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HDL-like discs for assaying membrane proteins in drug discovery

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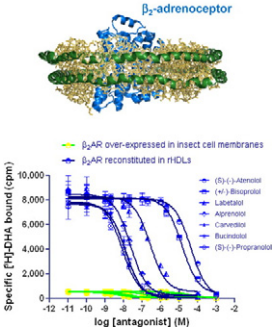
HIGHLIGHTS

- A SPA-based homogeneous assay allowing the study of ligand binding to the β_2AR.
- β_2AR has the same pharmacology in rHDLs than in cell membranes for a set of antagonists.
- Antagonist binding is right-shifted to lower affinities for the solubilized receptor.
- rHDL-like discs can be used for compound screening and lead optimization.

GRAPHICAL ABSTRACT



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ABSTRACT

To broaden the use of the recombinant high-density lipoprotein (rHDL) approach to the characterization of lead compounds, we investigated the pharmacology of the human beta-2-adrenoceptor in nanolipid bilayers (rHDL) with a broad set of beta-adrenoceptor antagonists. To that end, we developed a homogeneous copper-chelate scintillation proximity binding assay (SPA) in order to compare receptor-ligand binding affinities before and after reconstitution into rHDLs. Our results clearly show that the beta-2-adrenoceptor reconstituted in rHDLs display the same pharmacology as that in cell membranes and that rHDLs can be used not only to measure affinities for a range of ligands but also to study binding kinetics.

1. Introduction

Membrane-spanning proteins are important drug targets, as their activities and the pathways they mediate are relevant in regulating disease processes. There are technical limitations in the mechanistic understanding and application of membrane proteins to drug discovery because of the difficulties in isolating them from the membrane while maintaining function. Thus, drug discovery on membrane proteins is mainly carried out using cells expressing the membrane protein of interest or membrane preparations carrying a mixture of various endogenously expressed proteins. Therefore, the execution and interpretation of biochemical assays on membrane proteins is hampered by the presence of endogenous, related proteins and indirect effects. Furthermore, thermodynamic and kinetic binding studies are powerful tools in drug discovery but interpretation is complex and further complicated by the use of recombinant membrane preparations or tissue which may contain related receptor subtypes.

To study the function of isolated membrane proteins, a new technology has emerged: the so-called membrane discs or reconstituted

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high density lipoprotein (rHDL) particles [1–10]. In this approach, purified membrane proteins are assembled into a nanoscale phospholipid bilayer which is highly soluble in aqueous solution. These rHDLs are composed of approximately 160 phospholipids organized in a bilayer, which is encircled by two apolipoprotein A-I molecules (Fig. 1). The technology has been mainly applied to understand the function of single receptors [6–9] versus dimers [11] or other oligomeric species [12]. However, all these ligand-receptor studies were each limited to a few compounds only. An application to drug discovery has been hampered by the difficulty of preparation of rHDLs and the lack of suitable, higher throughput assays. The human β_2-adrenoceptor (β_2AR) is a frequently used model system in these studies [6,9]. β_2AR is an integral membrane, G-protein coupled receptor (GPCR), that is the focus of intense efforts in the field of asthma and COPD drug discovery and tools that allow detailed studies of receptor-ligand interaction are thus of great interest.

Our ultimate objective is to broaden the rHDL-approach to the characterization of lead compounds in order to facilitate membrane protein drug discovery. Here, we describe a strategy that overcomes these obstacles by combining optimized receptor expression, purification and reconstitution protocols with a commonly used assay technology (SPA). The resulting, novel, assay format is highly sensitive and high throughput-compatible. It not only allowed to measure binding affinities for a range of ligands of the β_2AR, but also to study binding kinetics.

Following their extraction from the membrane, receptors cannot be studied by classical filtration techniques as solubilized receptors in a detergent micelle or in a rHDL are about 10 nm in diameter and pass through the filter pores. As a consequence, the determination of receptor function in detergents or in nanobilayers has been limited to laborious equilibrium dialysis or gel-filtration methods. Here, we describe the development of a copper-chelate affinity-based scintillation proximity assay (SPA) to study ligand binding to the β_2AR in the membrane-like environment of a rHDL. This homogeneous radiotracer-binding assay was suitable for quick measurements and further used to optimize the reconstitution conditions of the purified receptor in rHDLs. In SPA, scintillant is incorporated into small fluomicrospheres or beads. If a radioactive molecule is bound to the bead, it is brought into close enough proximity that it can stimulate the scintillant contained within to emit light. Otherwise, the unbound radioactivity is too distant, the energy released is dissipated before reaching the bead, and these disintegrations are not detected. SPA binding assays avoid the usual filtration or washing procedures and are therefore often used in drug discovery where high throughput and ease of automation are required.

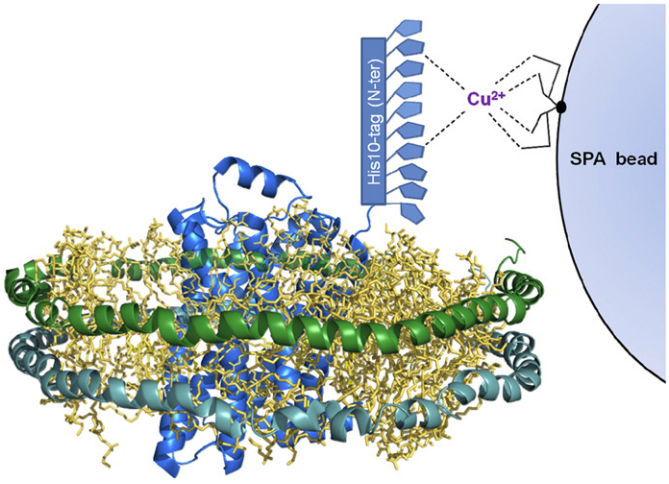

Fig. 1. Molecular model of the β_2AR reconstituted into a recombinant HDL particle (Protein Data Bank ID: 3D4S, 3J00). The receptor is rendered in blue color and the phospholipids are shown in orange. rHDLs are composed of a dimer of an amphipathic scaffold protein, called apolipoprotein (apoA-I), wrapped around a phospholipid bilayer composed of ~160 phospholipid molecules. Each apoA-I protein (cyan and green) is depicted as a ribbon diagram. Apolipoproteins have been shown to associate with phospholipids in vitro, self-assembling in discoidal-shaped particles very similar to the HDL vesicles produced by the liver, which enable lipids like cholesterol and triglycerides to be transported within the bloodstream. Recombinant HDLs can incorporate single molecules of integral membrane protein targets like the β_2AR through chemical self-assembly (see Methods). The resultant nanostructures, with diameters ranging from 10 to 20 nm, represent a highly stable and homogeneous population with an aqueous solubility in the millimolar range. Copper-chelate SPA beads (2–5 μM in diameter) are used to trap and quantify the association of radiolabelled ligands to the His_{10}-tagged β_2-adrenoceptor. Figures were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.).

2. Materials and methods

2.1. Cloning of human apoA-I and β_2AR

A codon optimized version of human apolipoprotein A-I (Uniprot: P02647) was cloned into a pET41a (Merck) derived *E. coli* expression vector using Gateway recombination technology [13]. A DNA fragment encoding APOA1 amino acids L68-Q267 was made by PCR with the primer pairs fwd-gatccCTGAAGCTGTTGGACAAT/rev-gcATTACTGGGTATTCAGCTT and fwd-cCTGAAGCTGTTGGACAAT/rev-ggccgcATTACTGGGTATTCAGCTT, respectively. The two PCR products were gel purified (NucleoSpin Extract II, Macherey-Nagel), unified, denatured and reannealed [14]. The resulting sticky-end PCR product was subsequently ligated with the large *Bam*HI-*Not*I fragment of the entry vector pGENT-PE. In a Gateway LR reaction, the gene of interest was subcloned into the expression vector pHisKan5. DNA encoding human β_2-adrenoceptor (RefSeq: NM_000024) was subcloned into a mammalian expression vector and into a baculovirus donor vector. Both vectors were previously prepared. The mammalian vector pACA-M11 was derived from pCMVSport1 (Invitrogen) whereas the baculo vector pACA-BG11 was derived from pBacPAK8 (Clontech) and features EGFP co-expression [15]. Both plasmids have a cleavable N-terminal Flag-His_{10} tag with a hemagglutinin signal peptide for protein secretion. The target gene was amplified by PCR using the primer pairs fwd-gggcCAACCAGGTAACGGCAG/rev-ttaCAGCAGTGAGTCATTTGTACTA and fwd-CAACCAGGTAACGGCAG/rev-aaccttaCAGCAGTGAGTCATTTGTACTA, respectively. Cloning was completed as described above via the type IIs restriction sites *Esp*3I of the expression vectors.

2.2. Expression and purification of apoA-I

Briefly, 50 ml of LB medium containing 50 μg/ml kanamycin were inoculated with a single colony from a fresh plate and the culture was grown at 37 °C with shaking (200 rpm) until OD_{600} reached 0.6–0.8 after 4–6 h. Two liters of sterilized TB medium, 50 μg/ml kanamycin were then immediately inoculated with 20 ml of starting culture and incubated at 37 °C with shaking. When OD_{600} reached 0.6 (~3 h), the temperature was lowered to 25 °C for 30 min before the culture was induced overnight with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were then harvested by centrifugation at 8000 ×*g* for 20 min and stored at −80 °C. For purification, 15-20 g of cells were resuspended in 200 ml of 50 mM sodium phosphate buffer pH 8.0 containing 300 mM NaCl, 20 mM imidazole, 5000 U benzonase (Merck) and complete protease inhibitor cocktail tablets (Roche). The cells were homogenized for 30 s with a Polytron PT-2100 homogenizer and lysed by two passages through an Avestin cell disruptor (10,000-15,000 psi). 0.5 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (Roche) and 1% Triton X-100 (Fluka) were added (30 min incubation with shaking at 4 °C) and the lysate was cleared by centrifugation at 25,000 ×*g* for 30 min. The cleared lysate was loaded onto 2×5 ml HisTrap HP columns (GE Healthcare). The columns were washed with 10 bed volumes (BV) of 50 mM sodium phosphate, pH

8.0, 300 mM NaCl, 20 mM imidazole and the apoA-I was eluted with a step gradient of 300 mM imidazole. Fractions containing apoA-I were pooled and mixed with 300 μL of PreScission protease (GE Healthcare) for His-tag cleavage and dialyzed overnight at 4 °C against 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole. Separation of the cleaved tag was achieved by a reverse HisTrap chromatography and the cleaved apoA-I was then loaded onto a Superdex 75 HiLoad 26/60 size exclusion column (GE Healthcare) in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol. ApoA-I fractions were pooled, concentrated to at least 10 mg/ml and stored at −80 °C.

2.3. Expression and purification of β_2AR

For transient expression, HEK293F cells were seeded at 1 million per ml in Free-Style medium (Invitrogen) and transfected with 1.5 mg plasmid DNA and 1.5 mg JetPEI (Polyplus-transfection) complexes per liter of suspension culture. Cells were then grown at 37 °C, 8% CO_2 for 72 h with constant shaking. For insect cell expression, high titer baculoviruses (10^9–10^{10} plaque-forming units/ml) were used to infect *Sf*21 suspension cultures at a multiplicity of infection of ~1. Cells were grown at 27 °C for 72 h in Ex-Cell 420 medium (Sigma) supplemented with 5% fetal calf serum (BioConcept Amimed). HEK293F and *Sf*21 cells were harvested at 1500 ×g for 15 min, washed with PBS buffer and stored at −80 °C until further use. Cell pellets (~20–30 g) were resuspended in 150 ml of 100 mM sodium phosphate, pH 7.5, 10% glycerol, 0.1 mM EDTA, complete protease inhibitor cocktail tablets, homogenized for 10 s with a Polytron PT-2100 homogenizer (speed set at 22,000 rpm) and disrupted by a single passage through an Avestin cell disruptor (10,000–15,000 psi). After a low-speed centrifugation step (3000 ×g, 4 °C, 10 min), cell membranes were isolated by centrifugation at 100,000 ×g for 1 h and washed once by homogeneization in a Potter-Elvejhem homogenizer (20 strokes) with 100 ml of 25 mM sodium phosphate, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, complete protease inhibitor cocktail tablets. Membranes were then recovered by centrifugation (100,000 ×g for 30 min) and resuspended in 90 ml of the same buffer. Solubilization of the receptor was initiated by addition of a mixture of *n*-dodecyl-β-D-maltopyranoside (DDM) (Anatrace) and cholesteryl hemisuccinate (CHS) (Sigma) to 1% and 0.1% concentration, respectively, to the membrane suspension. After homogeneization and 1 h incubation at 4 °C under stirring, NaCl was added to a final concentration of 0.5 M, and insolubilized material was removed by centrifugation at 100,000 ×g for 1 h. The supernatant was added to 5 ml of TALON Cobalt resin (Clontech) previously equilibrated with 10 bed volumes of 25 mM sodium phosphate, pH 7.5, 0.5 M NaCl, 10% glycerol, 0.1% DDM, 0.01% CHS (buffer A). After an overnight incubation at 4 °C under gentle agitation and extensive washing of the cobalt resin with buffer A (3× 10 BV, 5 min incubations, 700 ×g, 4 °C, 2 min in between each washing step), the receptor was eluted by 5× 1 BV of 200 mM imidazole in buffer A (10 min incubations). Fractions containing β_2AR were pooled and allowed to mix 2 h with 2 ml anti-Flag resin (Sigma) equilibrated on a gravity-flow column with 25 mM sodium phosphate, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% DDM, 0.01% CHS (buffer B). The resin was extensively washed in batch with buffer B (3× 10 BV, 10 min incubations), and the β_2AR was eluted with 0.2 mg/ml of the Flag peptide (Sigma) in buffer B (5× 1 BV, 10 min incubations). Elution fractions were concentrated down to 600 μl with an Amicon Ultra-15 (100,000 Da MWCO, Millipore). Four liters of HEK293/*Sf*21 cells typically yield 1 mg β_2AR, *i.e.* 500 μl of a 40 μM solution of receptor (the protein concentration was determined by using $\varepsilon_{280} = 67{,}840\ M^{-1}\ cm^{-1}$, established by amino acid analysis). All along the purification, the concentration of functional, purified receptor was determined using a scintillation proximity assay (SPA) and a saturating concentration (10 nM) of (−)-[^{3}H]-dihydroalprenolol (104.4 Ci/mmol, PerkinElmer). Receptor protein integrity was controlled using immunoblotting.

2.4. β_2AR–rHDL reconstitution procedure

Recombinant purified β_2AR was reconstituted with apoA-I and the lipid 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) (Avanti Polar Lipids) according to previously reported protocols [6,8]. Briefly, lipids were dried under nitrogen from a chloroform solution and placed in a vacuum dessicator for 30–60 min to remove residual traces of chloroform. Lipids were then solubilized as a 20 mg/ml stock solution in 25 mM Tris–HCl, pH 7.4, 75 mM NaCl, 100 mM sodium cholate (Sigma). Purified apoA-I was added to the β_2AR solution followed by the solubilized POPC. DDM and CHS were added from a 10% stock to make a final DDM and CHS concentrations of 0.1% and 0.01%, respectively. The apoA-I:POPC ratio was around 1:120 and the ratio of apoA-I:β_2AR was 10:1. The final volume of the reconstitution mixture was of 2 ml and the amount of receptor added was no more than 10% of the final reconstitution volume. Final concentration of POPC was 8 mM and well above the 4 mM minimum lipid concentration determined for efficient formation of disc complexes. The reconstitution mixture was incubated on ice for 1 h followed by addition of Bio-Beads SM-2 (Bio-Rad) at 0.5 g per ml reconstitution mixture to initiate the self-assembly process by removal of detergents. The mixture was incubated with Bio-Beads for 2 h on ice with gentle agitation, and the beads were removed by centrifugation at 3000 ×g for 2 min. A Superdex 200 HR 10/30 size exclusion chromatography column (GE Healthcare) was pre-equilibrated in a cold room cabinet with 25 mM sodium phosphate, pH 7.4, 150 mM NaCl and the β_2AR–rHDL reconstitution mixture was concentrated with an Amicon Ultra-4 (100,000 Da MWCO, Millipore) to 0.5 ml and injected onto the column with 0.5 ml fractions. The fractions containing rHDL complexes, as assessed by [^{3}H]-DHA SPA, were pooled and concentrated with an Amicon Ultra-15 (100,000 Da MWCO) to 1 ml. β_2AR-rHDLs were used immediately for homogeneous scintillation proximity assays (SPA) or stored at −80 °C until further use.

2.5. SPA

Binding reactions were performed in 100 μl volumes in a buffer containing 20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.1% BSA, 0.02% pluronic acid F-68 (Invitrogen) and 0.1% DDM for assays performed on solubilized receptors. For SPA saturation binding experiments, [^{3}H]-dihydroalprenolol (DHA) (104.4 Ci/mmol; PerkinElmer) or [^{125}I]-cyanopindolol (CYP) (2200 Ci/mmol; PerkinElmer) was added at various concentrations (0.1–25.0 nM for [^{3}H]-DHA and 0.015–1.435 nM for [^{125}I]-CYP) to copper-chelate YSi scintillation SPA beads (PerkinElmer, 150 μg/well). This suspension was then mixed with the solubilized (10 μl) or reconstituted β_2AR (25–50 fmol) in individual wells of a 96-well white-wall clear-bottom plate (Wallac). The plate was gently shaken on a vibrating platform for one hour at room temperature before counting in a photomultiplier tube MicroBeta counter (Wallac) (SPA cpm mode). Non-specific binding was determined in the presence of 1 μM (S)-(−)-propranolol or 1 μM ICI-118551 (Sigma). Saturation binding assays on membrane fractions prepared from HEK293F or *Sf*21 cells expressing β_2AR (2–10 μg total protein) were conducted as above using PVT PEI-treated wheat germ agglutinin coupled SPA beads instead (PerkinElmer, 50 μg/well). Competition binding assays were performed under similar conditions as used in the saturation binding assays with [^{3}H]-DHA (5 nM) co-incubated with increasing concentrations of β-adrenoceptor antagonists (1 pM to 1 mM based on Fig. 3). Kinetic binding assays on β_2AR incorporated into rHDL particles were also conducted using copper-chelate SPA beads in 100 μl final volumes but with only two different concentrations of [^{3}H]-DHA (0.36 and 0.72 nM) and 1 μM (S)-(−)-propranolol for the determination of non-specific binding. All experiments were repeated at least in duplicate and the data were fit to a one-site binding model using Prism 5.0 (GraphPad).

3. Results and discussion

The biochemistry of membrane proteins is difficult as each step necessary to obtain stable, active protein needs to be optimized. First, a recombinant N-terminally Flag-His_{10}-tagged human β_2-adrenoceptor was generated and transiently expressed in HEK293 cells. The receptor was extracted from its native hydrophobic environment, the cell membrane, by a *n*-dodecyl-β-D-maltopyranoside/cholesterol hemisuccinate (DDM/CHS) detergent/lipid mixture. The transmembrane receptor, solubilized in a detergent micelle, was purified to near homogeneity through a series of chromatographic steps (metal chelate followed by an anti-Flag affinity column) and reconstituted with the apolipoprotein A-I (apoA-I) and the lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). Protected by CHS, β_2AR could be purified and successfully reconstituted in a rHDL (Fig. 1). In this study, two highly potent labeled β-adrenoceptor antagonists, [^{3}H]-dihydroalprenolol (DHA) and [^{125}I]-cyanopindolol (CYP), were used to assess receptor functionality. β_2AR folding was tracked with the [^{3}H]-DHA SPA along the purification and reconstitution processes, whereas receptor protein integrity was controlled using immunoblotting. The resultant β_2AR–rHDL complex displayed a comparable K_d for [^{3}H]-DHA (0.26 nM) as the β_2AR in the HEK293 cell membranes (0.46 nM), whereas the intermediate preparation in detergent showed a 10-fold lower K_d (2.86 nM). In the work published by Whorthon et al. [6], a similar comparison of [^{3}H]-DHA binding to β_2AR also showed that the receptor isolated in rHDL particles had a binding affinity more consistent with those observed for the receptor in biological membranes rather than in detergent micelles. To further explore this behavior, we applied in the course of this study the copper-chelate SPA assay to the receptor solubilized in DDM/CHS micelles. For the broad set of β-adrenoceptor antagonists used in this work, ligand affinities were systematically right-shifted for the receptor solubilized in DDM/CHS micelles (next paragraph, Table 1). This loss of affinity also increased with decreasing ligand potency. This significant disruption of high-affinity ligand binding for the soluble receptor suggests that detergent micelles allow a degree of conformational flexibility that is different from a lipid bilayer [8,16]. However, replacement of detergents with phospholipids in rHDLs reversed the deleterious effects of detergents and restored the β_2AR conformation to one that binds antagonists with native, membrane-bound affinity (Fig. 2, Table 1).

Our ultimate goal was to evaluate whether or not a reconstituted GPCR can be used for compound screening and lead optimization. For this purpose, large quantities of β_2AR were needed, and hence the expression of the receptor was optimized in insect cells, a system which is known to produce such type of proteins in the submilligram range. Baculoviruses were generated using transfer vectors encoding an N-terminally Flag-His_{10}-tagged β_2AR. β_2AR was expressed in *Spodoptera frugiperda* (*Sf*21) insect cells, detergent extracted, purified and reconstituted as described above for the receptor transiently expressed in HEK293 cells. By doing so, the reconstituted β_2AR showed in miniaturized SPA assays K_d values for [^{3}H]-DHA and [^{125}I]-CYP of 1.66 nM and 0.08 nM, respectively. These values are in good agreement with those reported in literature for the receptor expressed in natural tissues and in insect cell membranes,~2.0 nM for [^{3}H]-DHA and ~0.17 nM for [^{125}I]-CYP [17–19].

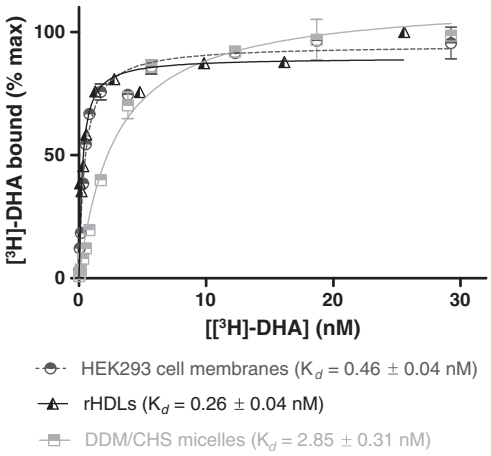

Fig. 2. Functional reconstitution of β_2AR (transiently expressed in HEK293 cells) into rHDLs. Saturation binding SPA experiments were performed on β_2AR in HEK293 cell membranes (3 μg of total membrane protein per well, grey dashed line), in DDM/CHS micelles (10 μl of purified receptor, light grey full line), or in rHDL lipid bilayers (50 fmol of receptor per well, black full line). Data shown are specific binding values.

To investigate the pharmacology of β_2AR in rHDL lipid bilayers, a set of seven β-adrenoceptor antagonists was tested in competition binding SPA experiments on the receptor in *Sf*21 insect cell membranes, in DDM/CHS micelles and in nanoparticles (Fig. 3). IC_{50} (50% inhibitory concentrations) were converted to inhibition constants (K_i), and are listed in Table 1. Inhibition of [^{3}H]-DHA with the non-labelled β-adrenoceptor antagonists showed similar pharmacological behavior in rHDLs compared to the membranes. Moreover, K_i values found for β_2AR–rHDLs are consistent with those of DHA binding to β_2-adrenoceptors in rat cardiomyocytes or when over-expressed in CHO cells [20–22]. These results clearly suggest that β_2AR in rHDLs displays the same pharmacology as in cell membranes and that rHDLs might be used to confirm direct binding to a membrane protein target. β_2AR–rHDLs also revealed a dramatically increased assay

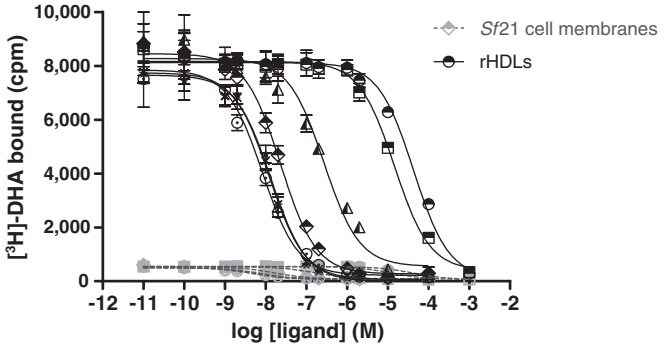

Fig. 3. SPA competition binding experiments on β_2AR expressed in Sf21 insect cell membranes (grey dashed lines) and after reconstitution in rHDLs (black full lines). β_2AR in Sf21 cell membranes (10 μg of total membrane protein per well) and β_2AR-rHDLs (25 fmol of receptor per well) were incubated with ~5 nM of [^{3}H]-DHA at room temperature in the presence of increasing concentrations of different antagonists: (S)-(−)-atenolol (circles), (+/−)-bisoprolol (squares), labetalol (triangles), alprenolol (diamonds), carvedilol (stars), bucindolol (crosses), (S)-(−)-propranolol (open circles). The compounds were selected based on their prevalent use in pharmacological studies and clinical settings. IC_{50} values were converted to inhibition constants (K_i) using the Cheng-Prusoff equation ($K_i = IC_{50}/1 + [L]/K_d$).

Table 1
Compounds tested and their displacement constants (K_i, nM) for β_2AR in Sf21 insect cell membranes ($K_{d,\ [3H]\text{-}DHA} = 1.59 \pm 0.46$ nM), in DDM/CHS micelles ($K_{d,\ [3H]\text{-}DHA} = 2.86 \pm 0.31$ nM), and in rHDL lipid bilayers ($K_{d,\ [3H]\text{-}DHA} = 1.66 \pm 0.19$ nM).

	*Sf*21 cell membranes	Solubilized β_2AR	β_2AR–rHDLs	Literature [20–22]
Carvedilol	1.99 ± 0.20	2.6 ± 0.19	2.96 ± 0.30	1.1
Bucindolol	1.17 ± 0.15	3.74 ± 0.18	2.70 ± 0.23	1.2
(S)-(−)-Propranolol	1.17 ± 0.17	5.98 ± 0.41	1.86 ± 0.26	0.96
Alprenolol	4.05 ± 0.56	19.97 ± 1.63	5.12 ± 0.59	0.2–0.4
Labetalol	39.72 ± 5.35	164.99 ± 20.15	59.61 ± 12.81	19
(S)-(−)-Atenolol	10,747 ± 2165	52,149 ± 10,018	9504 ± 1824	7193
(+/−)-Bisoprolol	2939 ± 350	76,774 ± 10,057	3209 ± 493	1150

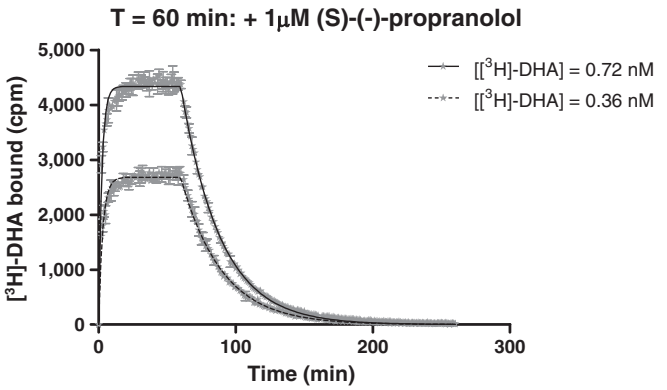

Fig. 4. Characterization of the kinetic parameters of [^{3}H]-DHA on β_2AR-rHDLs. To determine k_{on}, β_2AR–rHDLs (~50 fmol of receptor per well) were incubated with two different concentrations of [^{3}H]-DHA (0.36 and 0.72 nM) until equilibrium was reached (T = 60 min). Dissociation of [^{3}H]-DHA was then initiated by the addition of 1 μM (final concentration) (S)-(−)-propranolol. Data were best-fitted using a one-phase exponential decay function to produce $t_{1/2}$ estimates. These were converted into k_{off} values (0.0337 min^{-1} for [[^{3}H]-DHA] = 0.36 nM, 0.0349 min^{-1} for [[^{3}H]-DHA] = 0.72 nM) by using Prism 5.0 (GraphPad). The k_{on} values were then calculated using a one-phase exponential association function (8.101 x 10^8 M^{-1} min^{-1} for [[^{3}H]-DHA] = 0.36 nM, 5.333×10^8 M^{-1} min^{-1} for [[^{3}H]-DHA] = 0.72 nM). SPA experiments were conducted at room temperature and non-specific binding levels were determined in the presence of 1 μM (S)-(−)-propranolol.

window in the SPA binding assay as compared to β_2AR in cell membrane preparations due to an increased signal amplitude and neglectable non-specific binding (Fig. 3). These findings lead to believe that an important potential advantage of rHDLs in drug discovery is that they could significantly improve assay performance.

We measured the association (k_{on}) and dissociation (k_{off}) rates of [^{3}H]-DHA on β_2AR–rHDLs. The k_{on} was in good agreement with data previously reported for isolated adipocyte or skeletal muscle membrane preparations (5.33×10^8 M^{-1} min^{-1} in rHDLs versus 1.9 to 2.6×10^8 M^{-1} min^{-1} as reported [23–26]). The k_{off} (0.035 min^{-1}) was in excellent agreement with those already published (0.02 to 0.05 min^{-1}) [23–26] (Fig. 4). As it is sometimes difficult to produce accurate dissociation constants when working with membrane preparations derived from natural tissues or expressed clone material (receptor antagonists may have distinct dissociation rates from different receptor subtypes), the use of homogeneous receptor-rHDL complexes represent a significant advantage for kinetic studies. Furthermore, with the association and dissociation constants of a labeled antagonist fully characterized, it is possible to calculate the k_{on} and k_{off} of unlabeled compounds by using a method previously described and validated by Dowling and Charlton for the human muscarinic M_3 receptor [27]. Determining the kinetic parameters of unlabelled ligands early in the drug discovery process is extremely beneficial, as achieving slow dissociation kinetics for novel receptor antagonists is key for increasing duration of drug action in the target tissue.

4. Conclusion

In conclusion, we optimized the rHDL technology for utilization in drug discovery. Using the β_2AR, we have shown that rHDL provide a membrane like environment for purified recombinant membrane proteins that is compatible with assay technologies such as SPA, that are routinely used in drug discovery. Based on these results we anticipate that biophysical methods such as Surface Plasmon Resonance (SPR) can be used to study membrane protein ligand binding and kinetics in a more natural environment than detergent micelles [28–31], and this indeed will be the focus of our future efforts.

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References

[1] T.H. Bayburt, S.G. Sligar, Self-assembly of single integral membrane proteins into soluble nanoscale phospholipid bilayers, Protein Science 12 (11) (2003) 2476–2481.
[2] N.R. Civjan, T.H. Bayburt, M.A. Schuler, S.G. Sligar, Direct solubilization of heterologously expressed membrane proteins by incorporation into nanoscale lipid bilayers, Biotechniques 35 (3) (2003) 556–560 562–3.
[3] B.J. Baas, I.G. Denisov, S.G. Sligar, Homotropic cooperativity of monomeric cytochrome P450 3A4 in a nanoscale native bilayer environment, Archives of Biochemistry and Biophysics 430 (2) (2004) 218–228.
[4] A.J. Leitz, T.H. Bayburt, A.N. Barnakov, B.A. Springer, S.G. Sligar, Functional reconstitution of β_2-adrenergic receptors utilizing self-assembling Nanodisc technology, Biotechniques 40 (5) (2006) 601–602 604, 606.
[5] T.H. Bayburt, A.J. Leitz, G. Xie, D.D. Oprian, S.G. Sligar, Transducin activation by nanoscale lipid bilayers containing one and two rhodopsins, Journal of Biological Chemistry 282 (20) (2007) 14875–14881.
[6] M.R. Whorton, M.P. Bokoch, S.G. Rasmussen, B. Huang, R.N. Zare, B. Kobilka, R.K. Sunahara, A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein, Proceedings of the National Academy of Sciences of the United States of America 104 (18) (2007) 7682–7687.
[7] M.R. Whorton, B. Jastrzebska, P.S. Park, D. Fotiadis, A. Engel, K. Palczewski, R.K. Sunahara, Journal of Biological Chemistry 283 (7) (2008) 4387–4394.
[8] A.J. Kuszak, S. Pitchiaya, J.P. Anand, H.I. Mosberg, N.G. Walter, R.K. Sunahara, Purification and functional reconstitution of monomeric μ-opioid receptors: allosteric modulation of agonist binding by G_{i2}, Journal of Biological Chemistry 284 (39) (2009) 26732–26741.
[9] X.J. Yao, G. Vélez-Ruiz, M.R. Whorton, S.G. Rasmussen, B.T. DeVree, X. Deupi, R.K. Sunahara, B. Kobilka, The effect of ligand efficacy on the formation and stability of a GPCR-G protein complex, Proceedings of the National Academy of Sciences of the United States of America 106 (23) (2009) 9501–9506.
[10] C. Yoshiura, Y. Kofuku, T. Ueda, Y. Mase, M. Yokogawa, M. Osawa, Y. Terashima, K. Matsushima, I. Shimada, NMR analyses of the interaction between CCR5 and its ligand using functional reconstitution of CCR5 in lipid bilayers, Journal of the American Chemical Society 132 (19) (2010) 6768–6777.
[11] L.Z. Mi, M.J. Grey, N. Nishida, T. Walz, C. Lu, T.A. Springer, Functional and structural stability of the epidermal growth factor receptor in detergent micelles and phospholipid nanodiscs, Biochemistry 47 (39) (2008) 10314–10323.
[12] T. Boldog, S. Grimme, M. Li, S.G. Sligar, G.L. Hazelbauer, Nanodiscs separate chemoreceptor oligomeric states and reveal their signaling properties, Proceedings of the National Academy of Sciences of the United States of America 103 (31) (2006) 11509–11514.
[13] F. Freuler, T. Stettler, M. Meyerhofer, L. Leder, L.M. Mayr, Development of a novel Gateway-based vector system for efficient, multiparallel protein expression in *Escherichia coli*, Protein Expression and Purification 59 (2) (2008) 232–241.
[14] G. Zeng, Sticky-end PCR: new method for subcloning, Biotechniques 25 (2) (1998) 206–208.
[15] B. Philipps, D. Rotmann, M. Wicki, L.M. Mayr, M. Forstner, Time reduction and process optimization of the baculovirus expression system for more efficient recombinant protein production in insect cells, Protein Expression and Purification 42 (1) (2005) 211–218.
[16] M.J. Serrano-Vega, C.G. Tate, Transferability of thermostabilizing mutations between β-adrenergic receptors, Molecular Membrane Biology 26 (8) (2009) 385–396.
[17] C. Mukherjee, M.G. Caron, D. Mullikin, R.J. Lefkowitz, Structure-activity relationships of adenylate cyclase-coupled β-adrenergic receptors: determination by direct binding studies, Molecular Pharmacology 12 (1) (1976) 16–31.
[18] D.C. U'Prichard, D.B. Bylund, S.H. Snyder, (+/−)-[^{3}H] Epinephrine and (-)-[^{3}H] dihydroalprenolol binding to β_1- and β_2-noradrenergic receptors in brain, heart, and lung membranes, Journal of Biological Chemistry 253 (14) (1978) 5090–5102.
[19] G. Kleymann, F. Boege, M. Hahn, W. Hampe, S. Vasudevan, H. Reiländer, Human β_2-adrenergic receptor produced in stably transformed insect cells is functionally coupled via endogenous GTP-binding protein to adenylyl cyclase, European Journal of Biochemistry 213 (2) (1993) 797–804.
[20] P.J. Pauwels, W. Gommeren, G. Van Lommen, P.A. Janssen, J.E. Leysen, The receptor binding profile of the new antihypertensive agent nebivolol and its stereoisomers compared with various β-adrenergic blockers, Molecular Pharmacology 34 (6) (1988) 843–851.
[21] K. Pönicke, I. Heinroth-Hoffmann, O.E. Brodde, Differential effects of bucindolol and carvedilol on noradenaline-induced hypertrophic response in ventricular cardiomyocytes of adult rats, Journal of Pharmacology and Experimental Therapeutics 301 (1) (2002) 71–76.
[22] C. Hoffmann, M.R. Leitz, S. Oberdorf-Maass, M.J. Lohse, K.N. Klotz, Comparative pharmacology of human β-adrenergic receptor subtypes – characterization of stably transfected receptors in CHO cells, Naunyn-Schmiedeberg's Archives of Pharmacology 369 (2) (2004) 151–159.

[23] L.T. Williams, L. Jarett, R.J. Lefkowitz, Adipocyte β-adrenergic receptors. Identification and subcellular localization by (−)-[^{3}H]dihydroalprenolol binding, Journal of Biological Chemistry 251 (10) (1976) 3096–3104.
[24] E.M. Dax, J.S. Partilla, R.I. Gregerman, The (−)-[^{3}H]dihydroalprenolol binding to rat adipocyte membranes: an explanation of curvilinear Scatchard plots and implications for quantitation of β-adrenergic sites, Journal of Lipid Research 23 (7) (1982) 1001–1008.
[25] H.J. Mersmann, R.L. McNeel, Ligand binding to the porcine adipose tissue β-adrenergic receptor, Journal of Animal Science 70 (3) (1992) 787–797.
[26] M.E. Spurlock, J.C. Cusumano, S.E. Mills, (−)-[^{3}H]-dihydroalprenolol binding to β-adrenergic receptors in porcine adipose tissue and skeletal muscle membrane preparations, Journal of Animal Science 71 (7) (1993) 1778–1785.
[27] M.R. Dowling, S.J. Charlton, Quantifying the association and dissociation rates of unlabelled antagonists at the muscarinic M_3 receptor, British Journal of Pharmacology 148 (7) (2006) 927–937.
[28] I. Navratilova, M. Dioszegi, D.G. Myszka, Analyzing ligand and small molecule binding activity of solubilized GPCRs using biosensor technology, Analytical Biochemistry 355 (1) (2006) 132–139.
[29] R.L. Rich, A.R. Miles, B.K. Gale, D.G. Myszka, Detergent screening of a G-protein-coupled receptor using serial and array biosensor technologies, Analytical Biochemistry 386 (1) (2009) 98–104.
[30] R.L. Rich, J. Errey, F.H. Marshall, D.G. Myszka, Biacore analysis with stabilized G-protein-coupled receptors, Analytical Biochemistry 409 (2) (2011) 267–272.
[31] M. Congreve, R.L. Rich, D.G. Myszka, F. Figaroa, G. Siegal, F.H. Marshall, Fragment screening of stabilized G-protein-coupled receptors using biophysical methods, Methods in Enzymology 493 (2011) 115–136.