Functional Immobilization of a Ligand-Activated G-Protein-Coupled Receptor

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G-protein-coupled receptors (GPCRs) mediate the majority of cellular responses to hormones and neurotransmitters. They are the largest family of receptors in the human genome and constitute the largest class of targets for drug discovery. To facilitate studies of GPCR activation and interactions with other proteins, we developed a simple method to immobilize a functional, detergent-solubilized GPCR on gold and glass surfaces. The β_2 adrenergic receptor (β_2AR), a prototypical GPCR, was purified and labeled with a reporter fluorophore at a conformationally sensitive site. The detergent-soluble fluorescent β_2AR was immobilized through its amino-terminal FLAG epitope on a surface layered with biotinylated bovine serum albumin, avidin, and biotinylated M1 antibody. Agonist activation of the β_2AR was monitored in real time by fluorescence microscopy. This approach will make it possible to study conformational dynamics of single immobilized receptors and to generate arrays of functional GPCRs for novel high-throughput screening strategies.

KEYWORDS:

fluorescence spectroscopy · G-protein-coupled receptors · immobilization · protein chip · receptors

Introduction

There is growing interest in chip-based, high-throughput screening strategies for characterization of the function and interactions of proteins encoded by the human genome. Protein immobilization in combination with sensitive optical detection facilitates investigations of protein–protein interactions and protein activation mechanisms.[1] These are powerful tools for pharmaceutical sciences[2] and basic biological research,[3] which includes studies at single-molecule resolution.[4–6] However, immobilized proteins may not retain their function because of nonspecific interactions between the protein and the surface. This is particularly true for integral membrane proteins such as G-protein-coupled receptors (GPCRs). GPCRs are the largest family of receptors in the human genome and constitute the largest class of targets for drug discovery. Over 50% of drugs on the market or in development are targeted at GPCRs.[7] Therefore, there is considerable commercial interest in the development of chip-based approaches for both basic biological research and commercial high-throughput screening of GPCRs.

The β_2 adrenergic receptor (β_2AR) is a prototypical ligand-activated GPCR. This transmembrane protein mediates the effects of catecholamines released by the sympathetic nervous system and is one of the most extensively characterized members of the GPCR family. It was recently shown that agonist-induced conformational changes can be monitored directly by using conventional fluorescence spectroscopy on β_2AR labeled with fluorescein at Cys265.[8] Cys265 is adjacent to a critical G-protein-coupling site at the cytoplasmic end of transmembrane peptide 6. Fluorophores bound to Cys265 sense agonist-induced conformational changes that lead to G protein activation.[9, 10] Here we report the use of this fluorescence-based detection approach to monitor agonist-induced conformational changes in β_2AR immobilized on a glass surface.

Results

Strategy for detection of β_2AR activation

Agonist-induced conformational changes in β_2AR can be monitored directly in purified, detergent-solubilized receptors labeled at Cys265 with fluorescein-5-maleimide (FMβ_2AR). In the presence of the full agonist (−)-isoproterenol (ISO), the fluorescence intensity of FMβ_2AR decreases by 15% and this decrease can be reversed by displacment of ISO with the antagonist alprenolol (ALP).[8] These experiments were performed in a standard fluorometer with relatively low light intensities. Fluorescence-based detection of conformational changes in surface-immobilized β_2AR requires a fluorescence microscope and laser excitation of the sample. Therefore, we screened for dyes that are more photostable than fluorescein yet are able to detect agonist-induced conformational changes.

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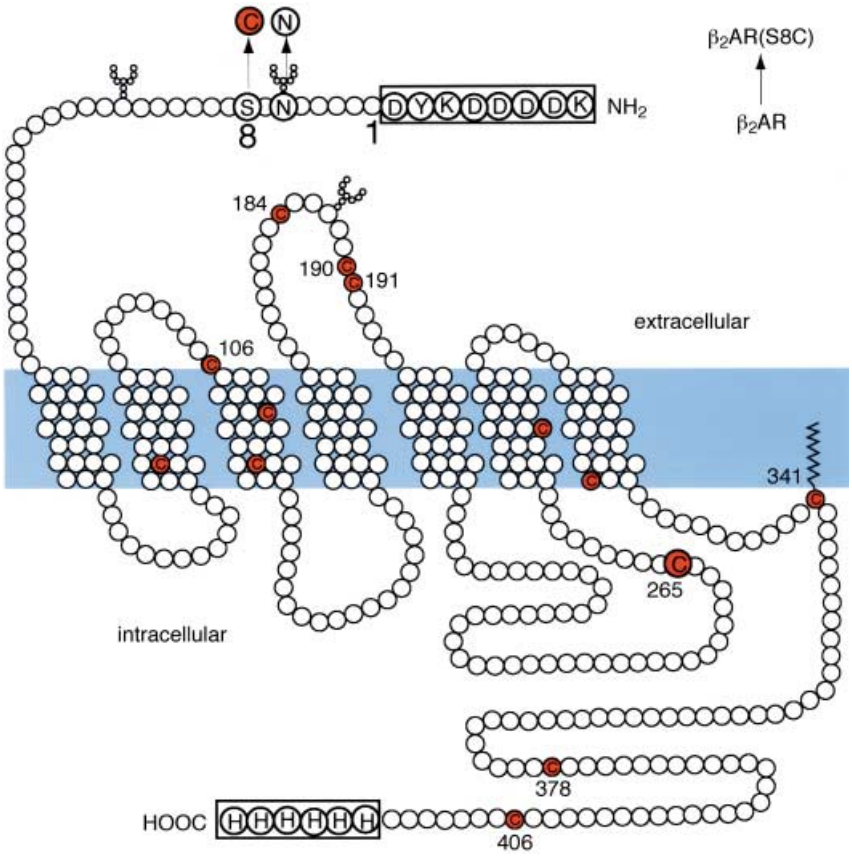

Figure 1. *Model of β_2AR. The receptor was equipped with an amino-terminal FLAG tag and a carboxy-terminal His_6 tag (shown in boxes) to allow efficient purification. Cysteine residues at positions 106, 184, 190, and 191 are involved in intramolecular disulfide bridges. During purification Cys378 and Cys406 form an additional disulfide bridge. Cys341 is palmitylated. With the exception of Cys265, all remaining natural cysteine residues are buried within the membrane or micelle. Thus, only Cys265 is labeled by hydrophilic flurophores. To introduce a specific extracellular labeling site, we generated β_2AR(S8C), which has an additional extracellular cysteine residue at position 8 and lacks the glycosylation consensus sequence NXS at positions 6, 7, and 8 (indicated by arrows).*

Purified β_2AR was labeled at Cys265 (see Figure 1) with a variety of cysteine-reactive fluorophores, which included versions of BODIPY fluorophores, Alexa dyes, CyDyes, 1-(3-(succinimidyloxycarbonyl) benzyl)-4-(5-(4-methoxyphenyl) oxazol-2-yl)pyridinium bromide (PyMPO), and tetramethylrhodamine-maleimide (TMR). These labeled receptors were then tested for changes in fluorescence after ISO exposure (data not shown). Only β_2AR labeled with tetramethylrhodamine-5-maleimide (TMR-5-β_2AR) was found to combine sufficient photostability with a clearly detectable and specific change of fluorescence intensity upon agonist binding. As shown in Figure 2, the fluorescence of solubilized and purified TMR-5-β_2AR measured in a standard fluorometer increases by 23% following the addition of a saturating concentration of the full agonist ISO. Approximation of fluorescence traces with an exponential fit yielded an $t_{1/2}$ of 164 ± 13 s, which is in agreement with values found for FM-β_2AR.[8] The mechanistic implications of the slow kinetics of the agonist-induced conformational changes have been discussed previously.[8] Additionally, the agonist epinephrine (EPI) and the partial agonist salbutamol (SAL) cause the fluorescence to increase by 19 and 8%, respectively (Figure 2). The smaller amount of fluorescence change after EPI or SAL exposure is consistent with their lower efficiencies at triggering coupling between β_2AR and the G protein.[8] These agonist-induced increases in fluorescence are reversed by displacment of ISO with the high-affinity antagonist ALP (Figure 2), and are significantly reduced when the receptor is pre-incubated with ALP before the addition of ISO (Figure 2). The opposing fluorescence response (fluorescence decrease/increase) of FM-β_2AR and TMR-5-β_2AR upon ISO exposure can be explained by differences in the photophysics of these dyes. This behavior does not imply that the fluorescence change of TMR-5-β_2AR is based on a different mechanism from that of FM-β_2AR. In fact, the comparable activation kinetics and the identical efficacies of different agonists for FM-β_2AR[8] and TMR-5-β_2AR (Figure 2) argue that TMR senses the same conformational transition as fluorescein.

Immobilization of β_2AR

The first step of the immobilization procedure is the deposition of bovine serum albumin (BSA) – biotin, which adsorbs spontaneously onto glass and gold surfaces, blocks nonspecific binding, and creates a layer of biotin residues. The adsorbed BSA – biotin is resistant to both high and low salt washes, and to washes with *N*-dodecyl-β-D-maltoside (DDM), the detergent

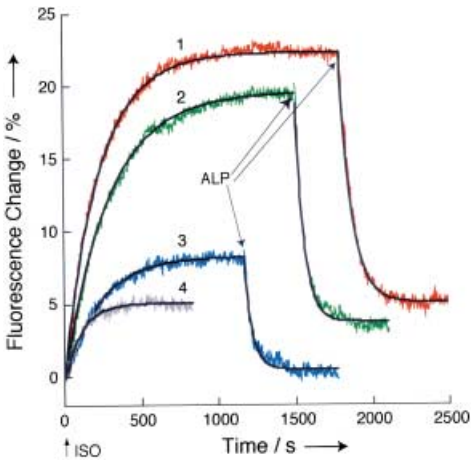

Figure 2. *Activation of TMR-5-β_2AR (35 nM) monitored in a standard fluorometer. 1) Fluorescence emission was recorded over time after addition of ISO, EPI, or SAL (100 μM; time = 0 sec). Typical fluorescence traces are shown after exposure with ISO (1), EPI (2), and SAL (3). The fluorescence intensity increased by 23, 19, and 8%, with $t_{1/2}$ values of 177 ± 1, 167 ± 1, and 144 ± 2 s, respectively, for each particular experiment. After equilibrium was reached, ALP (10 μM) was added, which induced the reversal of the fluorescence increase. 4) Pre-incubation of TMR-5-β_2AR with ALP (10 μM) significantly reduces the fluorescence increase induced by ISO (100 μM). Excitation and emission wavelengths were 551 and 569 nm, respectively. Data were acquired every 3 s with an integration time of 3 s (coloured lines). Monoexponential functions (black lines) were fitted to the data.*

used to solubilize the β_2AR. We then added excess avidin (or streptavidin) to build a layer of unsaturated avidin that can be used for the immobilization of other biotinylated proteins. Two different approaches were used to immobilize the TMR-β_2AR. In Approach 1 (Figure 3 A), the monoclonal antibody M1 against the amino-terminal FLAG tag is biotinylated (M1 – biotin) and bound to the avidin layer. The receptor is then immobilized through its FLAG tag. In Approach 2 (Figure 3 B), we use a modified receptor, β_2AR(S8C), which can be directly biotinylated and thereby allows immobilization without antibody. β_2AR(S8C) has an additional cysteine residue on the extracellular side of the β_2AR at position 8 (see Figure 1). Incubation of intact cells that express β_2AR(S8C) with biotin – maleimide allows specific labeling of the cysteine residue at position 8 without modification of Cys265. After purification and fluorescence labeling of Cys265, the TMR-5-β_2AR(S8C) is directly bound to the avidin layer. As a result of the S8C mutation the glycosylation consensus sequence NXS at positions 6 – 8 was removed, which avoided potential steric hindrance between sugar residues and avidin. Nonspecific binding does not exceed 20 % for either approach. The order and specificity of protein layer formation was confirmed by surface plasmon resonance (SPR, Figure 4)

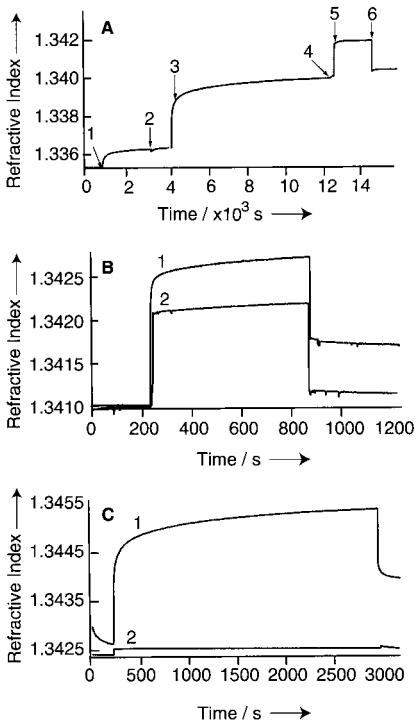

Figure 4. *SPR sensorgrams showing refractive index changes: A) Sequential binding of BSA – biotin, avidin, and M1 – biotin. At time 0 s, phosphate-buffered saline (PBS) was injected onto an unmodified gold sensing surface. At (1), BSA – biotin (0.25 mg mL^{-1}) in PBS was injected and allowed to adsorb onto the surface until (2), when PBS was reintroduced to remove any nonadhering BSA – biotin. At (3), avidin (0.3 mg mL^{-1}) in HS buffer (defined in the Experimental Section) was added and biotin/avidin binding was allowed to occur until a stable refractive index signal indicated equilibrium had been reached. At (4), HS buffer was added. At (5), M1 – biotin (2.1 mg mL^{-1}) was added and biotin/avidin binding was allowed to occur until the refractive index stabilized. Unbound M1 – biotin was rinsed away by the injection of HS buffer at (6). The refractive index response has been corrected for the changes caused by the introduction of protein-free buffer solutions, and one washing step has been removed for clarity. B) Binding of the β_2AR through a specific interaction of its FLAG tag with immobilized M1 antibody. Sensorgram 1 shows the binding of β_2AR to biotinylated M1 that has been immobilized through avidin/biotin linkages as shown in (A). Sensorgram 2 shows the same β_2AR introduced to a sensor that was treated with nonbiotinylated M1. C) Binding of biotinylated β_2AR(S8C). Sensorgram 1 shows the binding of biotinylated β_2AR(S8C) to an immobilized layer of avidin. Sensorgram 2 shows a nonbiotinylated β_2AR(S8C) introduced to a sensor prepared with avidin.*

Detection of β_2AR activation by fluorescence microscopy

Fluorescence from TMR-5-β_2AR or TMR-5-β_2AR(S8C) immobilized on a glass coverslip was recorded by using an intensified-charge-coupled device (ICCD) camera. Homogenous parts of the surface were chosen for evaluation of fluorescence intensities over time (Figure 5). After the addition of a saturating concentration of the agonist ISO, the intensity of TMR-5-β_2AR immobilized using Approach 1 (Figure 3 A) increased by up to 30 %, depending on the quality of the receptor preparation (Figure 5 B). The fluorescence increase of immobilized receptor derived from one individual protein preparation varies by $\pm$38 % around the average value. This agonist-induced increase in fluorescence could be reversed by displacement of ISO with the high-affinity antagonist ALP (Figure 5 C). To further evaluate the functionality of the immobilized β_2AR, we measured the kinetics of the

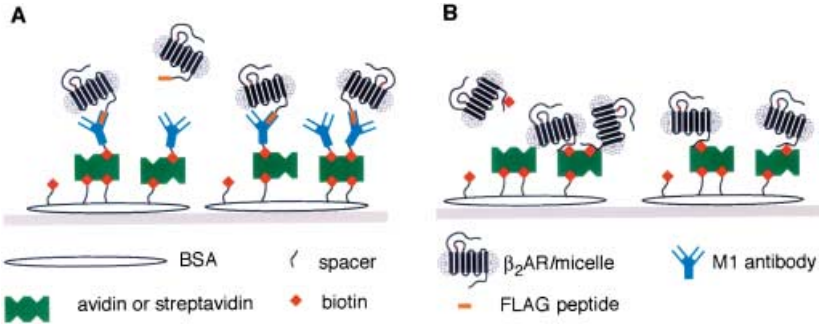

Figure 3. *Immobilization of the β_2AR on gold or glass surfaces. The surfaces were coated sequentially with BSA – biotin and avidin (or streptavidin). Two approaches were used to immobilize the receptor on avidin. A) A biotinylated monoclonal antibody (M1) against the amino-terminal FLAG tag was bound to the avidin layer. The solubilized TMR-5-β_2AR was then immobilized through its amino-terminal FLAG epitope. B) The TMR-5-β_2AR(S8C) mutant was biotinylated at the cysteine residue at position 8 and then bound directly to the avidin layer.*

agonist-induced change in fluorescence (Figure 6 A). ISO induced an increase in fluorescence with a $t_{1/2}$ of 202 $\pm$ 66 s for receptor immobilized by Approach 1 (Figure 3 A). This value is in good agreement with the kinetics measured for nonimmobilized β_2AR in detergent solution at room temperature (Figure 2). The ISO-induced fluorescence increase was blocked when the receptor was pre-incubated with ALP. The time-dependent decrease in fluorescence of receptor pre-incubated with ALP alone is caused by photobleaching (Figure 6 A). We observed significantly smaller ISO-induced increases in fluorescence for receptor immobilized by using Approach 2 (10.6 $\pm$ 7.8 % (maximum 13 %), $t_{1/2}$ = 141 $\pm$ 131 s). As additional evidence of the functional activity of the immobilized β_2AR, we demonstrated that the ago-

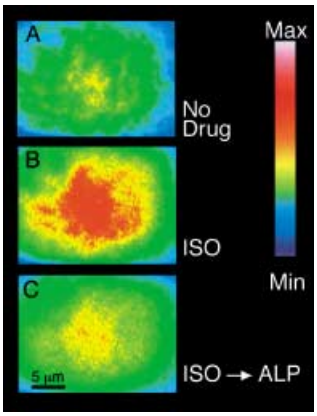

Figure 5. *False-color CCD images of immobilized TMR-5-β_2AR showing intensity changes upon agonist (ISO) and subsequent antagonist (ALP) addition. A) Freshly prepared surface of TMR-5-β_2AR; B) same surface location, picture taken 12 minutes after ISO addition; C) same surface location, picture taken 6 minutes after ALP addition. The pictures clearly show the fluorescence intensity increase upon agonist addition and the subsequent reversal of this fluorescence increase when a high-affinity antagonist displaces the agonist.*

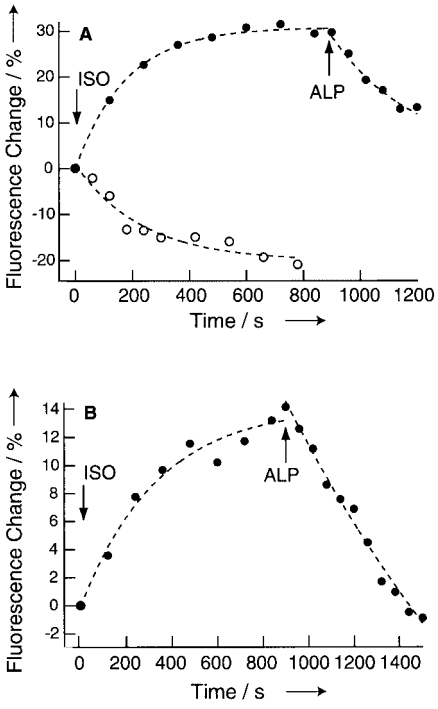

Figure 6. *Fluorescence intensity measurements of immobilized TMR-5-β_2AR as a function of time after addition of the agonist ISO. A) TMR-5-β_2AR immobilized by Approach 1 (Figure 3A). The fluorescence intensity rose 30% after the addition of 100 μM ISO. Filled circles represent intensity data; the dotted line is an exponential fit with an $t_{1/2}$ of 122 ± 9 s. Pre-incubation with the competitive antagonist ALP (10 μM; open circles) blocks the ISO-induced increase in fluorescence. B) Biotinylated TMR-5-β_2AR(S8C) immobilized by Approach 2 (Figure 3B). Fluorescence intensity increased by 13% after the addition of 100 μM ISO. Filled circles represent intensity data; the dotted line is an exponential fit with an $t_{1/2}$ of 248 ± 57 s for the particular experiment shown. Data are not corrected for photobleaching.*

nists ISO and EPI and the partial agonist SAL possess nearly identical relative efficacies for activation of immobilized (Figure 7) and solubilized TMR-5-β_2AR (Figure 2). As in solution

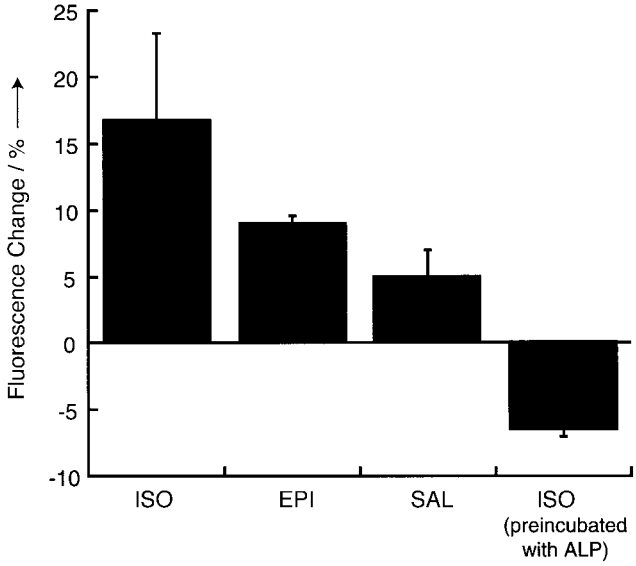

Figure 7. *Fluorescence response of immobilized TMR-5-β_2AR measured after exposure to saturating concentrations (100 μM) of ISO, EPI, or SAL. As a control, TMR-5-β_2AR was pre-incubated with ALP (10 μM) before treatment with ISO (100 μM).*

experiments, the largest fluorescence increase of immobilized TMR-5-β_2AR was measured after ISO exposure, whereas EPI induces a less pronounced change in fluorescence. SAL exposure results in the smallest fluorescence increase (Figure 7).

Discussion

Surface immobilization of proteins has a wide variety of applications ranging from the study of protein structure and function at the single molecule level, to chip-based high-throughput screening strategies for characterization of the function and interactions of a large series of proteins. However, immobilization may lead to loss of function as a result of nonspecific interactions (either polar or nonpolar) between the protein and the surface. This is particularly true for polytopic membrane proteins, which have both polar and nonpolar surfaces. To minimize these nonspecific interactions, we sequentially layered the glass surface with biotinylated BSA and avidin. Attachment of the receptor to this two-layer protein buffer was accomplished either directly by biotinylation of the amino terminus of the TMR-5-β_2AR(S8C) (Figure 3B), or through biotinylated M1 antibody (Figure 3A), which recognizes the amino terminal FLAG epitope on the receptor. The functional response of the immobilized receptor to the agonist ISO was assayed by monitoring the intensity of the conformationally sensitive fluorescent reporter in real time by fluorescence microscopy.

We observed an agonist-induced increase in fluorescence in TMR-5-β_2AR(S8C) immobilized by direct biotinylation and in TMR-5-β_2AR immobilized through biotinylated M1 antibody. Immobilization mediated by M1 antibody resulted in a functional response (Figure 6 and 7) nearly identical to that observed

with TMR-5-β_2AR in solution (Figure 2). Moreover, the agonist-induced signal is large enough to detect by visual inspection of an ICCD camera image. Thus, this approach could be used for drug screening of receptor arrays. Moreover, immobilization by M1 antibody should be readily applicable to other GPCRs.

The β_2AR could also be efficiently immobilized by direct biotinylation of the modified protein TMR-5-β_2AR(S8C) (Figure 3B, 4C). However, the agonist-induced response in TMR-5-β_2AR(S8C) was smaller and less consistent than for the receptor immobilized through the antibody (Figure 6B). The smaller response observed in directly immobilized TMR-5-β_2AR(S8C) is most likely caused by the quality of the protein. Expression of TMR-5-β_2AR(S8C) was about 50% of that of the wild-type β_2AR. This lower expression may be caused by the S8C mutation, but may also be a result of the fact that it was expressed under the control of the basic protein promoter, while the wild type receptor was expressed under the control of the polyhedrin promoter. The basic protein promoter is less efficient than the polyhedrin promoter, but when TMR-5-β_2AR(S8C) was expressed under the control of the polyhedrin promoter most of the protein was retained in the endoplasmic reticulum/Golgi. When TMR-5-β_2AR(S8C) was expressed under the control of the basic protein promoter most of the receptor was localized on the plasma membrane. Since cell surface expression is essential for selective biotinylation of the amino terminus of TMR-5-β_2AR(S8C), we had to use the less efficient promoter.

The approach described here for immobilization of TMR-5-β_2AR differs from the previously reported immobilization of rhodopsin[11] and the CCR5 receptor[12] in several ways. The method reported here does not require the formation of a lipid bilayer, which may limit access to one surface of the receptor. Immobilization of receptors in detergent micelles facilitates access to both cytoplasmic and extracellular surfaces of the receptor. Thus, by using this approach it may be possible to study agonist-dependent interactions between the immobilized receptor and soluble proteins such as G proteins and arrestins. This approach does not require the use of purified G proteins or fluorescent ligands to detect receptor activation. Agonist-induced conformational changes in TMR-5-β_2AR can be detected directly by fluorescence microscopy (Figure 5). However, the applicability of this technique is dependent on the quality of the immobilized protein and the presence of an environmentally sensitive fluorophore in a critical region of the GPCR. Therefore, this approach will be limited to those GPCRs that can be purified and labeled in a functional state.

This work addresses the special challenges of generation of arrays of integral membrane proteins by demonstration of a simple method for the immobilization of a functional, detergent-solubilized, ligand-activated GPCR on glass and gold surfaces. These technical developments demonstrate the feasibility of chip-based approaches for drug screening and the monitoring of interactions between GPCRs and other signaling proteins. In addition, it may be possible to use single molecule spectroscopy to study conformational changes in single receptor molecules over time, which will yield new insights into the mechanism of agonist activation.

Experimental Section

Materials: Strepatvidin, avidin, biotin–maleimide, benzamidine, Igepal, (−)-isoproterenol, M1 FLAG antibody, and alprenolol were purchased from Sigma; tetramethylrhodamine-5-maleimide, BODIPY 507/545 IA, Alexa 488 maleimide, and PyMPO maleimide from Molecular Probes (Eugene, OR); Cy5 maleimide from Amersham Biosciences (Piscataway, NJ); leupeptin from Boehringer (Mannheim, Germany); nickel-chelating Sepharose from Pharmacia (Uppsala, Sweden); BSA–biotin, EZ-Link *N*-hydroxysulfosuccinimidobiotin (sulfo-NHS-biotin) from Pierce (Rockford, Il); *N*-dodecyl-β-D-maltoside (DDM) from Anatrace (Maumee, OH). The vectors pVL1392 and pAcMP2 were ordered from Pharmigen (San Diego, CA).

Buffers: HS buffer: NaCl (0.5 M), tris(hydroxymethyl)aminomethane (20 mM), pH 7.4. HS/DDM buffer: HS buffer, DDM (0.1%). HS/DDM/Ca buffer: HS/DDM buffer, $CaCl_2$ (2 mM).

Expression, purification, and labeling of β_2AR: Expression of human β_2AR in Sf-9 insect cells, membrane preparation, solubilization, and purification were performed as described previously.[8] The wild-type receptor was cloned into vector pVL1392 and the mutant β_2AR(S8C) was cloned into vector pAcMP2. In order to biotinylate β_2AR(S8C), intact Sf-9 cells (5×10^6) were resuspended in PBS (100 mL) and stirred with biotin–maleimide (1 mM) for 1 hr at room temperature. The addition of a ten-fold molar excess of cysteine terminated the biotinylation reaction. For fluorescence labeling, purified β_2AR (1 μM) was mixed with an equimolar concentration of tetramethylrhodamine-5-maleimide in HS/DDM. After incubation for 2 hr at room temperature, the labeling reaction was terminated by addition of a 100-fold excess of cysteine. ALP-affinity chromatography was used to separate functional from nonfunctional β_2AR as previously described.[13] The eluent from the ALP resin was purified by Ni chromatography then dialyzed against HS/DDM buffer. Fluorescence spectroscopy was performed on a SPEX Fluoromax (Jobin Yvon Horiba, Edison, NY) in photon counting mode at room temperature, as previously described.[8]

Biotinylation of M1 antibody: M1 antibody (50 μM) was incubated with a 12-fold molar excess of EZ-Link sulfo-NHS-biotin in NaCl (100 mM), glycerol (10%), and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (50 mM, pH 8.0) for 1 hr at room temperature. Excess EZ-Link sulfo-NHS-biotin was removed by dialysis against HS buffer.

Protein immobilization: Coverslips (24 × 50 mm, thickness 0.17 mm, VWR Scientific, Media, PA) were cleaned by overnight incubation in sulfuric acid (5 M), and by subsequent ultrasonication in the detergent Igepal (2%) and Milli-Q water (Millipore, Bedford, MA) for 10 minutes each. Cover wells (Molecular Probes, Eugene, OR) were mounted on the coverslips. Each well held up to 80 μL. The wells were then incubated with BSA–biotin (1 mg mL^{-1}) in PBS (40 μL) for 5 minutes. After extensive rinsing with HS buffer, the wells were incubated with avidin or strepavidin (1 mg mL^{-1}) in HS buffer (40 μL) for 5 minutes. The TMR-5-β_2AR and TMR-5-β_2AR(S8C) samples were rinsed with HS buffer then immobilized as follows: Biotinylated TMR-5-β_2AR(S8C) (40 μL, 500–600 nM) was added to the wells and incubated for 5–10 minutes. The slides were then rinsed and filled with HS/DDM buffer (80 μL) before use. For immobilization of the TMR-5-β_2AR, the wells were incubated with M1–biotin (6 μM) in HS/Ca buffer (40 μL) for 15 minutes. The wells were then rinsed with HS/DDM/Ca buffer and were incubated with a solution of TMR-5-β_2AR (0.4–2.0 μM) in HS/DDM/Ca buffer for 30 minutes. The wells were finally rinsed and filled with HS/DDM/Ca buffer (80 μL) directly before measurement.

Surface plasmon resonance measurements: SPR measurements of the immobilized proteins were conducted on a Spreeta miniaturized SPR sensor (Texas Instruments, Dallas, TX). A flow cell with an approximate volume of 15 μL was positioned above an unmodified gold sensing surface and protein solutions were injected through the flow cell with a 0.5-mL syringe. Enough protein solution (approximately 25 μL) was injected to ensure that the entire sensing surface was covered. Refractive index (RI) changes of the solution adjacent to the surface were monitored over time. Protein solutions were allowed to remain in contact with the surface until a stable RI value indicated that the binding had reached equilibrium. Protein-free buffer solutions (0.25 mL) were then injected to rinse off nonspecifically bound proteins. Between experiments the sensing surface was cleaned by washing with NaOH (100 mM) and Triton-X (1%) in Milli-Q water, followed by several washes with Milli-Q water. This cleaning procedure effectively removed all the layers of deposited protein, as indicated by a return of the measured RI value to that of pure water (1.333).

Fluorescence measurements of immobilized receptors: Fluorescence measurements of immobilized proteins were conducted on a home-built microscope. Fluorescence excitation was achieved by use of the 514 nm emission line of an argon-krypton laser (Melles Griot, Irvine, CA). The laser beam was focused into the back aperture of a microscope objective (100×, Fluar, oil immersion, numerical aperture = 1.3, infinity corrected, Zeiss, Thornwood, NY) by use of a dichroic mirror (565DRLPXR, Omega Optical, Brattleboro, VT). The fluorescence emission of the immobilized proteins was collected by the microscope objective, passed through the dichroic mirror, filtered by a bandpass filter (580DF60, Omega Optical), and imaged (Achromat, $f = 160$ mm, Linos Photonics) onto an ICCD (I-PentaMAX, Princeton Instruments, Trenton, NJ). The laser power was set to 70–150 μW, as measured in front of the microscope objective. The intensifier of the ICCD and the length of the measurement (1–2 seconds per frame) were adjusted according to the emitted fluorescence intensity, but were kept constant during one measurement series. Pictures were taken at intervals of 1 (ALP) or 2 (ISO) minutes after addition of the ligand. A homogenous surface area comprised of 400–900 pixels (1 pixel ≈ 150 nm) was chosen for evaluation of fluorescence intensities. The values of the pixels in a chosen surface area varied typically by 5–10% (1 standard deviation). This variation results in a standard error in the mean of the average intensity of the surface area of usually less than 1%.

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