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Science Signaling **4** (185), ra53. [DOI: 10.1126/scisignal.2001681]

GPCR SIGNALING

A Polymorphism-Specific "Memory" Mechanism in the β_2-Adrenergic Receptor

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Signaling through G protein (heterotrimeric guanosine triphosphate–binding protein)–coupled receptors is affected by polymorphisms in receptor-encoding genes. Using fluorescence resonance energy transfer, we found that the β_2-adrenergic receptor (β_2AR) responded to repeated activation with altered activation kinetics. Polymorphic variants of the β_2AR displayed divergent changes of β_2AR activation kinetics that closely mimicked their different efficacies to generate cyclic adenosine 3′,5′-monophosphate. More efficacious variants became faster in their activation kinetics, whereas less efficacious variants became slower, compared to their initial activation. These differences depended on phosphorylation of the receptor by G protein–coupled receptor kinases. Our findings suggest an intrinsic, polymorphism-specific property of the β_2AR that alters activation kinetics upon continued stimulation and that may account for individual drug responses.

INTRODUCTION

G protein [heterotrimeric guanosine triphosphate (GTP)–binding protein]–coupled receptors (GPCRs) mediate cellular responses to various external stimuli and account for about 50% of all current drug targets. GPCRs share a set of distinct structural and functional properties, such as their seven-transmembrane domain structure and a conformational change that GPCRs undergo upon ligand binding, which is followed by rapid G protein coupling and activation of second messenger–generating enzymes (*1*). The crystal structures of large parts of the β_1- or β_2-adrenergic receptor (βAR) in the inactive state replaced previous assumptions that were based on its analogy to rhodopsin (*2*–*5*). Both the β_1AR and the β_2AR have successfully been crystallized in their agonist-bound active conformations, thereby providing a better understanding of ligand-induced rearrangements of transmembrane segments (*6*–*8*). In addition, optical detection of protein conformational changes has enabled assessment of key characteristics of GPCR activation such as their activation and deactivation kinetics (*9*–*11*). Despite this progress, it remains unclear why the therapeutic response of several drugs is altered by polymorphic variations in the primary structure of the target GPCR. In the β_2AR, the two most frequent polymorphisms are Arg^{16}→Gly and Gln^{27}→Glu (*12*, *13*), both of which have been implicated in the response to β agonists. For example, patients homozygous for Arg^{16} did not benefit from β agonist bronchodilator therapy for asthma, whereas Gly^{16} carriers did (*14*–*16*).

Using fluorescence resonance energy transfer (FRET) to determine ligand-induced conformational changes that the β_2AR undergoes during activation, we provide evidence that the activation kinetics of the β_2AR change in response to repeated stimulation and that this altered activation behavior is a major determinant of the different functionality observed for β_2AR variants.

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RESULTS

Visualization of β_2AR conformational changes reveals receptor "memory" of previous activation

To examine β_2AR conformational changes upon receptor activation, we inserted a yellow fluorescent protein (YFP) into the third intracellular loop and fused a cyan fluorescent protein (CFP) to the C terminus of the β_2AR to generate a sensor molecule capable of FRET (Fig. 1A). This β_2AR sensor localized to the cell membrane in human embryonic kidney (HEK) 293 cells and displayed considerable FRET as determined by acceptor photobleaching (Fig. 1B and fig. S1A). Agonist stimulation resulted in a decrease in the FRET ratio, which reflected receptor activation as previously described for a β_1AR FRET sensor (*9*) (Fig. 1C). This is consistent with observations derived from fluorescence spectroscopy and structural analysis, which predicted agonist-induced relative movements of the transmembrane helices. Furthermore, these data suggest that G protein engagement is required to stabilize the active receptor state (*7*, *17*–*19*). The pharmacological and signaling properties of the β_2AR sensor and the non-modified β_2AR were virtually identical (fig. S1, B to F). These experimental conditions assessed the rare event of a single stimulation of an agonist-naïve receptor. To overcome this limitation and to model more physiological conditions of receptor activation, we therefore sought to analyze βAR activation upon repeated stimulation. Upon repeated activation, we detected significant slowing of the second receptor activation compared to that of the first (Fig. 1, D and E). This change in the activation kinetics was also apparent when the first stimulation was as short as 5 s (fig. S1G) or upon additional stimulation (table S1). This suggests that the receptor "recalls" its first activation and alters its activation kinetics upon further stimulation, a phenomenon we referred to as receptor memory. The respective deactivation kinetics remained unchanged, indicating no alteration of k_{off} (table S1).

β_2AR polymorphisms determine receptor activation kinetics

Next, we assessed the activation characteristics of prestimulated β_2ARs with the frequently occurring polymorphism Arg^{16}→Gly or Gln^{27}→Glu.

These variants were properly targeted to the cell membrane (Fig. 2A), were expressed at similar abundances, and displayed similar ligand-binding affinities as determined by radioligand binding and approximated by FRET measurements at half-maximal concentrations (fig. S2A and table S1), which is in line with previous reports (*12*). Upon a single stimulation with agonist, they did not differ in FRET amplitude or activation and deactivation kinetics (table S1). In contrast, we observed polymorphism-specific differences in the kinetics of receptor activation upon repetitive agonist stimulation. Whereas the Arg^{16} Gln^{27} variant of the β_2AR became slower, the variants carrying Gly^{16} in combination with Gln^{27} or Glu^{27} showed significant acceleration in response to repetitive stimulation (Fig. 2, B to D, and fig. S2B). The following observations led us to believe that the observed differences reflected the characteristics of receptor activation within their native environment. First, these differences were also apparent upon stimulation with a low dose of epinephrine (fig. S2C). Second, the FRET ratio always returned to basal after washing, and changes in the activation kinetics for the different variants were in opposing directions. These findings effectively ruled out incomplete washout of the agonist as a reason for the observed differences (fig. S2A). Finally, none of the β_2AR variants internalized to a substantial extent within the time frame of the experiments, ruling out differential internalization as the basis for the observed differences (fig. S2D).

Receptor memory affects downstream signaling

To test whether receptor memory determines downstream signaling, we measured cAMP (cyclic adenosine 3′,5′-monophosphate) formation in HEK293 cells stably expressing the different β_2AR variants at comparable abundances (Arg^{16} Gln^{27}, 1.33 ± 0.11 pmol/mg; Gly^{16} Gln^{27}, 1.31 ± 0.20 pmol/mg; Gly^{16} Glu^{27}, 1.29 ± 0.17 pmol/mg; $n = 4$ experiments). Upon agonist stimulation for 5 min, the Gly^{16} variants produced more cAMP than the Arg^{16} variant, indicating greater functionality of the faster-activating Gly^{16} variants (Fig. 2E).

This different extent of cAMP formation was attenuated upon long periods (up to 1 hour) of agonist stimulation (fig. S3A), suggesting that the increased functionality of the Gly^{16} variants is associated with a stronger activation of receptor desensitization processes. To further pursue the idea that activation of Gly^{16} β_2AR is faster, more persistent, and thus more effective in downstream signaling, we investigated the agonist-dependent interaction of β_2AR and β-arrestin2 by FRET microscopy and receptor internalization. Although we did not observe differences with regard to receptor internalization among the three receptor variants (fig. S3D), we found that the (faster) Gly^{16} variants induced a more rapid translocation of β-arrestin2 to the membrane (fig. S3, B and C).

The stimulation-induced changes in β_2AR activation kinetics are dependent on soluble intracellular factor or factors

We next sought to investigate whether intracellular factors determine the stimulation-induced changes in β_2AR activation kinetics. To determine the effect of cytosolic proteins on receptor memory, we permeabilized cells expressing the polymorphic variants of the β_2AR sensors before and after the initial agonist stimulation. Permeabilization effectively enabled the rapid diffusion of cytosolic proteins as exemplified by the diffusion of a fluorescent indicator [red fluorescent protein (RFP)–tagged β-arrestin2] out of the cell into the medium (Fig. 3A). Permeabilization before the first stimulation completely abolished the differences in activation kinetics, suggesting a requirement of a cytosolic factor (Fig. 3B). In contrast, receptor memory was not affected when the permeabilization was carried

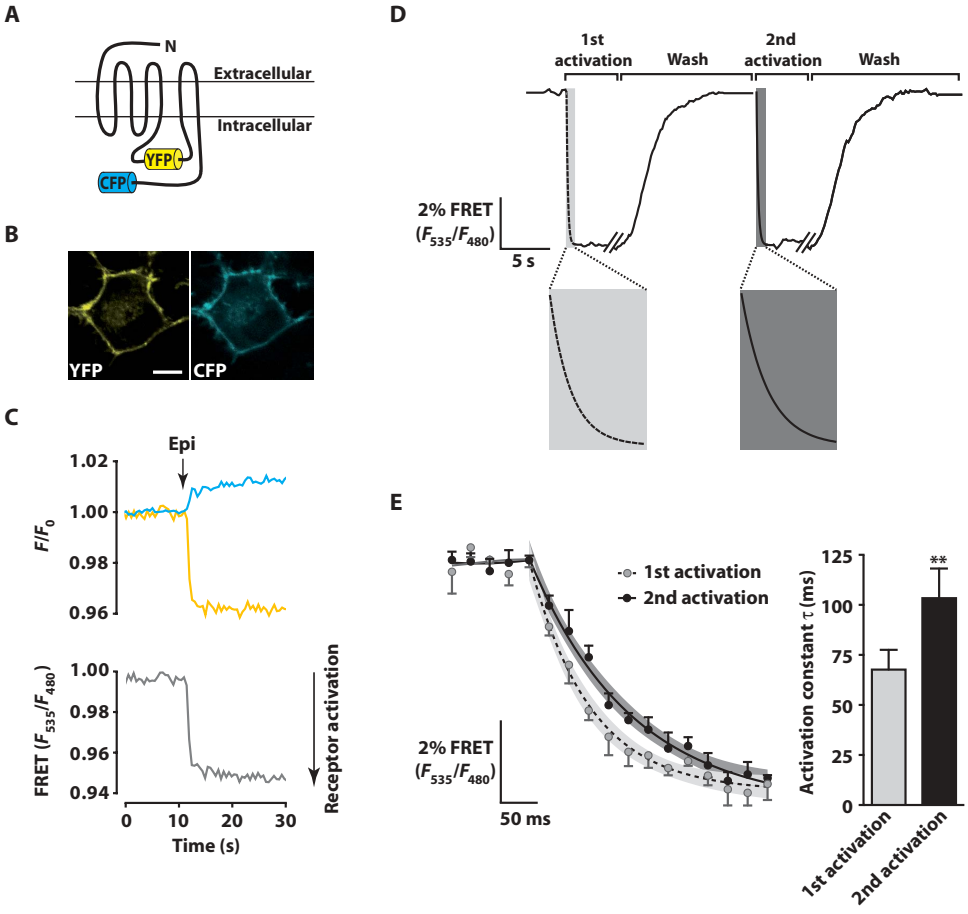

Fig. 1. β_2AR exhibits memory of previous activation. (**A**) Transmembrane topology of the β_2AR FRET sensor construct. (**B**) Subcellular localization of the β_2AR FRET sensor expressed in HEK293 cells as determined by confocal microscopy. Cell representative of >50 analyzed cells. Scale bar, 10 μm. YFP fluorescence is depicted in yellow, CFP in cyan. (**C**) Emission intensities of YFP (535 nm, yellow), CFP (480 nm, cyan), and the FRET ratio F_{535}/F_{480} recorded simultaneously upon superfusion with epinephrine (10 μM; arrow). The decrease in FRET ratio indicates activation of the β_2AR. (**D**) Schematic overview of the experimental approach investigating receptor activation kinetics during consecutive rounds of stimulation. (**E**) Activation kinetics of the β_2AR FRET sensor upon repeated stimulation with 100 μM epinephrine. The first stimulation was carried out for 5 min, the second stimulation directly after complete washout of the agonist. Time constants were determined by fitting monoexponential curves to the first (gray) and second (black) activation ($n = 7$ sets of cells). Curves were fitted to plot mean ± SEM of first (5 s) and second activation ($n = 5$). Ninety-five percent confidence intervals are displayed as shaded areas. $^{**}P < 0.01$ (Student's t test).

out after the initial agonist exposure (Fig. 3C). The addition of guanosine 5′-*O*-[γ-thio]-triphosphate (GTP-γ-S) (to achieve permanent G protein activation) did not alter the kinetics of β_2AR activation. These findings indicate that the interaction of a cytosolic protein with the β_2AR is essential for the observed kinetic differences and that this memory effect, once established, is conserved independently of G protein activation.

The stimulation-induced changes in β_2AR activation kinetics are phosphorylation-dependent

Finally, we assessed receptor phosphorylation as a candidate mechanism underlying receptor memory. Using an antibody directed against β_2AR residues phosphorylated by G protein–coupled receptor kinases (GRKs), we determined β_2AR phosphorylation by Western blotting of HEK293 cell lysates.

Although basal phosphorylation of β_2AR was barely detectable, we found that phosphorylation of the receptor increased after agonist application; furthermore, cells expressing the Gly^{16} variants showed greater GRK phosphorylation (Fig. 4A). Accordingly, the FRET sensors also showed agonist-induced GRK phosphorylation (fig. S4, A and B).

To determine whether GRK-mediated phosphorylation of the β_2AR is necessary for receptor memory, we chose three different approaches to deplete phosphorylation of the β_2AR. First, we mutated all GRK phosphorylation sites in the β_2AR sensors; second, we cotransfected a dominant-negative mutant of GRK2; or third, we transfected a small interfering RNA (siRNA) cocktail against GRK2, GRK3, GRK5, and GRK6 (Fig. 4, B and C). These interventions did not affect receptor activation after a single agonist exposure (fig. S4C), but abolished receptor memory for all three variant β_2ARs (Fig. 4D), thus pointing toward a crucial role for receptor phosphorylation by GRKs for receptor memory.

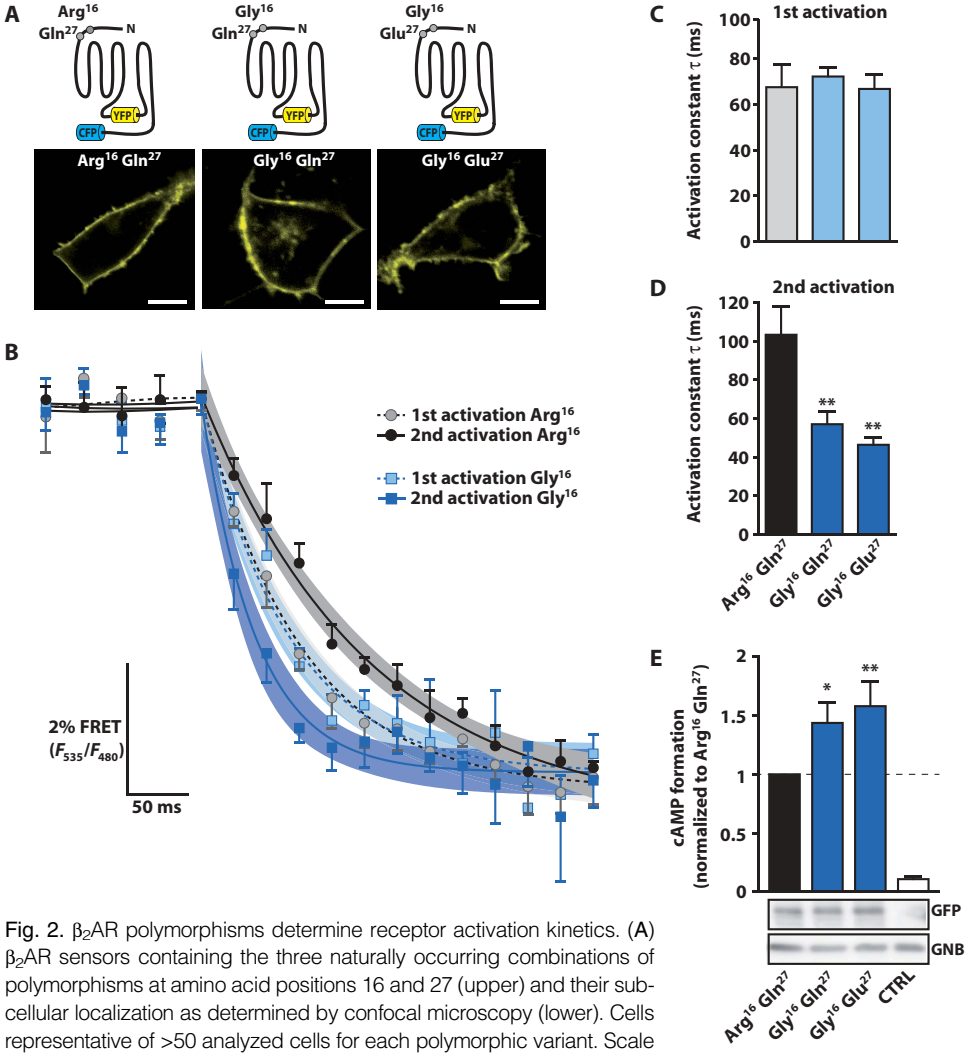

Fig. 2. β_2AR polymorphisms determine receptor activation kinetics. (**A**) β_2AR sensors containing the three naturally occurring combinations of polymorphisms at amino acid positions 16 and 27 (upper) and their subcellular localization as determined by confocal microscopy (lower). Cells representative of >50 analyzed cells for each polymorphic variant. Scale bars, 10 μm. (**B** to **D**) Repetitive stimulation of the polymorphic β_2AR sensor variants with 100 μM epinephrine. Response of β_2AR variants with Arg^{16} or Gly^{16} to 5-s stimulation and corresponding second responses were fitted to plot mean ± SEM of first and second activation (95% confidence interval; see Fig. 1E; $n = 5$ sets of cells) (B). Time constants of the first (5 min, $n = 7$ sets of cells) (C) and second activation ($n = 7$ sets of cells) (D) (analyzed by one-way ANOVA followed by Bonferroni test). (**E**) cAMP formation in HEK293 cells stably expressing the β_2AR FRET sensors at comparable abundances or in untransfected control cells. Stimulation with 100 nM epinephrine for 5 min. Data are normalized to Arg^{16} Gln^{27}–β_2AR ($n = 7$ sets of cells, one-way ANOVA followed by Bonferroni test) (upper). Western blot using antibodies directed against GFP (for β_2AR sensor expression) and G protein β subunits 1 to 4 (GNB) as a loading control (representative of $n = 2$ blots) (lower). $^*P < 0.05$, $^{**}P < 0.01$.

DISCUSSION

Signaling through some GPCRs displays considerable polymorphism-dependent variation, which, according to our findings, might be partially related to their different activation kinetics. Our data indicate that βARs respond to ligand activation with a change of their activation kinetics during subsequent stimulations. Variants with increased functionality became faster in their activation kinetics, whereas variants with decreased functionality became slower compared to their initial activation.

These data link βAR activation kinetics to receptor efficacy. We see two possible ways to interpret our findings in this regard. First, the different activation kinetics may reflect distinct receptor conformations or return of the receptor to an alternative "basal" state after dissociation of the ligand. Such different states might not be detected with our FRET sensor because they may display a similar FRET ratio, but they might cause differences with regard to downstream signaling processes. Second, the kinetic differences themselves may constitute the mechanistic basis for the differences in efficacy. With regard to the latter, the summation of multiple short-lived and repetitive receptor-ligand interactions under native conditions (nonsaturating ligand concentrations) sums up to the downstream signal (namely, cAMP formation). Given that these cycles of activation and deactivation are sufficiently

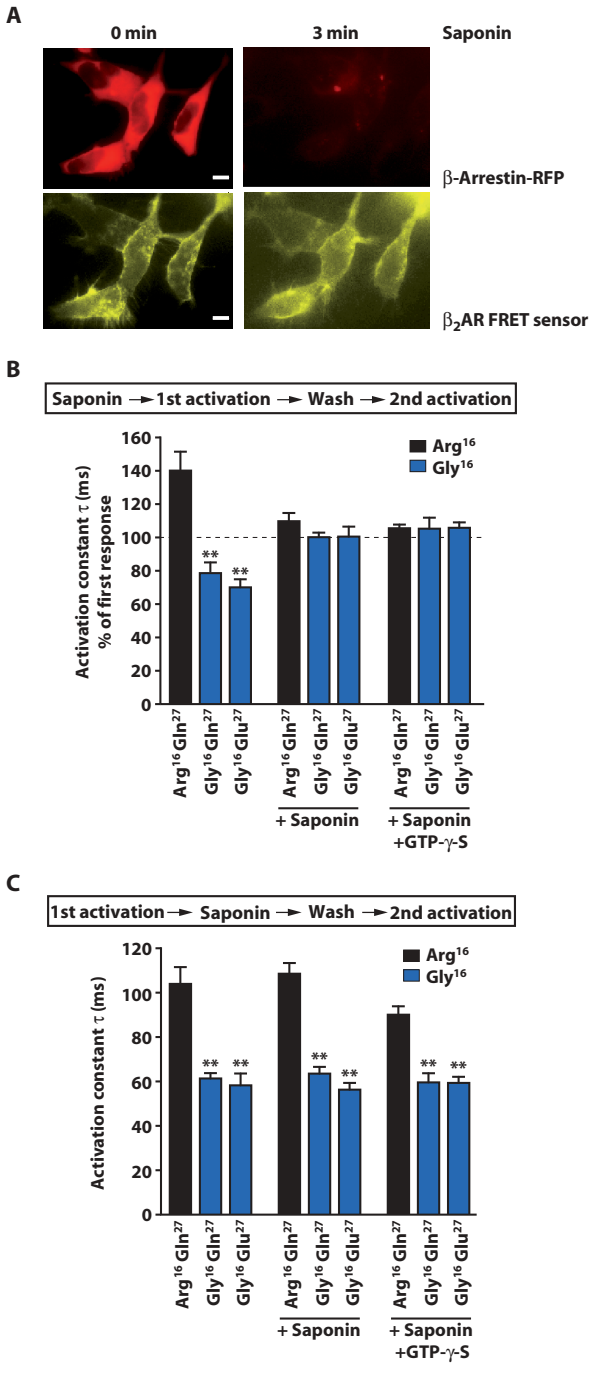

Fig. 3. The differences in activation kinetics depend on soluble cytosolic factors. (A) HEK293 cells expressing a β_2AR FRET sensor at the plasma membrane and β-arrestin2–RFP in the cytosol before and after permeabilization of the cells with 0.05% saponin for 3 min. n = 20 cells. Scale bar, 10 μm. (B and C) Activation kinetics of the polymorphic β_2AR sensors upon permeabilization with 0.05% saponin to release intracellular proteins before (B) and after (C) the first activation. In some experiments, 100 μM GTP-γ-S was added to all solutions. The experimental approach is shown above the graphs. n = 4 to 6 sets of cells for control cells; n = 5 to 10 sets of cells for (A) and n = 10 to 15 sets of cells for (B). Statistical significance was determined by ANOVA and Bonferroni tests. $^{**}P < 0.01$.

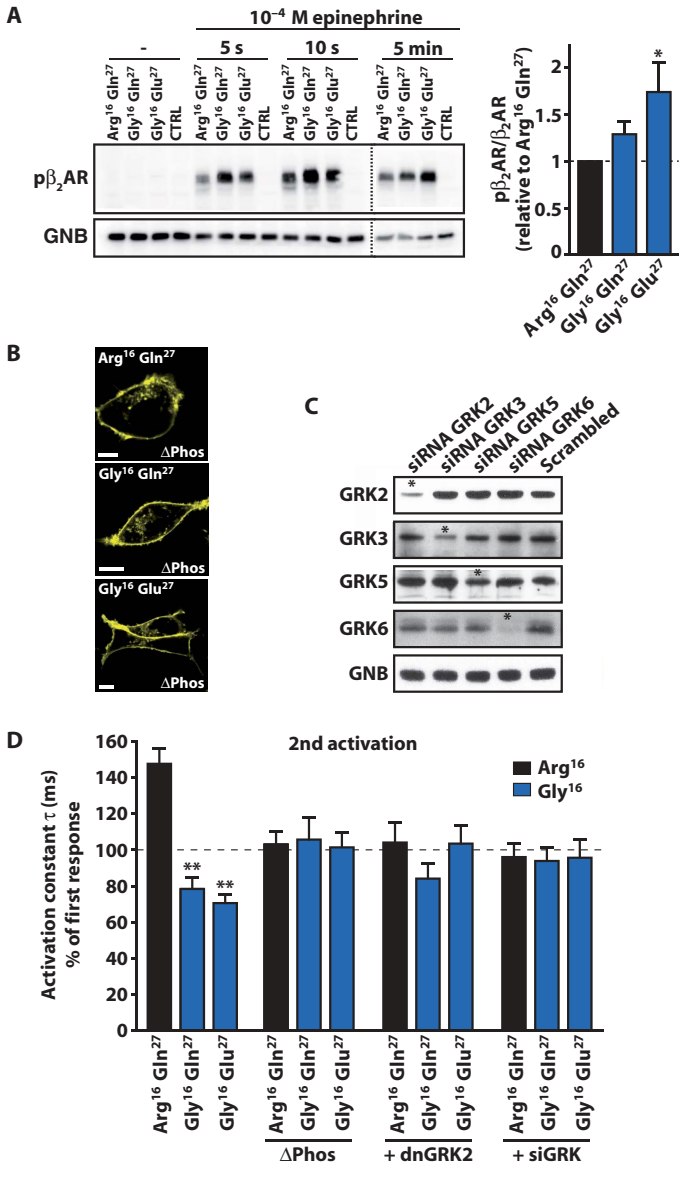

Fig. 4. The stimulation-induced changes in β_2AR activation kinetics are phosphorylation-dependent. (A) Determination of phosphorylation of the β_2AR. Lysates from HEK293 cells transfected with the different receptor variants were immunoblotted with an antibody directed against β_2AR phosphorylated at Ser^{355} and Ser^{356} by GRK ($p\beta_2$AR) and G protein β subunits 1 to 4 (GNB) as the loading control (left). Quantification of β_2AR phosphorylation after 5-min stimulation with 100 μM epinephrine (n = 5 Western blots) (right). (B) Subcellular localization of phosphorylation-deficient β_2AR sensor mutants (ΔPhos) expressed in HEK293 cells as determined by confocal microscopy. Images representative of >20 cells. Scale bars, 10 μm. (C) Lysates of HEK293 cells transfected with siRNAs targeted against GRK2, GRK3, GRK5, or GRK6 were immunoblotted with GRK subtype-specific antibodies (n = 2 Western blots), with GNB as the loading control. (D) Activation kinetics of the polymorphic β_2AR sensors without further intervention (Control; see also Fig. 2D), of the polymorphic β_2AR sensors with mutations in GRK phosphorylation sites (ΔPhos), cotransfection of a dominant-negative form of GRK2 (dnGRK2), or an siRNA cocktail targeting GRK2, -3, -5, and -6 (siGRK). n = 6 to 8 sets of cells. Statistical significance was determined by ANOVA and Bonferroni tests. $^{**}P < 0.01$.

short, a substantial change in activation speed may result in altered downstream signaling.

The elucidation of crystal structures for the βAR has yielded important insights relevant to our findings. The extracellular loop 2 of the β_2AR forms an α helix followed by a β strand that is constrained by two disulfide bonds such that the binding pocket is readily accessible to the extracellular milieu, thereby enabling ready diffusion of water-soluble ligands (*4*, *20*). In contrast to the well-defined and highly conserved ligand-binding pocket, the extracellular surface is structurally diverse with a flexible N terminus (*21*), and structural data for this part of the receptor (which contains the polymorphic position 16 residue) are lacking to date. Although ligand-binding affinities of the polymorphic β_2ARs were unaltered, our data suggest that the N-terminal part of the β_2AR critically determines receptor activation and intracellular phosphorylation and signaling. This finding is unexpected and warrants further experimentation assessing the conformation of the N terminus. Crystal structures of an active conformation of the β_2AR predict G protein engagement as an essential step for achieving the stabilized active state of the receptor (*7*), suggesting the possibility that G protein interaction might alter the conformational changes recorded by the βAR sensors.

Also unexpected was the lack of a clear-cut biphasic response of receptor activation, assuming that during the time frames studied, only a fraction of the receptors will be phosphorylated. However, the temporal resolution of our recordings (5 ms for solution exchange and a sampling rate of 25 ms) might not suffice to determine the biphasic nature of a response with the necessary accuracy. Experiments using coexpression of β_2AR constructs tagged with either CFP or YFP alone at identical sites as in the CFP/YFP-β_2AR sensor indicated that receptor dimerization does not substantially contribute to the FRET changes recorded by the β_2AR sensor (0.5% change in FRET ratio).

Our results for the β_2AR provide a molecular basis to explain several of the seeming inconsistencies reported regarding the signaling properties of the receptor variants investigated here. Previous studies have reported that individuals carrying the Arg^{16} variant showed less bronchodilation after inhaling short-acting β_2 agonists (*22*), whereas studies in isolated membranes did not find that the receptor variants produced different amounts of cAMP (*12*, *23*). Both results agree with our finding that the increased functionality of the Gly^{16} variants depends on receptor phosphorylation (which does not occur in isolated membranes). Our data also suggest (see minor differences in cAMP after agonist exposure >30 min in fig. S3A) that the differences in downstream signaling affect short-term receptor activation and will be less important in situations in which the receptor is chronically exposed to agonists and desensitization compensates for these differences (*24*, *25*).

Future studies should take into account the altered activation behavior that may occur once a receptor has been exposed to a ligand and should determine the structural differences of the native receptor variants. Together, our findings suggest an intrinsic, polymorphism-specific memory mechanism for a GPCR that may account for individual drug responses.

MATERIALS AND METHODS

Molecular biology and cell culture

β_2AR and β_2AR FRET sensors were generated by polymerase chain reaction (PCR) amplification from $pcDNA_3$ plasmids encoding the complementary DNA (cDNA) of the human β_2AR, EYFP-F46L, and CFP, respectively, using Accu Prime DNA Polymerase (Invitrogen). EYFP-F46L (*26*) cDNA was amplified with a reverse primer that encoded an additional flexible linker of 10 amino acids (GSGEGGSGEG) (*27*) and introduced into the third intracellular loop of the β_2AR at amino acid position 247. CFP was fused to the C terminus of the β_2AR (position 413), separated by a linker of five glycines. Site-directed mutagenesis at amino acid positions 16 and 27 of the β_2AR was carried out with the QuikChange II Site-Directed Mutagenesis kit (Stratagene). The phosphorylation-deficient (*28*) β_2AR FRET sensors were generated analogously. β-Arrestin2 was amplified from cDNA of HEK293 cells. EYFP-F46L or RFP was fused to the C terminus of β-arrestin2 by conventional ligation separated by a linker of five glycines.

All constructs were verified by sequencing and subcloned into the pT-REx DEST30 vector (Invitrogen) for eukaryotic expression. Native β_2AR and the βAR sensor constructs, β_2AR-CFP, and β-arrestin2 constructs as well as the dominant-negative GRK2 (K220R) were transfected into HEK293 cells (Invitrogen) for transient expression with Effectene (Qiagen). Cell lines that stably expressed polymorphic β_2AR sensors were selected in the presence of G-418 (0.4 mg/ml) and characterized by radioligand-binding assays (see below).

Adenovirus generation and infection

Recombinant adenoviral vectors were generated according to standard procedures. The human β_2AR and the β_2AR FRET sensor sequences as well as Epac1-camps (*29*) were introduced into an adenoviral vector (pAD/CMV/V5, Invitrogen) by homologous recombination. Adenovirus titers were determined by plaque assays in HEK293 monolayer cultures embedded in agarose. To analyze the functionality of the sensor, we coinfected Chinese hamster ovary (CHO)–K1 cells, which lack endogenous βARs, with Epac1-camps and either the β_2AR or the β_2AR FRET sensor at a multiplicity of infection (MOI) of 100.

siRNA-mediated silencing of gene expression

Chemically synthesized double-stranded siRNA duplexes (with 3′ dTdT overhangs) were purchased from Sigma-Aldrich for the following mRNA targets: GRK2 (5′-GAAAUUCAUUGAGAGCGAU-3′), GRK3 (5′-CAGUUUAUGAAGCAGUAAA-3′), GRK5 (5′-GCAGUAUCGAGUGCUAGGA-3′), and GRK6 (5′-CACCUUCAGGCAAUACCGA-3′). A scrambled RNA duplex (5′-GCUUAGGAGCAUUAGUAAA-3′) served as a negative control. HEK293 cells were transfected with a concentration of each siRNA duplex of 200 nM with HiPerFect (Qiagen) according to the manufacturer's protocol, and cells were subsequently incubated for 3 days. Silencing was verified by Western blotting.

Membrane preparation and radioligand-binding assay

Stably transfected HEK293 cells or nontransfected control cells were washed with phosphate-buffered saline (PBS), and membrane fractions were prepared as described (*30*). The resulting membrane pellets were resuspended in 50 mM tris buffer (pH 7.4). Protein concentrations were determined by the Bradford method with bovine serum albumin (Sigma-Aldrich) as standard. Radioligand-binding experiments were performed in 50 mM tris-HCl (pH 7.4) in the presence of 100 μM GTP to ensure monophasic competition curves for agonists (*31*). Nonspecific binding was determined in the presence of 10 μM alprenolol (Sigma). Receptor number (femtomoles) was normalized to milligrams of membrane protein.

Radioligand-binding assay on intact cells

HEK293 cells expressing the polymorphic β_2AR variants were seeded in 24-well plates coated with poly-D-lysine. Radioligand-binding experiments on intact cells were performed in serum-free Dulbecco's modified Eagle's medium (DMEM) with [^{3}H]CGP-12177 (Hartmann Analytic, 1 nM). After incubation for 1 hour at 37°C, cells were washed twice with PBS, and lysed with 1 M NaOH. Nonspecific binding was determined in the presence of 10 μM alprenolol (Sigma).

Permeabilization of HEK293 cells

For permeabilization of HEK293 cells attached to poly-D-lysine–coated coverslips, the cells were incubated with internal buffer [100 mM K^+-aspartate, 30 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 5 mM EGTA, and 10 mM Hepes (pH 7.35)] containing 0.05% saponin (Sigma) for 3 min and subsequently superfused with internal buffer for FRET measurements. Optionally, 100 μM GTP-γ-S (Sigma) was added to the internal buffer and saponin solution.

Nonconfocal images of β_2AR FRET sensor expressing cells before and after permeabilization were taken with a Zeiss Axio Observer Z1 inverted microscope with a 40× oil-immersion objective and a Retiga 4000DC camera (Qimaging).

FRET measurements

HEK293 cells were split 24 hours after transfection, seeded on poly-D-lysine–coated coverslips, and kept in culture for additional 24 hours in DMEM containing 10% fetal calf serum (FCS). Cells grown on coverslips were maintained at room temperature in FRET buffer [140 mM NaCl, 4.5 mM KCl, 10 mM Hepes, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 7.4)] during the assay. Agonists and antagonists were purchased from Sigma-Aldrich. Stock solutions of 10^{-1} M epinephrine and 10^{-2} M propranolol were prepared in double-distilled H_2O. Isoproterenol at 10^{-1} M was prepared in a solution of ascorbic acid (1 mg/ml) to prevent oxidation.

FRET experiments were performed with a Zeiss inverted microscope (Axiovert 200) equipped with an oil-immersion 100× objective and a dual-emission photometric system equipped with a polychrome IV light source (TILL Photonics). FRET was monitored as the emission ratio of YFP to CFP, F_{535}/F_{480}, with F_{535} and F_{480} denoting the emission intensities at 535 ± 15 nm and 480 ± 20 nm, respectively [505-nm dichroic long-pass filter (DCLP) beam splitter], upon excitation at 436 ± 10 nm (455-nm DCLP beam splitter). The emission ratio was corrected for the spillover of CFP into the 535-nm channel and for direct excitation of YFP at 436 nm. For the determination of pharmacological stimulation–induced FRET changes, cells were continuously superfused with FRET buffer, and ligands were applied with the computer-assisted solenoid valve–controlled rapid superfusion device ALA-VM (ALA Scientific Instruments; solution exchange, 5 to 10 ms). Signals detected by amplified photodiodes (TILL Photonics) were digitalized with an analog digitizer converter (Digidata 1322A; Axon Instruments) and recorded and analyzed with Clampex 9.0 (Axon Instruments) and Origin6 software, respectively. For repetitive stimulation, we applied a first agonist exposure for either 5 s (short-term) or 5 min. After complete washout of the agonist, the cell was stimulated for a second time.

Acceptor photobleaching was carried out by direct excitation of YFP at 490 nm for 8 min. Emission intensities for CFP and YFP for excitation at 436 nm were recorded before and after bleaching of the YFP.

β_2AR–β-arrestin interaction studies were performed with a Polychrome 5000 (TILL Photonics) and a Zeiss Axio Observer Z1 inverted microscope equipped with an oil-immersion 100× objective, DualView2, and Evolve camera (Photometrics). FRET was monitored with the MetaFluor Software (Visitron Systems). To study agonist-induced changes in FRET, we continuously superfused cells with buffer and ligand solutions by means of the ALA VC3-8 (ALA Scientific Instruments) system for perfusion.

Confocal microscopy

Confocal analysis was performed with a Leica TCS SP2 system, a Zeiss LSM 510 system, or an Olympus Konfokal FV1000 system with an Attofluor holder (Invitrogen). YFP was excited with the 514-nm line of an argon laser, CFP with 436 nm. Images were taken with a 63× objective using the factory settings for YFP or CFP fluorescence, respectively.

Western blot analysis

After treatment with epinephrine at 37°C, cells were washed once with PBS and directly lysed on ice in a 50 mM tris buffer (pH 6.7) containing 2% SDS, 1 mM Na_3VO_4, protease inhibitor Complete Mini (Roche), and an optional phosphatase inhibitor, PhosSTOP (Roche), for detection of phosphorylated proteins. Protein (5 to 20 μg) was separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) in gels containing 10 or 12% acrylamide, followed by Western blotting as described previously (*32*). Primary antibodies against pβ_2AR (Ser^{355}/Ser^{356})-R (sc-16719-R), β_2AR (sc-569), GRK2 (sc-562), GRK3 (sc-563), GRK5 (sc-565), GRK6 (sc-566), green fluorescent protein (GFP) (sc-8334), and G β subunits 1 to 4 (GNB, sc-378) were obtained from Santa Cruz Biotechnology. Secondary antibodies were obtained from Dianova.

Measurement of cAMP accumulation

HEK293 cells stably expressing the β_2AR FRET sensors were seeded in 24-well plates, incubated for 24 hours, and starved in 0% FCS for 8 hours before they were stimulated with 100 nM epinephrine for the respective time. Subsequently, intracellular cAMP was quantified with the Parameter Cyclic AMP Assay kit according to the manufacturer's protocol (R&D Systems). The protein content was determined in parallel using the Pierce BCA Protein Assay Kit (Thermo Scientific).

Statistics

Average data are presented as means ± SEM. Statistical analysis was carried out with the Prism software package (version 4.0; GraphPad Software). Analysis of variance (ANOVA) followed by Bonferroni test and Student's *t* test were used as appropriate and indicated in the figure legends. $P < 0.05$ was considered statistically significant.

SUPPLEMENTARY MATERIALS

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Fig. S1. Characterization of the β_2AR FRET sensor.

Fig. S2. β_2AR activation at half-maximal agonist concentrations, kinetics upon short-term stimulation, and assessment of internalization.

Fig. S3. β_2AR desensitization.

Fig. S4. Phosphorylation of the polymorphic β_2AR sensors and activation kinetics.

Table S1. Characteristics of ligand binding and β_2AR sensor expression in intact cells, FRET responses for submaximal (K_i), and maximal agonist concentrations.

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33. **Acknowledgments:** We thank C. Dees (Universitaet Wuerzburg) for his support with radioligand binding, F. Rodewald [Technische Universitaet Muenchen (TUM)] for help with cloning of single-tagged receptor constructs, B. Laggerbauer (TUM) and M. J. Lohse (Universitaet Wuerzburg) for critical discussions, and T. Misgeld and M. Brill (TUM) for their support with confocal microscopy. We thank B. K. Kobilka (Stanford) for helpful advice on experiments. **Funding:** S.E. is supported by grants from the Deutsche Forschungsgemeinschaft and the Bundesministerium für Bildung und Forschung. **Author contributions:** A.A. performed the experiments. A.A. and S.E. designed the experiments, analyzed the data, and wrote the manuscript. F.R. contributed to the generation of the β_2AR FRET sensor. T.F. and M.B. contributed to the design of the experiments. **Competing interests:** M.B. holds a patent on the technology of measuring receptor activation by FRET (EP 1581811B1).

Submitted 15 November 2010
Accepted 27 June 2011
Final Publication 9 August 2011
10.1126/scisignal.2001681
Citation: A. Ahles, F. Rochais, T. Frambach, M. Bünemann, S. Engelhardt, A polymorphism-specific "memory" mechanism in the β_2-adrenergic receptor. *Sci. Signal.* **4**, ra53 (2011).