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Activation of G-Protein-Coupled Receptors in Cell-Derived Plasma Membranes Supported on Porous Beads

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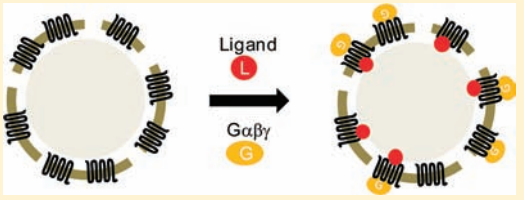
Supporting Information

ABSTRACT: G-protein-coupled receptors (GPCRs) are ubiquitous mediators of signal transduction across cell membranes and constitute a very important class of therapeutic targets. In order to study the complex biochemical signaling network coupling to the intracellular side of GPCRs, it is necessary to engineer and control the downstream signaling components, which is difficult to realize in living cells. We have developed a bioanalytical platform enabling the study of GPCRs in their native membrane transferred inside-out from live cells to lectin-coated beads, with both membrane sides of the receptor being accessible for molecular interactions. Using heterologously expressed adenosine A_{2A} receptor carrying a yellow fluorescent protein, we showed that the tethered membranes comprised fully functional receptors in terms of ligand and G protein binding. The interactions between the different signaling partners during the formation and subsequent dissociation of the ternary signaling complex on single beads could be observed in real time using multicolor fluorescence microscopy. This approach of tethering inside-out native membranes accessible from both sides is straightforward and readily applied to other transmembrane proteins. It represents a generic platform suitable for ensemble as well as single-molecule measurements to investigate signaling processes at plasma membranes.



INTRODUCTION

The seven transmembrane (7TM) helical G-protein-coupled receptors (GPCRs) detect a variety of physical and chemical extracellular signals (photons, neurotransmitters, hormones, and odorants to mention a few) and transduce them across the plasma membrane to induce intracellular reactions of utmost importance for proper cell functioning.[1−9] Due to their central role in cellular signaling, GPCRs are among the most important targets for modern medicine.[5,6] Typically, a GPCR is activated by the binding of an agonist, initiating a signaling cascade with the formation of a ternary complex composed of agonist, GPCR, and guanosine diphosphate (GDP)-bound G protein. This complex has a short lifetime in living cells, depending on the kinetics of nucleotide exchange at the G protein, which is modulated by the cellular spatial concentration distributions of the different signaling components involved.[5,10−12] *In vitro*, such ternary signaling complexes are stable in the absence of nucleotides and have been investigated in isolated cell membranes[11,13,14] or using detergent-solubilized components.[15−19]

Several conceptually different approaches have been shown valuable to investigate GPCR-mediated signaling, each having its specific advantages and disadvantages: (i) Live primary cells comprising native GPCRs and their signaling network are the biologically most relevant systems to measure cellular responses after receptor activation although in this case it is challenging to access the intracellular effectors with probes to monitor their participation in signal transduction.[20,21] (ii) Mammalian cells heterologously expressing GPCRs and relevant signaling proteins[4,22−25] are an alternative because the receptors together with their intracellular signaling proteins can be co- and post-translationally labeled with optical probes for optical imaging.[26−28] However, it remains very cumbersome to control the concentration of all expressed and/or labeled components and to distinguish them from endogenous competing cellular components. (iii) Cell-derived plasma membrane fragments comprising the desired GPCRs and parts of the cellular signaling proteins offer the possibility to access the intracellular plasma membrane surface, e.g., for controlled labeling or reconstitution of missing downstream secondary messengers.[11,13,29−34] (iv) Reducing cellular complexity by reconstituting signaling complexes in micelles, artificial lipid bilayers, or lipid nanodiscs is an attractive complementary approach.[14−18,35−37] However, this requires purification of the receptors and their interacting partners, which is challenging and time-consuming.[37,38]

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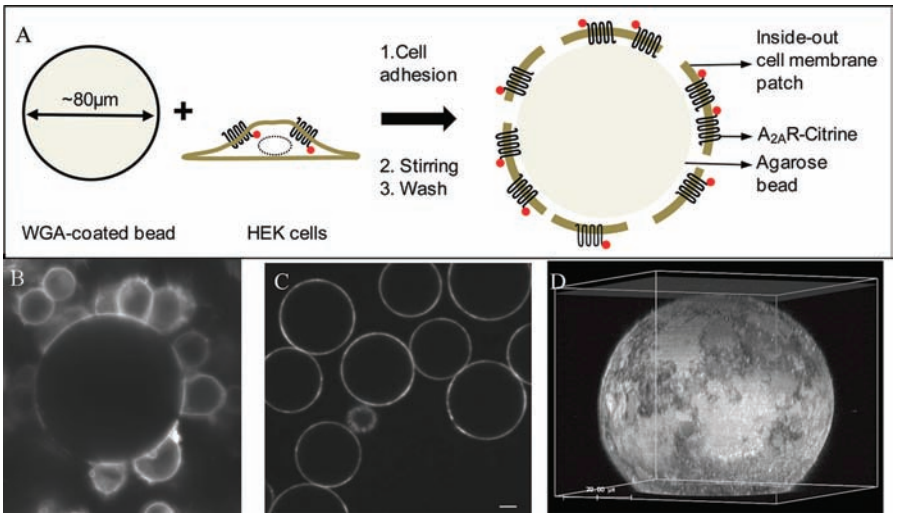

Figure 1. Preparation and imaging of cell membrane-coated beads. (A) Strategy to transfer plasma membranes from living cells to wheat germ agglutinin (WGA)-coated agarose beads. (B) Confocal fluorescence micrograph showing the cross-section of a single bead with densely packed HEK cells attached to its surface. The emitted fluorescence stems from A_{2A}R-Citrine expressed at the cell plasma membrane. (C) Confocal fluorescence micrograph of beads after cell disruption. The fluorescence at the circumference of the beads results from A_{2A}R-Citrine in plasma membranes coating the beads. (D) 3D reconstitution of a bead coated with A_{2A}R-Citrine containing plasma membrane sheets. The image was obtained by deconvolution of a z-stack of confocal cross-sections.

Solid supported membranes are of utmost importance in the present context.[39−48] Classically, they have been used to investigate artificial proteo-lipid membranes by microscopic and surface-sensitive techniques, allowing the selective observation of membrane processes in real time without interference from the bulk. Considering the fact that the function of GPCRs critically depends on the composition of their lipid environment, GPCR signaling studies in native supported membranes are particularly interesting. Different methods have been developed to transfer native cell plasma membranes to planar[48−57] and spherical[58−68] solid supports. They are principally based on unspecific interactions of the surface of the plasma membrane with the surface of the solid support by either (i) binding living cells to the supporting material and subsequently disrupting the cell by applying mechanical shear forces[50,51,54,55,57,59−61,64,65] or (ii) first preparing plasma membrane vesicles from cells which are subsequently bound to solid surfaces under conditions in which they open to planar membrane sheets.[49,52,53,56,58,63,64,67−69]

Until now, supported native membranes were used to isolate cell membranes for biochemical analysis or characterization by optical and atomic probe microscopies, yet no studies have been reported using supported membranes with freely accessible intra- and extracellular membrane surfaces, enabling investigation of GPCR activation and downstream signaling reactions under controlled conditions in real time.

Here, we demonstrate how binding of ligands to GPCRs and subsequent interactions with G proteins can be observed on single micrometer-sized beads. For this, GPCR-containing plasma membranes have been transferred from live cells and tethered through their cell surface-exposed glycosylated proteins to lectin-coated porous agarose beads, as illustrated in Figure 1. Our approach offers a number of advantages which are generally applicable for the investigation of membrane proteins and their interactions with other components in a membrane environment: (i) GPCRs remain in their native plasma membrane, displaying native functionality and avoiding cumbersome detergent-solubilization, purification, and membrane reconstitution. (ii) Both intra- and extracellular sides of the receptors are accessible to signaling molecules, allowing quantification of interactions. (iii) The inside-out orientation of the tethered plasma membrane makes its intracellular surface accessible for post-translational modifications. (iv) The bead geometry enhances fluorescence signal-to-noise ratios because confocal imaging elevated above the coverslip avoids the detection of fluorescent molecules nonspecifically adsorbed to the glass surface. (v) The high affinity between the glycosylated extracellular plasma membrane surface and the lectin on the beads yields long-term mechanical stability for storage and analysis. (vi) Down-scaling the bioanalytics to single micrometer-sized beads saves substantially the consumption of material and opens possibilities for extending the analysis to an array of beads with potential applications in high-content, high-throughput formats of functional drug screening.

In our present study, we used a prototypical GPCR, the A_{2A} adenosine receptor (A_{2A}R) genetically fused to the yellow fluorescent protein citrine for optical monitoring. Receptor-specific ligands and $G\alpha o_B$ proteins were labeled with different fluorescent probes, allowing us to study the interaction of agonists, antagonists, and G proteins with the A_{2A}R under defined conditions. Although A_{2A}R signaling is usually mediated by $G\alpha s$ proteins, we show here that the assembly and disassembly of ternary complexes can be observed in real time upon addition of $G\alpha o_B$-$\beta\gamma$ to liganded A_{2A}Rs. For simplicity, we use in the following the term $G\alpha$ to represent $G\alpha o_B$.

EXPERIMENTAL SECTION

Preparation of Cell Membrane-Coated Beads. A suspension of wheat germ agglutinin (WGA)-coated beads was added dropwise to a suspension of HEK293 TRex cells expressing the A_{2A}R fused to mCitrine (A_{2A}R-Citrine) at the C terminus (see Supporting Information SI0). The sample was incubated for 20 min at 4 °C under gentle stirring to allow binding of the cells to the beads (Figure 1B). Unbound cells were removed by two cycles of centrifugation (2 min at 50 rcf). Vigorous vortexing for 2 min sheared off bead-attached cells, leaving plasma

membrane sheets oriented inside-out tethered on the beads. The cell membrane-coated beads were washed by three centrifugation/washing cycles (2 min, 18 rcf). The average number of A_{2A}R-Citrine receptors expressed per cell was assessed by measuring the binding of the radioligand ^{3}H-ZM 241385 (Supporting Information SI1). The potential presence of fragments of endoplasmic reticulum (ER) or Golgi on cell membrane-coated beads was tested using HEK293 cells transfected with either EYFP-ER or EYFP-Golgi, respectively (Supporting Information SI0).

Competitive Ligand Binding. A 0.1 nM concentration of receptors on cell membrane-coated beads was incubated for 2 h at 4 °C under gentle agitation in 60-μL samples comprising a 50 or 100 nM concentration of the fluorescent antagonist XAC-Atto655 in the presence of 1 nM to 10 μM of either ZM241385 (ZM) or XAC as competitor in buffer comprising 20 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 0.2 mg/mL BSA (TNMEB buffer). Under these conditions, the concentrations of free ligand and competitor could be approximated by their total concentrations. ZM binding was measured in duplicate; XAC binding was measured from single data points. Nonspecific binding was determined on beads coated with cells lacking the heterologous expression of A_{2A}R-Citrine. Competitive binding data analysis is described in Supporting Information SI0.

Binding of G Proteins to GPCRs on Native Membrane-Coated Beads. Fluorescent heterotrimeric G proteins (G$\alpha\beta\gamma$-Atto647) were reconstituted using purified His_6-Gα, purified G$\beta\gamma$ proteins containing their native membrane anchor, and the fluorescent probe Tris-NTA-Pro8-Atto647N that specifically binds to the polyhistidine sequence on Gα (hereafter named Atto647)[70] (Supporting Information SI3.1). The binding of 0.1−113 nM labeled heterotrimeric G protein to 0.1 nM A_{2A}R-citrine on the beads was studied in 30 μL of TNMEB buffer in the presence of a final concentration of 1 μM GDP. Two separate binding curves were obtained using different batches of cells and beads. Nonspecific binding of G$\alpha\beta\gamma$-Atto647 to the membranes was determined using beads coated with plasma membranes devoid of receptors and was subtracted from total binding. The experimental points were fitted as described in Supporting Information SI3.2 to obtain the dissociation constant K_D of the complex G$\alpha\beta\gamma$-A_{2A}R.

Kinetics of Assembly and Disassembly of Ternary Complexes between Ligand, GPCR, and G Protein on Beads. The fluorescent probe Tris-NTA-Pro8-Atto565 (hereafter named Atto565) was used to label the G protein in order to enable the simultaneous detection of APEC-Atto633 and G$\alpha\beta\gamma$-Atto565 binding to A_{2A}R-Citrine. A 0.3 nM concentration of A_{2A}R-Citrine on beads was incubated with a 60 nM concentration of APEC-Atto633 in TNMEB buffer for 2 h. APEC-bound beads in 20 μL of buffer were placed on a coverslip and mixed with 40 μL of a solution containing 17 nM G$\alpha\beta\gamma$-Atto565 and 7 nM of G$\alpha\beta\gamma$, together with 0.5 μM GDP. The fluorescence in the three separate detection channels (Citrine, Atto565, and Atto633) was recorded every 34 s over a period of 45 min. The experiment was repeated three times, and the depicted fluorescence intensity time courses were normalized by the initial value of fluorescence monitored just after addition of G proteins. The control experiment involved addition of TNMEB buffer to beads coated with cell membranes comprising liganded A_{2A}Rs. To determine the apparent association and dissociation rate constants of APEC and G proteins, fluorescence time courses were fitted by monoexponential functions.

Citrine Quenching. Changes of the fluorescence of A_{2A}R-Citrine upon addition of G$\alpha\beta\gamma$-Atto647, G$\alpha\beta\gamma$, or G$\beta\gamma$ were monitored on beads comprising receptor-G protein complexes in 30 μL of TNMEB buffer with GDP at a final concentration of 1 μM.

Image Acquisition and Analysis. Binding of the different components to the beads was measured by confocal imaging at room temperature of samples transferred onto 0.16-mm-thick glass coverslips. Cell membrane-coated beads were preincubated with G proteins and ligands at 4 °C for 1 or 2 h, respectively, in polypropylene reaction tubes. Four images representing the cross sections of at least two beads were analyzed per sample. A procedure was established to automate the analysis on the confocal cross sections of the beads (Igor Pro, WaveMetrics) (Supporting Information SI0). The error bars depicted in the graphs correspond to the standard errors of the values of fluorescence intensities on the images. Unless stated otherwise, scale bars correspond to 20 μm.

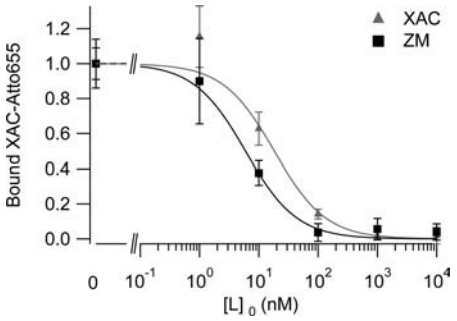

Figure 2. Competitive ligand binding experiments on cell membrane-coated beads. XAC-Atto655 bound to A_{2A}R-Citrine on beads was replaced by competitive binding of nonfluorescent ligands ZM and XAC. One-site binding model fit yields IC_{50} values of 6 ± 5 nM (ZM) and 17 ± 7 nM (XAC).

RESULTS

1. GPCRs on Cell Membrane-Coated Beads. *1.1. Characterization of Cell Membranes Immobilized on Beads.* Cells attach densely packed to agarose beads (Figure 1B). Application of mechanical shear disrupted the cells and yielded beads covered with inside-out plasma membranes (Figure 1C,D and Supporting Information SI4). Considering that (80 ± 5)% of the bead surface is covered by cell membranes (Figure 1D) and a receptor cell surface density of 1.5 × 10^3 A_{2A}R-Citrine/μm^2 (Supporting Information SI1), a bead comprises about 2 × 10^7 A_{2A}R-Citrine in the absence of multilamellar membrane sheets. The absence of fragments of the ER and of the Golgi apparatus, which both could contain nonfunctional receptors, was demonstrated using plasma membrane-coated beads prepared with cells expressing EYFP-ER and EYFP-Golgi (Supporting Information SI0). As no EYFP fluorescence signal could be detected, the receptors on the beads originate exclusively from the plasma membrane.

1.2. Ligand Binding to A_{2A}R-Citrine on the Beads. Incubation of the antagonist XAC-Atto655 with A_{2A}R-Citrine-containing membrane-coated beads resulted in the appearance of a bright fluorescence signal at the surface of the beads. Unspecific binding of XAC-Atto655 was assessed on beads coated with plasma membranes devoid of A_{2A}R-Citrine. We found that 20−30% of total binding stemmed from nonspecific binding (Supporting Information Figure SI2). Similar observations were made for the agonist APEC-Atto633. Hence, nonspecific binding has been systematically measured and subtracted from total binding for quantitative analysis.

The potential of cell membrane-coated beads in pharmacological ligand-screening assays is demonstrated in Figure 2: XAC-Atto655 bound to A_{2A}R-Citrine on beads was replaced by competitive binding of the nonfluorescent antagonists ZM241385 or XAC, yielding IC_{50} values of 6 ± 5 or 17 ± 7 nM, respectively. We calculated dissociation constants of 2 ± 2 nM for XAC-Atto655 and 0.3 ± 0.1 nM for XAC using the

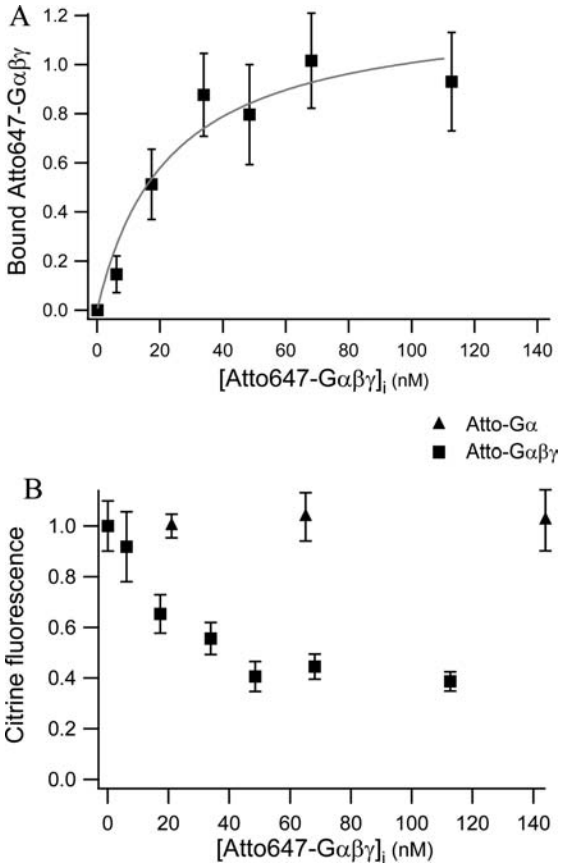

Figure 3. Binding of heterotrimeric G proteins to $A_{2A}R$ on beads. (A) Specific binding of G$\alpha\beta\gamma$-Atto647 to beads, yielding $K_D = 23 \pm 9$ nM ($\chi^2 = 0.007$). (B) Quenching of $A_{2A}R$-Citrine fluorescence upon addition of either G$\alpha\beta\gamma$-Atto647 or Gα-Atto647 to beads.

dissociation constant of the radioligand ^{3}H-ZM241385 measured on cells (Supporting Information SI1). These values are in good agreement with native ligand affinities.[32,71] Thus, although the extracellular membrane leaflet is oriented toward the bead surface, the ligand-binding site of GPCRs is accessible, and its main properties are preserved.

2. Reconstitution of the Ternary Complex between Ligand, GPCR, and G Protein on Beads. Since the native membranes on the beads expose their intracellular side to the bulk aqueous phase, they offer a unique possibility to reconstitute genetically engineered (e.g., fluorescent), purified heterotrimeric G proteins at defined concentrations and to examine in real time the formation and dissociation of the ternary complex between an activating ligand, its cognate GPCR, and a G protein.

2.1. Equilibrium Binding of Fluorescent Heterotrimeric G Proteins to Membrane-Coated Beads. The fluorescent heterotrimeric G proteins G$\alpha\beta\gamma$-Atto647 bound to cell membrane-coated beads either containing or devoid of $A_{2A}R$-Citrine (Supporting Information SI3.3). However, in the presence of $A_{2A}R$-Citrine, the binding of G proteins was significantly increased, demonstrating specific interaction between the GPCR and the G protein. After subtraction of the nonspecific binding of the G protein to receptor-devoid membranes, a K_D value of 23 ± 9 nM was calculated for the interaction between Atto647-G$\alpha\beta\gamma$ and $A_{2A}R$-Citrine (Figure 3A). Interestingly, Gα alone was not able to bind to the receptors (Supporting Information SI3.3).

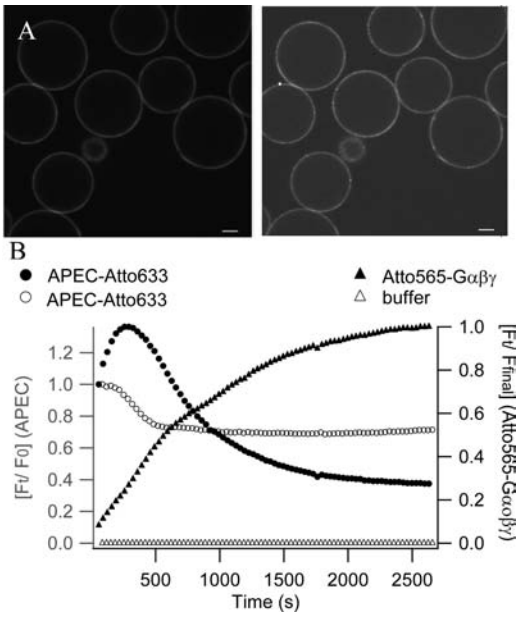

Figure 4. Binding of agonists and G proteins to cell membrane-coated beads. (A) Confocal microscope images showing simultaneous binding of 25 nM G$\alpha\beta\gamma$-Atto565 (left) and 20 nM APEC-Atto633 (right) to beads coated with cell membranes comprising $A_{2A}R$-Citrine. The corresponding citrine fluorescence is displayed in Figure 1C. (B) Kinetics of the binding of G$\alpha\beta\gamma$-Atto565 and APEC-Atto633 to beads coated with cell membranes comprising liganded $A_{2A}Rs$. Control experiment to estimate the effect of dilution involved addition of buffer to beads coated with cell membranes comprising liganded $A_{2A}Rs$. This is a representative experiment out of three used for quantitative analysis.

2.2. G Proteins as Modulators of Ligand–Receptor Affinity. The kinetics of the binding of G$\alpha\beta\gamma$ to $A_{2A}R$-Citrine on beads was studied using receptors preincubated with agonist. The binding of ligands and of G proteins to the $A_{2A}R$ on the beads was measured simultaneously using G$\alpha\beta\gamma$-Atto565 and APEC-Atto633 (Figure 4A). The association of G$\alpha\beta\gamma$-Atto565 to the membranes is indicated by the increase of Atto565 fluorescence on the beads (Figure 4B). The presence of G proteins changed the initially established binding equilibrium between APEC-Atto633 and $A_{2A}R$-Citrine: first APEC-Atto633 fluorescence on the beads increased, reaching a maximal ligand binding, and then the signal decreased, leading to a new steady-state equilibrium (Figure 4B). In control experiments adding only buffer to beads coated with cell membranes comprising liganded $A_{2A}Rs$, no enhancement but rather a 34% decrease of the initial fluorescence intensity of APEC-Atto633 was observed due to the increase of the sample volume and a concomitant dissociation of the ligand (Figure 4B). From this a K_D value of 12 ± 3 nM was calculated for the ligand–receptor complex. This is in good agreement with the value of $K_D = 20 \pm 2$ nM we obtained from competitive binding of APEC-Atto633 and ^{3}H-ZM241385 to $A_{2A}R$ (not shown).

Fitting the kinetics of G protein binding to the supported membranes monoexponentially yielded an apparent association rate constant of $(2 \pm 2) \times 10^{-3}$ s^{-1}. Similarly, we determined APEC-Atto633 apparent rate constants for the initial association and following dissociation as $(2 \pm 1) \times 10^{-2}$ and $(2.6 \pm 0.1) \times 10^{-3}$ s^{-1}, respectively.

2.3. Quenching $A_{2A}R$-Citrine Fluorescence upon Addition of G$\beta\gamma$ Interestingly, upon addition of Atto647-G$\alpha\beta\gamma$, but not of

Atto647-Gα, to the membrane-coated beads, the citrine fluorescence of A_{2A}R-Citrine was quenched in a concentration-dependent manner (Figure 3B) for yet unknown reasons.

DISCUSSION

Taking these results together, we have developed a bioanalytical platform for investigating membrane protein receptors and their signaling cascades in supported native plasma membranes. Typical major hurdles encountered by receptors integrated in membranes tethered to a solid support are the conservation of their functionality and the accessibility of their extra- and intracellular binding sites.[36] Here we have solved these problems by transferring native plasma membranes from live cells to porous agarose beads. Selective and high-affinity binding of the glycosylated extracellular plasma membrane surface to the lectin-coated beads yielded an inside-out membrane orientation. The cellular membranes covering the beads comprise fully functional GPCRs: the receptor's extracellular binding site was accessible at the membrane−bead interface, as demonstrated by the binding of agonists, while the intracellular membrane surface could be accessed by G proteins from the bulk aqueous phase. Our method is simple, and preliminary results show that it can be readily applied to other transmembrane receptors, such as the neurokinin 1 receptor, a GPCR, and the 5-HT_3 receptor, a serotonin-gated ion channel (Supporting Information SI5).

We have demonstrated the versatility of our system using a representative GPCR, the A_{2A}R, which is endogenous in the central nervous system and in endothelial cells, and can be functionally expressed in heterologous HEK cells.[72] The specific affinities (K_D values) of the fluorescent agonist APEC-Atto633 and the antagonist XAC-Atto655 to the A_{2A}R-Citrine determined here for the tethered membranes were similar to those reported elsewhere in heterologous cells.[29,32] This shows that the receptor's ligand binding activity is preserved in the native membranes tethered to the beads.

A central result of our study is the functional reconstitution of the complex between a heterotrimeric Gα_{0B}-$\beta\gamma$ protein and the A_{2A}R-Citrine in the tethered native membranes. Formation of this complex could be quantified by measuring the appearance of the fluorescently labeled G protein on the bead. Although the Gα protein subunit alone was not able to bind to the receptors on the beads, the heterotrimeric G$\alpha\beta\gamma$ proteins did bind with high affinity. The measured K_D value of 23 ± 9 nM is comparable to affinities reported elsewhere for other G-protein/GPCR systems.[15,18,33,73,74]

Interesting in this context are reports showing that (i) non-palmitoylated and non-myristoylated Gα subunits are not able to bind to the plasma membrane in the absence of G$\beta\gamma$ subunits,[75−77] and (ii) G$\beta\gamma$ subunits are required to reconstitute high-affinity agonist binding and receptor-catalyzed GTPγS binding using purified Gα proteins and GPCRs in native membranes.[30,31,74−76,78,79]

Heterotrimeric G$\alpha\beta\gamma$ proteins were binding also to cell membrane-coated beads in the absence of A_{2A}R-Citrine but to a much smaller extent than in the presence of A_{2A}R, presumably as a result of favorable lipophilic interactions of the G$\beta\gamma$ lipid anchor with the tethered membranes.

The reconstitution of G proteins into the tethered membranes on beads induced a biphasic response of the fluorescence of the receptor-bound agonist. The receptor first adopts a high ligand-affinity state during formation of the ternary complex between agonist, receptor, and G protein, followed by a destabilization of this complex, as manifested by the dissociation of the fluorescent ligand. The kinetics of the release of the ligand (k_{diss} = 2.6 × 10^{-3} s^{-1}) compares well with ligand dissociation rates from ternary complexes measured with solubilized N-formyl peptide receptor (k_{diss} = 6.5 × 10^{-3} s^{-1})[15] or in permeabilized neutrophil cells (k_{diss} = 1 × 10^{-3} s^{-1}).[80] Transient dissociation of such ternary complexes has been observed in other systems,[15,19,80] but previous studies did not investigate ternary complex formation in real time. As the concentration of the individual partners can be controlled independently in a defined manner and under physiological relevant conditions, our system is ideally suited for investigating the thermodynamics and kinetics of such complex biochemical networks in more detail in the future.

CONCLUSION

The fact that the inside-out oriented plasma membranes on porous agarose beads can be accessed from both the extra- and the intracellular surface offers a unique possibility to reconstitute complex signaling networks on the intracellular plasma membrane side using isolated native or recombinant signaling proteins which can be equipped with suitable probes to be distinguished in situ by optical and scanning probe microscopies or after fixation/freezing by electron microscopies. The field of applications can be extended beyond GPCR signaling toward membrane protein signaling networks in general. Ensemble or single-bead analysis can be performed in bulk as well as in microfluidic volumes, with potential for further miniaturization and interesting bioanalytical applications for pharmaceutical, pharmacological, and medical screening and diagnostics.

ASSOCIATED CONTENT

Supporting Information. Materials, experimental methods, and additional experimental data; Figures SI1−5; complete refs 9, 21, 43, and 64. This material is available free of charge via the Internet at http://pubs.acs.org.

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Panel B of Figure 1 and panel A of Figure 4 have been revised and this paper reposted ASAP October 4, 2011.