptor activation.

Figure 3A shows representative traces obtained for se-

quential activation of the three receptor constructs by a weak partial agonist (octopamine), a strong partial agonist (dopamine), and the full agonist, norepinephrine. In the I3-C construct, all three compounds caused FRET changes that corresponded to their known ability to activate receptor signaling. In contrast, in the two constructs that carried the FlAsH label more N-terminally (I3-M and I3-N). Octopamine caused no change in FRET, and dopamine caused only a small change. The results of a large series of experiments with all ligands are presented Fig. 3B. Clonidine-induced FRET-changes were ≈50% in amplitude relative to norepinephrine in all three constructs. Dopamine also induced FRET changes in all three constructs, but here the amplitudes (relative to the norepinephrine signal) were not equal but ranged from 22.3 ± 1.9% for I3-N to 36.2 ± 1.9% for I3-C ($p < 0.01$). Finally, for norphenephrine and octopamine, clear FRET changes were measured only with construct I3-C (≈20% of the norepinephrine signal), whereas no FRET changes were observed for the other two constructs (Fig. 3, A and B).

A similar trend in the susceptibility to effects of partial agonists was made with respect to receptor activation kinetics. Although norepinephrine induced conformational changes for all constructs with similar kinetics (Fig. 3C), dopamine-induced effects were significantly slower in construct I3-N (Fig. 3C), whereas they were only slightly slower in the other two constructs. The difference in dopamine versus norepinephrine kinetics is better visualized if one compares the ratio of the τ values for the two compounds, which differ by a factor of ≈3 for the I3-N construct but only by a factor of ≈1.5 for the I3-M and I3-C constructs (Fig. 3D).

Discussion

The concepts of different agonist-induced conformations of G-protein-coupled receptors and of their sequential switching by partial and full agonists have gained increasing popularity in recent years (Perez and Karnik, 2005; Kenakin, 2007; Kobilka and Deupi, 2007). Based on well established data for rhodopsin (Okada et al., 2001; Arshavsky et al., 2002; Hub-

TABLE 1

Binding characteristics for the α_{2A}-adrenergic receptor constructs

For saturation binding, cell membranes were incubated for 90 min with 1 to 110 nM [^{3}H]RX821002 in binding buffer (5 mM Tris-HCl and 2 mM EDTA, pH 7.4). Average data from two independent experiments are presented for saturation experiments. Competition binding was then performed with 4 nM [^{3}H]RX821002 and different concentrations of each agonist. Three to four competition experiments were performed for each compound. Nonspecific binding was determined in the presence of 1 μM atipamezole. Saturation and competition binding studies were analyzed with the program Origin to calculate K_D and K_i values and the 95% confidence intervals.

Compound	Wild Type		I3-N		I3-M		I3-C	
	K_i	95% CI	K_i	95% CI	K_i	95% CI	K_i	95% CI
	μM		μM		μM		μM	
NE	15.6	10.5–23.1	18.9	14.5–24.7	15.5	12.4–19.2	31.2	29.9–32.6
DA	47.5	45.1–50.1	52.4	36.8–74.7	35.7	28.8–44.3	55.9	42.1–74.4
CL	0.10	0.08–0.13	0.15	0.14–0.17	0.12	0.10–0.14	0.24	0.18–0.32
OC	41.1	34.5–48.8	65.8	43.5–96.1	93.6	69.0–126.9	70.4	65.7–75.4
NF	30.4	17.0–54.2	40.3	33.7–48.3	50.3	20.0–126.5	52.3	29.1–94.1
[^{3}H]RX821002 (nM)	3.59	N.D.	2.58	N.D.	4.38	N.D.	3.80	N.D.

CI, confidence interval; N.D., not determined.

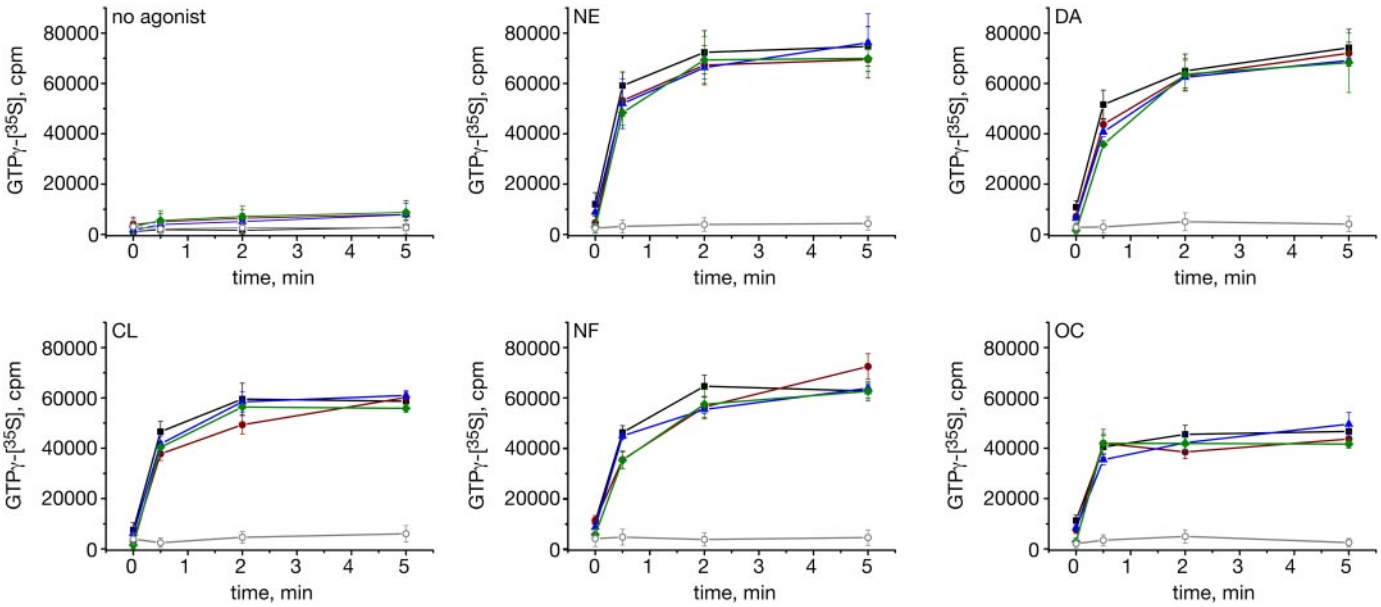

Fig. 2. G-protein activation by the α_{2A}-adrenergic receptor constructs. The ability to activate G-proteins was measured for wild-type α_{2A}-adrenergic receptors, and the receptor constructs shown in Fig. 1 by determining their ability to stimulate [^{35}S]GTPγS binding. Membrane preparations of nontransfected HEK-293 cells (gray) or membranes from cells expressing the I3-N construct (black), I3-M construct (red), I3-C construct (blue), and wild-type (green) were tested for [^{35}S]GTPγS binding. Membrane preparations (10-μg membrane protein) were incubated at 20°C for the indicated times without (control) or with a saturating concentration of the following agonists: NE (1 mM), CL (10 μM), DA (1 mM), NF (1 mM), or OC (1 mM). Each point represents the mean ± S.E.M. of three independent experiments.

bell et al., 2003) and more recent models for the purified β_2-adrenergic receptor (Yao et al., 2006; Kobilka and Deupi, 2007), it has been suggested that intermediate or partially active conformations might exist that are capable of interacting with only some of the downstream proteins, whereas a fully active conformation—corresponding to metarhodopsin II—would only be induced by full agonists. Kinetic FRET experiments with α_2-adrenergic receptors in intact cells showed vastly different time constants of activation for full, partial, and inverse agonists, suggesting that different conformations were attained with different speeds (Vilardaga et al., 2005; Nikolaev et al., 2006).

Our study addressed the question of multiple agonist-induced conformations in the α_2-adrenergic receptor using

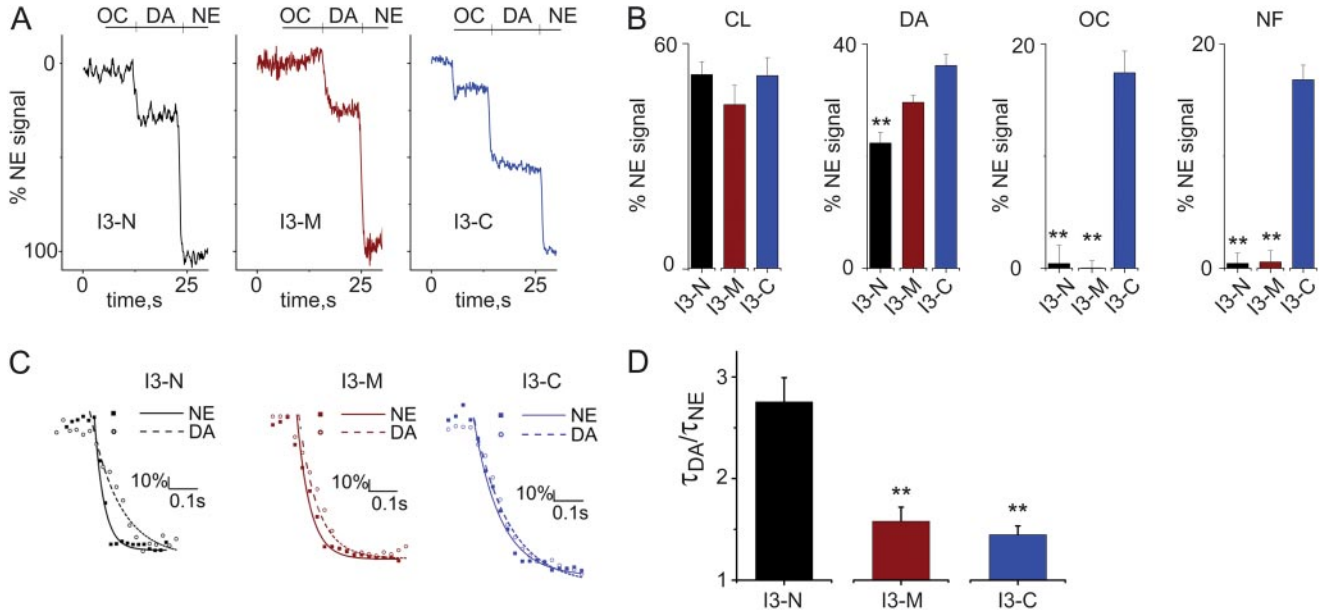

Fig. 3. Agonist effects and kinetics of FRET signals in α_{2A}-adrenergic receptor constructs. A, representative normalized FRET ratio traces from single HEK-293 cells transfected with the indicated receptor construct and stimulated with different agonists: left, I3-N construct (black); center, I3-M construct (red); right, I3-C construct (blue). Cells were superfused with buffer containing 1 mM agonists for 10 s each: OC, DA, and NE. All traces were normalized to the effects of norepinephrine (=100%). B, averaged effects of agonists from experiments as in A. FRET-changes were calculated as the percentage of the change induced by NE. Between 6 and 15 cells were analyzed for each ligand and construct. Color codes are as in A (I3-N, black; I3-M, red; I3-C, blue). All values are normalized to the effects of norepinephrine (=100%). **, significantly different from I3-C, $p < 0.01$ (analysis of variance). C, comparison of the kinetics FRET changes for NE and DA. The panels show sample traces (normalized to the relative maximal effect) for the three α_{2A}-adrenergic receptor constructs, color-coded as above. D, comparison of the rate constants of the FRET change, τ, for DA and NE from experiments as shown in C. Given are the ratios τ_{DA}/τ_{NE}, color-coded as before. For each construct, between five and seven cells were used for the data calculation. **, significantly different from I3-N, $p < 0.01$ (analysis of variance).

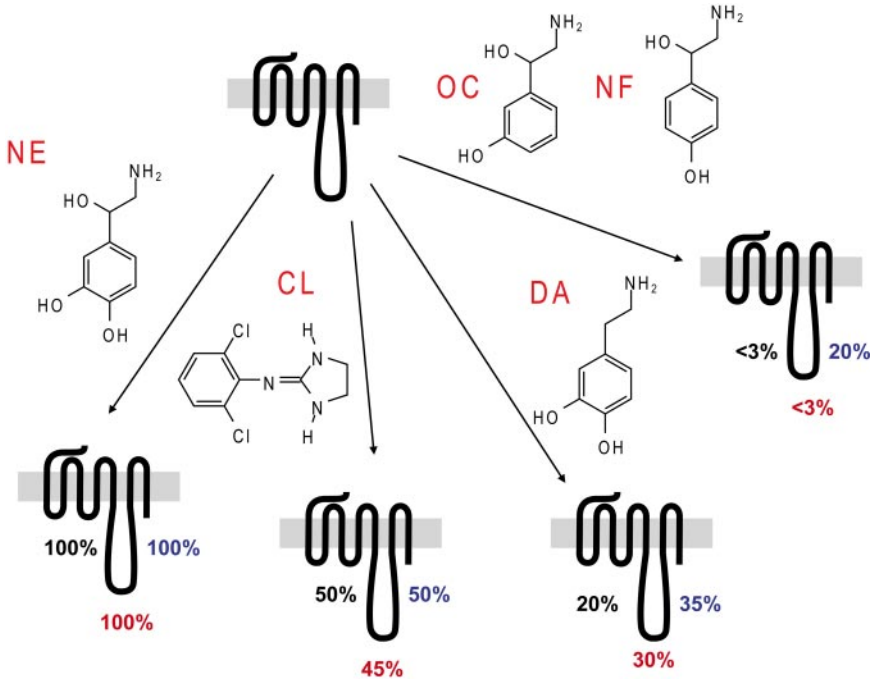

Fig. 4. Scheme of agonist-selective conformations in α_{2A}-adrenergic receptor constructs. The unoccupied α_{2A}-adrenergic receptor is in a resting conformation. Binding of NE, CL, DA, NF, or OC leads to a conformational rearrangement of the receptor, reported by the depicted percentage of FRET changes between the C terminus and different sites in the third intracellular loop in the three receptor constructs. All values are normalized to the effects of norepinephrine (=100%). The data suggest different conformations for different ligand as indicated by the distinct changes in FRET of the three constructs in response to the different agonists.

FRET as a readout, with FlAsH labels in three different positions of the third intracellular loop to sense movements relative to a C-terminally attached CFP. These constructs retained normal ligand binding and signaling properties. Their FRET signals in response to full and partial agonists are summarized in Fig. 4. All three constructs responded to the full agonist norepinephrine with a robust change of similar size in the FRET signal, indicating a conformational change that is visible in all three reference points. We have shown earlier that such changes are due to intramolecular FRET and do not involve intermolecular FRET that might occur in a receptor dimer (Vilardaga et al., 2003; Hoffmann et al., 2005). Smaller but again similar FRET changes were found for the structurally distinct strong partial agonist clonidine.

In contrast, the other partial agonists induced stronger changes in the I3-C construct but weaker (dopamine) or no (octopamine and norphenephrine) changes in the I3-M and I3-N constructs. These three partial agonists lacked different OH groups, but interestingly, those compounds that lacked either of the two catechol OH groups (i.e., octopamine and norphenephrine) failed to cause movements that were visible in the regions close to TMV. Furthermore, the kinetic differences between the (slower) partial agonist dopamine and the (faster) full agonist norepinephrine were clearly more pronounced for the I3-N construct than for the other two constructs (Fig. 3C). All of these data are compatible with the notion that the N-terminal region of the third intracellular loop shows smaller and slower reactions to most partial agonists than the center or the C-terminal region of this loop. This interpretation suggests that partial agonists do not induce a smaller proportion of the same active receptor conformation compared with full agonists but instead evoke ligand-selective conformations.

The "mechanics" of agonist-induced conformational changes in G-protein-coupled receptors are just beginning to be unraveled (Hoffmann et al., 2008). Based on mutagenesis, biochemical, and biophysical data, a general consensus has evolved that activation of these receptors occurs by movements mainly of TMVI versus TMIII (Gether, 2000; Okada et al., 2001; Hubbell et al., 2003). In addition, several reports describe movements of TMV upon ligand binding. This has been proposed for the M_1 muscarinic receptor (Allman et al., 2000), the herpesvirus 8-encoded CXC-chemokine receptor ORF74-HHV8 (Rosenkilde et al., 2006), and specifically for the α_{2A}-adrenergic receptor (Marjamaki et al., 1999; Nyronen et al., 2001). TMV binds to the catechol OH groups via several serine residues in the α_{2A}- and the β_2-adrenergic receptor (Peltonen et al., 2003; Xhaard et al., 2006). This is compatible with our observation that agonists lacking one of the catechol groups fail to induce FRET signals close to TMV (I3-C construct).

In experiments with purified labeled β_2-adrenergic receptors, norepinephrine and dopamine induced similar conformational changes for both the toggle-switch in TMVI and the ionic-lock between TMIII and TMVI (Yao et al., 2006). Therefore, it was suggested that their different efficacy must be determined by a different part of the receptor. Our data suggest the region of the third intracellular loop adjacent to TMV as a potential site to determine partial versus full efficacy.

Eason and Liggett (1995, 1996) suggested that in addition to canonical coupling to G_i via the C-terminal region of the third intracellular loop, α_2-adrenergic receptors can also couple to G_s via the N-terminal third intracellular loop. It is noteworthy that they observed such G_s coupling only for full (norepinephrine) but not for partial agonists (octopamine and norphenephrine) and not with receptors lacking the conserved serines in TMV. This is fully compatible with our observation that the I3-N label failed to report changes in response to these partial agonists, whereas the I3-C label did. It further suggests that G_i and G_s might recognize distinct conformations of the third intracellular loop.

To address this issue, we have done experiments to search for G_s-mediated cAMP increases induced by the different compounds via α_{2A}-receptors. To this end, we transfected Chinese hamster ovary cells with cDNAs both for the receptor and $G\alpha_s$, treated the cells with pertussis toxin to eliminate G_i-mediated signals, and measured cAMP with the very sensitive epac1-camps fluorescent indicator (Nikolaev et al., 2004). However, although the general direction of the resultant cAMP increases was in line with greater effects of norepinephrine compared with octopamine and dopamine, these effects were too small and variable to draw firm conclusions on possible G_s-coupling of α_{2A}-receptors (data not shown).

The recently determined X-ray structures of the β_2-adrenergic receptor (Rasmussen et al., 2007; Rosenbaum et al., 2007), the β_1-adrenergic receptor (Warne et al., 2008), and the partially active opsin (Park et al., 2008), which can be compared with the inactive rhodopsin (Palczewski et al., 2000; Li et al., 2004), reveal two major changes in the partially active opsin that may be important in the context of this study (Fig. 5): the helix of TMV seems extended at its cytosolic end, and movements at the cytosolic face are most prominent for TMVI (>7Å), followed by TMV (>3Å). There are a number of reasons for great caution in linking these structural data to our experimental situation, most importantly, the size of our fluorescent labels, and the lack of X-ray structures containing the large third loop of receptors. However, it is remarkable that our data on partial agonist-

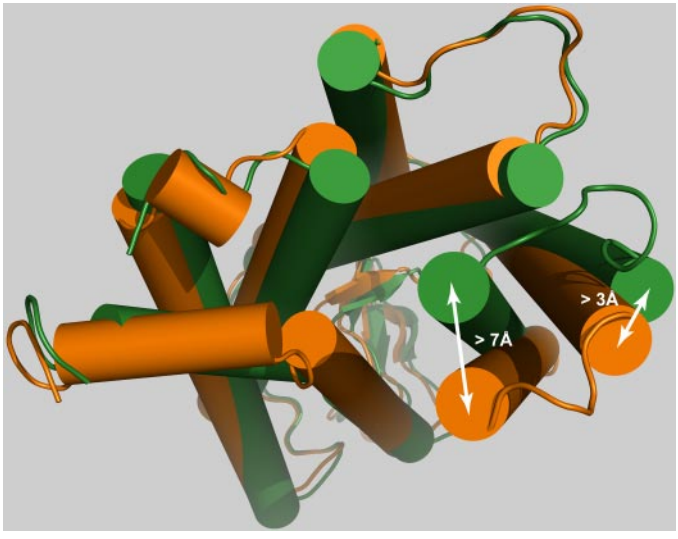

Fig. 5. Superposition of the structures of rhodospin and opsin. Shown are the X-ray structures of rhodopsin (=inactive; PDB 1GZM, green) and opsin (=partially active; PDB 3CAP, orange), viewed from the cytosolic face. The greatest differences between the two structures are visible at the cytosolic end of transmembrane domain VI (>7 Å) and smaller differences at the cytosolic end of transmembrane domain V (>3 Å).

induced changes being reported by labels adjacent to TMVI coincide with the structural evidence for a large change in this region in partially active opsin compared with inactive rhodopsin.

Much less is known about potential movements in the receptors' C terminus, the region that in many receptors undergoes agonist-dependent phosphorylation by G-protein-coupled receptor kinases and subsequently binds β-arrestins (Benovic et al., 1986; Lohse et al., 1990). A recent FRET study using purified β_2-adrenergic receptors labeled at the end of the third intracellular loop and in two sites in the C terminus (Granier et al., 2007) revealed a fairly extended structure of the C terminus and only small movements (1–2 Å) upon activation. Because these sites are similar to those used for labeling with FlAsH and CFP in the I3-C construct described here, this would argue for a smaller contribution of C-terminal movements to our FRET signals.

Our kinetic data support the contention that different agonists induce distinct active conformations. The I3-N construct was less responsive to partial agonists both in terms of amplitudes and of kinetics (Fig. 3). Differences in activation kinetics have been reported for the purified β_2-adrenergic receptor when norepinephrine and dopamine were compared (Swaminath et al., 2004), and it was speculated that these differences could be important for partial agonist activity. Likewise, we have shown earlier that the speed of activation correlated with efficacy for different agonists at the α_{2A}-adrenergic receptor (Nikolaev et al., 2006). Even though the kinetics of purified reconstituted receptors are much slower than those observed in intact cells, there is overall agreement between these two lines of experimentation.

Taken together, these data indicate that different agonists induce distinct changes in receptor conformation, which differ both in terms of the amplitudes and the rates of conformational changes. Our data further suggest that for different full and partial agonists, these conformations might differ in the third intracellular loop, particularly its N-terminal region. Our observations for α_2-adrenergic receptors in intact cells are compatible with those obtained for purified, reconstituted β_2-adrenergic receptors and furthermore agree with the X-ray structures of rhodopsin and opsin. They suggest that distinct changes in the receptor's third loop relative to the C terminus are linked to partial agonist properties relative to G-protein activation. Thus, a complex picture of receptor activation emerges in which distinct changes in different regions of a receptor may induce the ability to interact with different downstream proteins.

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