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# Functional studies with membrane-bound and detergent-solubilized $\alpha_2$-adrenergic receptors expressed in Sf9 cells

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**Abstract**

A chip-based biosensor technology using surface plasmon resonance (SPR) was developed for studying the interaction of ligands and G protein-coupled receptors (GPCRs). GPCRs, the fourth largest superfamily in the human genome, are the largest class of targets for drug discovery.

We have expressed the three subtypes of $\alpha_2$-adrenergic receptor ($\alpha_2$-AR), a prototypical GPCR as functional fusion proteins in baculovirus-infected insect cells. The localization of the expressed receptor was observed in intracellular organelles, as detected by eGFP fluorescence. In addition, the deletion mutants of $\alpha_{2B}$-AR, with a deletion in the 3rd intracellular loop, exhibited unaltered $K_d$ values and enhanced stability, thus making them more promising candidates for crystallization. SPR demonstrated that small molecule ligands can bind the detergent-solubilized receptor, thus proving that $\alpha_2$-AR is active even in a lipid-free environment. The $K_d$ values obtained from the biosensor analysis and traditional ligand binding studies correlate well with each other. This is the first demonstration of the binding of a small molecule to the detergent-solubilized state of $\alpha_2$-ARs and interaction of low-molecular mass-ligands in real time in a label-free environment. This technology will also allow the development of high throughput platform for screening a large number of compounds for generation of leads.


*Keywords:* G protein-coupled receptor; Baculovirus; Over-expression; Localization; *Spodoptera frugiperda* Sf9 cell; Surface plasmon resonance

## 1. Introduction

Solubilization is probably one of the most important steps in the integrated process of expression–purification–crystallization and structure determination of a membrane protein. However, monitoring the activity, conformational state and real time kinetics of a G protein-coupled receptor (GPCR) in the detergent-solubilized, lipid-free environment has proven very difficult. Here, we demonstrate a method of determining ligand binding in the detergent-solubilized state.

We have determined $K_d$ and other pharmacological properties for $\alpha_{2B}$-AR using the traditional harvester-based equilibrium binding system and have developed a Scintiplate™ based method for doing the same [1,2]. However, neither method could be used to demonstrate that ligand binds to detergent-solubilized receptor. This made it impossible to know whether detergent-solubilized $\alpha_{2B}$-AR bound ligand—whether it had a native conformation or not. The major drawbacks of the above techniques were: firstly,

*Abbreviations:* $\alpha_2$-AR, human $\alpha_2$-adrenergic receptor; $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$, subtypes of human $\alpha_2$-adrenergic receptor; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate; EDC, ethyl-3(3-dimethylamino) propyl carbodiimide; GFP, green fluorescent protein from *Aequorea victoria*; GPCR, G protein-coupled receptor; G protein, guanine nucleotide-binding regulatory protein; HA, hemagglutinin; HRP, horseradish peroxidase; HTS, high throughput screening; $K_d$, dissociation constant; NHS, *N*-hydroxysuccinimide; P20, polysorbate 20; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; SFV, Semliki Forest virus; SPR, surface plasmon resonance

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in the harvester method, upon solubilization, the receptor-detergent micelle is too small to be retained by the glass-fiber filters. Second, in the Scintiplate method, the detergent interferes, giving a high background signal and so masks the signal, if there is one, from the interaction between the ligand–receptor complex. We therefore developed a binding assay using surface plasmon resonance (SPR) technology in the hope that we would be able to observe ligand binding to detergent-solubilized $\alpha_{2B}$-AR.

SPR has additional advantages. Ligand binding is measured without labels and both association and dissociation rates can be directly measured. Recently, it has become possible to measure small molecules interacting with immobilized macromolecular targets [3,4]. One of the binding partners is immobilized on the sensor chip surface and the other flowed over it in solution. Binding causes a change in the refractive index at the biosensor surface.

This method has been used with recombinant human $\alpha_2$-adrenergic receptors. The $\alpha_2$-adrenergic receptors belong to the GPCR superfamily and mediate various functions of the central and peripheral nervous systems [5,6]. We have studied here the pharmacologically defined receptor subtypes $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$ [7–10]. The adrenergic receptor subtypes exhibit varied cellular and tissue distribution, mediate different physiological functions and hence are the possible targets for the development of subtype selective drugs. All three $\alpha_2$-adrenergic receptor subtypes were expressed in the baculovirus-insect cell system, as has been done for other GPCRs [11]. Sf9 insect cells from *Spodoptera frugiperda*, which do not express endogenous GPCR [12], provide a good environment for ligand binding studies. Studies on the detergent-solubilized $\alpha_2$-adrenergic receptor subtypes have still not been possible, as mentioned above. Here, we describe a simplified method for carrying out such studies based on the principles of SPR, which also allowed us for the first time to detect binding in detergent-solubilized states. We also examined the binding of various compounds having different affinities both to receptor in lipid vesicles and to detergent-solubilized receptor.

## 2. Materials and methods

### 2.1. Reagents

[methyl-$^3$H] Rauwolscine (specific activity 71.0 Ci/mmol) was from NEN Life Science Products (Boston, USA), and Anti-mouse IgG from Santa Cruz Biotechnology Inc. From Sigma (St. Louis, USA), we purchased clonidine, chlorpromazine, prazosin, phentolamine, dopamine and yohimbine. Detomidine, dexmedetomidine and l-medetomidine were gifts from Orion-Farmos (Turku, Finland). Guanfacine was purchased from Sandoz (Helsinki, Finland). Guanabenz was from Research Biochemicals Inc. (Natick, USA). The detergents Big CHAP and *n*-dodecyl-β-D-maltoside were from Calbiochem (San Diego, USA). PerkinElmer Life Sciences, Wallac Oy (Turku, Finland) supplied MeltiLex™ Wax, OptiPhase "Hisafe" III Liquid Scintillation Cocktail and Glass Fiber filtermat. Coupling reagents (EDC, NHS and ethanolamine HCl) were purchased from Biacore (Uppsala, Sweden). Anti-HA monoclonal antibodies were purchased from Nordic BioSite (Propellervägen, Sweden) and anti-His antibody (tetra-His) from Qiagen (Helsinki, Finland). Cellfectin reagent was purchased from Invitrogen (Helsinki, Finland).

### 2.2. Instrumentation

The SPR experiments were carried out using Biacore 2000 (University of Helsinki) and Biacore 3000 (Biocenter Oulu, University of Oulu) optical biosensors equipped with research-grade CM5 sensor chips (Biacore AB, Uppsala, Sweden).

### 2.3. Construction of expression plasmids and preparation of recombinant baculovirus

For $\alpha_{2A}$-AR, the cloning vector pUC19 was digested with *Eco*RI and *Sma*I. After 2 h incubation at 37 °C, enzymes were heat inactivated, 5 units of Klenow fragment and 0.5 mM (final) dNTP added, and incubated for 10 min at 37 °C. The excised fragment was gel purified and religated to generate the "pUC19-modified" plasmid. pREP4-HA-$\alpha_{2A}$-AR [13], containing the hemagglutinin (HA) tag, was amplified with a F1 forward and an R1 reverse primer (Table 1A for primer sequences). The amplified 1430 bp fragment was cut with *Bam*HI and *Xba*I and ligated into the *Bam*HI–*Xba*I fragment of the pUC19-modified plasmid, generating plasmid pUC19-HA-$\alpha_{2A}$-AR.

For $\alpha_{2C}$-AR, pUC19-HA-$\alpha_{2A}$-AR was digested with *Ngo*MIV and *Bsi*WI and the 2736 bp fragment isolated. The earlier published construct pcDNA-$\alpha_{2C}$-AR [13] was amplified by PCR with F2 forward and R2 reverse primers (Table 1A). The resulting product was digested with *Age*I and *Bsi*WI and the 1386 bp fragment isolated and inserted into the *Mgo*MIV–*Bsi*WI fragment of pUC19-HA-$\alpha_{2A}$-AR, generating pUC19-HA-$\alpha_{2C}$-AR.

pUC19-HA-$\alpha_{2A}$-AR and pUC19-HA-$\alpha_{2C}$-AR were cut with *Bam*HI and *Hin*dIII, and the resulting 1445 bp and 1481 bp fragments, respectively, were inserted into the vector pFastBacTM1 (Invitrogen), yielding the shuttle vectors for generating recombinant baculoviruses.

For the eGFP-tagged form of $\alpha_{2A}$-AR and $\alpha_{2C}$-AR, eGFP-C1 vector (Clontech Laboratories Inc, USA) was PCR amplified with forward and reverse primers F3 and R3 (Table 1A). After digesting the PCR product with *Bsi*WI and *Nhe*I, the fragment was ligated into the pUC19-HA-$\alpha_{2A}$-AR and pUC19-HA-$\alpha_{2C}$-AR cloning vectors; these newly created vectors carry the eGFP gene fused to the C-terminus of the $\alpha_{2A}$-AR and $\alpha_{2C}$-AR. The $\alpha_{2A}$-AR-eGFP

Table 1A
DNA sequences of the primers used in the engineering of different constructs

| Primer | Sequence |
|---|---|
| F1 | 5'-TTG GAT CCA TTA TGG GTA CCG GCA CTA GTG GCT ACC CAT ACG ACG TCC-3' |
| R1 | 5'-TTT CTA GAC GCT AGC CGC CGT ACG CAC GAT CCG CTT CCT GTC-3' |
| F2 | 5'-TTT TAC CGG TGC GTC CCC GGC GCT-3' (*Age*I) |
| R2 | 5'-TTT TCG TAC GCT GCC TGA AGC CCC TTC TCC-3' (*Bsi*WI) |
| F3 | 5'-GTT TCG TAC GAT GGT GAG CAA G-3' (*Bsi*WI) |
| R3 | 5'-AGA AGC TAG CTT GAG CTC GAG A-3' (*Nhe*I) |

and $\alpha_{2C}$-AR-eGFP genes were transferred to the shuttle vector as described above.

The previously published pUC19-HA-$\alpha_{2B}$-AR and pUC19-HA-$\alpha_{2B}$-AR-eGFP plasmids [2] were cleaved with *Kpn*I and *Hin*dIII. The resulting DNAs containing the coding regions of $\alpha_{2B}$-AR and $\alpha_{2B}$-AR translationally fused with eGFP were isolated and ligated using the *Kpn*I and *Hin*dIII sites of the pFastBacTM1 vector. The segment deletion mutants of $\alpha_{2B}$-AR were prepared as described elsewhere [14].

The various constructs (Table 1B) were sequenced and recombinant baculoviruses were generated in DH10Bac *E. coli* cells according to the Bac-to-Bac™ Baculovirus expression system (Life Technologies/Invitrogen) and subsequently used to transform Sf9 insect cells using Cellfectin reagent according to the manufacturer's instructions (Invitrogen).

### 2.4. Cell culture and expression of recombinant receptor protein

Sf9 insect cells grown in suspension culture were maintained at a density of $0.5-2.5\times10^6$ cells/ml and were passaged every 2–3 days. The optimal infection titer and the optimal time for harvest for each viral stock were determined by infecting several 5-ml cell suspensions ($2\times10^6$ cells/ml) in 100-ml flasks with different amounts of virus from a given stock, followed by radioligand binding assay or relative fluorescence on cells harvested 45–60 h post-infection. We initiated protein production by infecting 40–60 ml of cell suspension in a 250-ml flask with the optimal amount of virus (determined as above) and culturing at 27 °C for 45–48 h at 120 rpm. Cells were centrifuged and washed twice with 50 mM phosphate buffered saline (PBS) and stored at −20 °C until further use.

Table 1B
Constructs of $\alpha_2$-ARs expressed in the baculovirus system

| Construct ID | Construct | -N terminal tag | -C terminal tag | Deletion |
|---|---|---|---|---|
| I | $\alpha_{2A}$-AR | HA | eGFP | – |
| II | $\alpha_{2B}$-AR | HA | eGFP | – |
| III | $\alpha_{2C}$-AR | HA | eGFP | – |
| IV | $\alpha_{2B}$-AR-5D[a] | HA | – | 245–359 (in IL3) |
| V | $\alpha_{2B}$-AR-5D-His[a] | HA | His | 245–359 (in IL3) |
| VI | $\alpha_{2B}$-AR-6D[a] | HA | – | 206–359 (in IL3) |

[a] Cloning of 5D, 5D-His and 6D described in [14].

### 2.5. Preparation of insect cell membranes

All the membranes prepared were either on ice or at 4 °C unless otherwise mentioned. All buffers contained the Protease inhibitor cocktail from Roche. Cell pellets were resuspended in osmotic lysis buffer containing 5 mM Tris, pH 7.4. After breaking the cells open with a hand held homogenizer, we centrifuged them at $500\times g$ for 10 min to discard the cell debris, then re-centrifuged at $32{,}000\times g$ for 45 min to collect the membrane fraction. The supernatant was discarded, and the freshly prepared membrane fraction was immediately used for radioligand binding assays. Protein concentration was estimated using the Bio-Rad Bradford protein assay kit with bovine serum albumin as a standard.

### 2.6. Ligand binding assays

Membrane fractions from the three subtypes of $\alpha_2$-ARs and from the mutant constructs of $\alpha_{2B}$-AR were used for harvester-based radioligand binding assays (performed at least in duplicate) as described earlier [1]. Non-specific binding was determined by including 10 μM phentolamine, a nonselective $\alpha_1$ and $\alpha_2$ antagonist, in parallel assays. Data were fitted to a Scatchard plot by nonlinear regression analysis of the equation $[LR]=(B_{max}\times[L])/(K_d+[L])$ in order to calculate the equilibrium dissociation constant $K_d$ and the receptor density $B_{max}$. [LR] is the concentration of the specifically bound radioligand and $[L]$ is the total concentration of the ligand. Non-linear curve fitting was done with PRISM v 3.02 (GraphPad Software, San Diego, CA).

### 2.7. Fluorescence measurements and microscopy

We used the $\alpha_{2B}$-AR-eGFP construct for both receptor localization and to monitor the optimal harvest time. Fluorescent microscopy was done as described earlier [2]. For time course, the relative fluorescence counts were measured using Victor (PerkinElmer Life Sciences) with the FITC filter.

### 2.8. Electrophoresis and Western blotting

Membrane fractions from various constructs (either solubilized, unsolubilized or purified on a small scale with an $\alpha_{2B}$-AR-7G1 column [15] or -HA column (Sigma)) were analyzed on 12% SDS-PAGE [16] and electro-transferred onto Hybond ECL nitrocellulose membranes (Amersham Biosciences) for Western blotting [17] using the Bio-Rad semi-dry transfer system. For immunostaining, the primary antibodies used included $\alpha_{2B}$-AR-7G1 monoclonal antibody [15], monoclonal anti-HA antibody and monoclonal anti-His antibody and the signal detected as described earlier [2].

### 2.9. Biacore analysis studies

#### 2.9.1. Solubilization of $\alpha_2$-AR

Freshly thawed receptor preparations were solubilized in solubilization buffer (5 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% glycerol, complete protease inhibitor from Roche) with a final concentration of 67% *n*-dodecyl-β-D-maltoside and 33% of Big CHAP. The protein concentration of the solubilization mixture was maintained at 2.5 mg/ml. The mixture was placed on a rocker platform at 4 °C overnight. It was then centrifuged at 100,000 × *g* for 60 min at 4 °C to isolate the supernatant containing the solubilized receptor fraction.

#### 2.9.2. Immobilization of $\alpha_2$B-AR-7G1 mAb or anti-HA mAb

The CM5 sensor chip surface was prepared for $\alpha_{2B}$-AR-7G1 monoclonal antibody [15] or anti-HA monoclonal antibody immobilization using standard amine-coupling procedures [18]. HBS (Hepes-buffered saline, 20 mM Hepes, 150 mM NaCl, 0.005% P20, pH 7.4) was used as the basic buffer in the procedure. After activating the surface by injecting NHS:EDC for 7 min, the antibody solution (in 10 mM acetate buffer, pH 5) was injected for 15 min at 5 μl/min. Finally, ethanolamine was injected to block any remaining activated groups on the surface.

#### 2.9.3. Capture of the whole receptor and detergent-solubilized receptor

Receptor preparations (either whole or detergent-solubilized) were captured on the immobilized antibody surface by injecting at a flow rate of 10 μl/min for 15 min to attain maximum density. In case of the detergent-solubilized receptor, 0.05% detergent was included in the assay buffer. The antibody-receptor complex was stable for the duration of a ligand binding experiment.

#### 2.9.4. Binding of small molecules to the antibody-receptor complex surface

For calculation of the equilibrium dissociation constant values in detergent solubilized states for Constructs I–III, we injected rauwolscine over the antibody captured receptor. The controls were non-receptor coated surface and an empty surface. The ligand was allowed to associate with the receptor for 120 s and dissociation was monitored for a further 120 s. In a high throughput format, we injected a series of small molecular weight compounds at a flow rate of 5 μl/min over the antibody-captured receptor surface, the association and dissociation times being same as above. This was carried out in an automated mode.

For both of the above cases, the ligand–receptor complex was stripped from the antibody surface using the Biacore "short regeneration injection" mode to shift the pH with 0.1 M glycine–HCl, pH 2.5. Buffer blanks were injected at intervals for referencing of the base line.

#### 2.9.5. Data analysis

Biacore data were processed using the BiaEvaluation Software (Biacore) program. Bulk refractive index changes were corrected by subtracting the responses of the reference surfaces from the binding responses. To obtain the equilibrium dissociation constant ($K_d$) values, data from the reactions were fitted to 1:1 bimolecular interaction model, with baseline drift correction factor included when appropriate.

## 3. Results

### 3.1. Expression of the recombinant receptor protein in baculovirus cells and solubilization studies

We used fluorescence counts or radioactivity assays, as appropriate, to maximise virus titer or protein production.

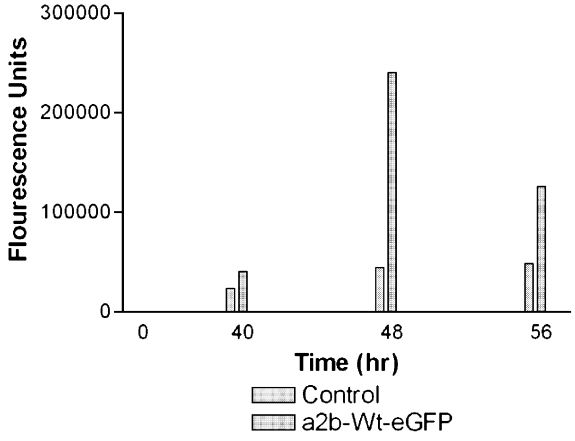


Fig. 1. Baculovirus expression fluorescence chase. Plot showing the increase of GFP-producing cells as a function of time. Fluorescence counts from live baculovirus/Sf9 cells producing the recombinant $\alpha_{2B}$-AR-eGFP were measured using a Victor instrument and the fluorescence units are plotted as a function of time. Non-infected cells were used as a control.

Table 2
Comparison of the expression data and equilibrium dissociation constants ($K_d$) for the various constructs expressed in the baculovirus system

| Construct ID | Construct | $K_d$ (nM) | $B_{max}$ (pmol/mg) | Optimal virus titer (μl/ml) | Optimal harvest time (h) |
|---|---|---|---|---|---|
| I | $\alpha_{2A}$-AR | 4.49 ± 0.74 | 63.8 | 1 | 48 |
| II | $\alpha_{2B}$-AR | 4.52 ± 0.16 | 42.32 | 0.1 | 48–50 |
| III | $\alpha_{2C}$-AR | 2.02 ± 0.78 | 13.33 | 1 | 48 |
| IV | $\alpha_{2B}$-AR-5D | 4.84 ± 0.04 | 21.33 | 1 | n.d. |
| V | $\alpha_{2B}$-AR-5D-His | 5.51 ± 0.58 | 51.33 | 3 | n.d. |
| VI | $\alpha_{2B}$-AR-6D | 2.73 ± 0.44 | 11.08 | 1 | n.d. |

n.d., not determined.

This showed that, as for other baculovirus expression studies [11], 48 h was the optimal harvesting time (Fig. 1). The optimal virus titer was between 0.1 and 3.0 μl/ml (Table 2). We therefore harvested the cells at 48 h. The membrane fractions were prepared as described earlier [1]. We observed fluorescence signals for $\alpha_{2B}$-AR-eGFP both in the membrane fraction and in the supernatant obtained upon the preparation of the membrane. This might indicate that the $\alpha_{2B}$-AR-eGFP is cleaved, as is also evident from the Western blot analysis (Fig. 2), when probed with two different antibodies. The various constructs produced the recombinant protein, as determined by binding activity assays (Table 2), at levels from 11 to 64 pmol/mg. We could express the C-terminally $His_6$-tag for the 5D deletion construct, but the same fusion at the N-terminal failed. For the wild type, we could express the N-terminal $His_6$-tagged protein, but it always disappeared when we tried to purify using Ni-affinity column. This result is similar to our earlier observation when we tried to express and purify $His_6$-$\alpha_{2B}$-AR, which also contained an N-terminal $His_6$ tag, in the SFV system (unpublished data).

### 3.2. Electrophoresis and Western blotting

Western blot analysis with appropriate antibodies showed that each construct expresses protein of the expected molecular weight (Fig. 2). Two different antibodies, however, show that $\alpha_{2B}$-AR is consistently cleaved into two major bands (Fig. 2B), with one of the cleavage sites being in the 3rd intracellular loop after the 7G1 antibody detection epitope, while the other site for cleavage lies in the C-terminal extramembranous tail. The cleavage is also in agreement with the fact that fluorescence is observed both in the membrane fraction and in the supernatant fraction obtained during the membrane preparation as described above. In contrast, $\alpha_{2A}$-AR and $\alpha_{2C}$-AR show only a single major band (Fig. 2A). As common for membrane proteins [19], the expressed receptors also appeared to migrate slowly, aggregate and form multimers. We also observed low molecular weight bands which may correspond to proteolytic fractions as well as non-specific binding to the secondary antibody.

### 3.3. Radioligand binding assays

The $K_d$ values (Table 2) for the three receptor subtypes are similar to literature values [2]. The mutants have essentially unaltered binding constants (Table 2). This indicates that the deletion has at least no deleterious effect on the ligand binding site and probably also no effect on protein folding. Non-infected cells, or cells infected with wild-type baculovirus, did not yield any specific binding (data not shown).

### 3.4. Localization of the expressed receptor

We localized $\alpha_{2B}$-AR-eGFP using the eGFP fluorescence with live cells. The receptor localized mainly to sub-cellular organelles, rather than on the plasma membrane (Fig. 3). This correlates well with our earlier results using SFV-CHO cells [2].

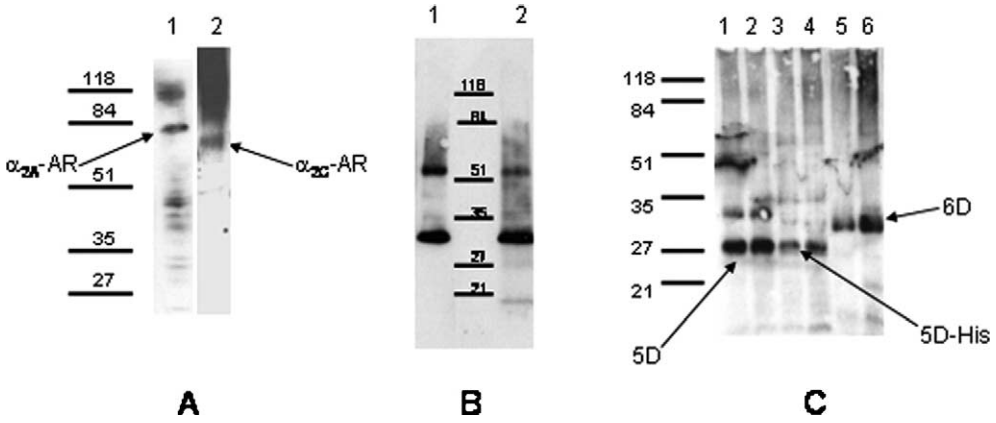


Fig. 2. Western blot of recombinant human $\alpha_2$-AR constructs. (A) Western blot of the membrane fractions of $\alpha_{2A}$-AR-eGFP (lane 1, 0.5 μg) and $\alpha_{2C}$-AR-eGFP (lane 2, 1.0 μg), both being probed with anti-HA monoclonal antibody. (B) Western blot of the purified membrane fractions of $\alpha_{2B}$-AR-eGFP (lane 1, 2 μg; lane 2, 2 μg); lane 1 being probed with $\alpha_{2B}$-AR-7G1 monoclonal antibody [15] and lane 2 with anti-HA monoclonal antibody. (C) Western blot of the whole membrane fractions of 5D (lane 1, 1.0 μg; lane 2, 1.5 μg), 5D-$His_6$ (lane 3, 1.0 μg; lane 4, 1.5 μg) and 6D (lane 5, 1.0 μg; lane 6, 1.5 μg), with all the three constructs being probed with anti-HA monoclonal antibody.

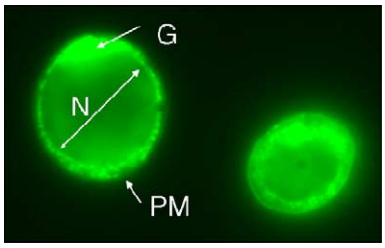


Fig. 3. Subcellular localization of $\alpha_{2B}$-AR-eGFP in baculovirus-infected Sf9 cells. The fluorescence observed from the $\alpha_{2B}$-AR-GFP construct showing the protein expression and the intracellular localization of $\alpha_{2B}$-AR-GFP in baculovirus-infected Sf9 cells, as observed by fluorescence microscopy at 48-h post-infection, are shown. The fluorescence intensity shows that the receptor is distributed throughout all the membrane compartments, in addition to the plasma membrane. N, nucleus; G, Golgi body; PM, plasma membrane.

### 3.5. Biacore results

#### 3.5.1. Solubilization of $\alpha_2$-AR subtypes

Previously, we tested various detergents to determine the best condition for solubilization (Sen et al., unpublished data) and observed that a mixture of detergents containing *n*-dodecyl-β-D-maltoside and Big CHAP produced the best solubilization results for the baculovirus system. With this detergent combination, we obtain a solubilization efficiency of about 50–60% as judged by Western blot. We used whole membrane fractions and detergent-solubilized $\alpha_2$-ARs in Biacore assays. The whole membrane fraction was used only for $\alpha_{2B}$-AR and the detergent-solubilized fractions for all three subtypes.

#### 3.5.2. Ligand binding on sensor surface

We immobilized $\alpha_{2B}$-AR-7G1 [15] or anti-HA monoclonal antibodies on the activated Biacore sensor chip, the former to capture $\alpha_{2B}$-AR and the latter to capture $\alpha_{2A}$-AR and $\alpha_{2C}$-AR (Fig. 4). The receptor subtypes were then captured either as membrane-bound or detergent-solubilized fraction. We could regenerate the surface by washing the antibody–receptor complex away by pH shift (0.1 M glycine–HCl, pH 2.5). The sensogram signal increased by 8000–12,000 RU upon the immobilization of the antibody and by a further 1600–3000 RU upon the capture of the receptor. The subsequent baseline drift, which lasted for about 600 s, may be due to excess receptor which was stripped off during the initial wash. Rauwolscine was then injected in different flow-cells over both the whole and solubilized receptor. The $K_d$ values were similar to each other (Table 3), to our harvester values (Table 2) and to the literature values.

Table 3
Comparison of $K_d$ values for rauwolscine binding to the membrane fraction (detergent-solubilized and non-solubilized) of $\alpha_2$-ARs expressed in baculovirus/Sf9 cells using filter-binding assay and Biacore/SPR assay

| Receptor subtype | $K_d$ (nM)/filter binding assay[a] | $K_d$ (nM) Biacore/SPR assay |
|---|---|---|
| $\alpha_{2A}$-AR | 4.49 ± 0.74 | n.d. |
| $\alpha_{2A}$-AR-detergent solubilized | – | 3.59/(2)/0.22[b] |
| $\alpha_{2B}$-AR | 4.52 ± 0.16 | 5.29 (5)/1.45[b] |
| $\alpha_{2B}$-AR-detergent solubilized | – | 2.29/10.2[b] |
| $\alpha_{2C}$-AR | 2.02 ± 0.78 | n.d. |
| $\alpha_{2C}$-AR-detergent solubilized | – | 4.7/(4)/0.104[b] |

n.d., not determined.
[a] Values from Table 2.
[b] $\chi^2$ value, standard statistical measure for closeness of the fit. Air spikes in the sensogram may have marked effect in the value. Experimental error is reported in parentheses.

#### 3.5.3. Rapid drug screening using SPR

Drug screening experiments using all three receptor subtypes expressed in the baculovirus system (Constructs I–III) were performed using 17 different compounds. Qualitative screening in the SPR mode showed that each of the solubilized antibody-captured $\alpha_2$-ARs bound a series of compounds with different affinities (Table 4 and Fig. 5). The rise in RU values was within the expected range, varying between the compounds depending upon their affinity towards the receptor and upon the total amount of captured receptor. The rank order of affinity of the compounds to $\alpha_{2B}$-AR was similar to that observed previously by Scintiplate™ and harvester methods [2]; we have not measured similar data for $\alpha_{2A}$-AR and $\alpha_{2C}$-AR. The screening was done in an automated way, and hence can be applied in a high-throughput format to identify lead compounds for the pharmaceutical industry.

## 4. Discussion

In the present study, immunological, radioligand binding and fluorescence studies demonstrate that we have success-

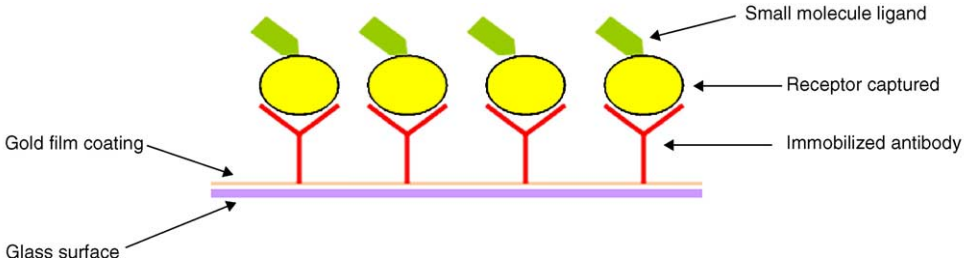


Fig. 4. Biacore capture chemistry. A schematic diagram showing the design for the Biacore/SPR assay, wherein the antibody is captured on the sensor chip. The receptor (either solubilized or non-solubilized) is then allowed to bind to the antibody, and the small molecule ligands are then added. The change in the refractive index at the sensor chip interface is recorded as the sensogram signal.

Table 4
Rank order of binding of the different compounds to the detergent-solubilized $\alpha_2$-AR subtypes expressed in baculovirus/Sf9 cells using the Biacore/SPR assay**

| Compound ID | Drugs/Compounds | $\alpha_{2A}$-AR | $\alpha_{2B}$-AR | $\alpha_{2C}$-AR |
|---|---|---|---|---|
| I | Adrenaline | 11 | 3 | 11 |
| II | Nor-adrenaline | 7 | 12 | 10 |
| III | UK 14,304 | 17 | 17 | 17 |
| IV | Spiperone | 15 | 15 | 15 |
| V | Spiroxatrine | 16 | 16 | 16 |
| VI | Prazosine | 13 | 11 | 12 |
| VII | Clonidine | 6 | 14 | 9 |
| VIII | Dexmedetomidine | 4 | 4 | 2 |
| IX | Oxymetazoline | 2 | 6 | 1 |
| XI | Yohimbine | 5 | 8 | 4 |
| XII | Chlorpromazine | 12 | 2 | 13 |
| XIII | Dopamine | 1 | 10 | 5 |
| XIV | (*R*)-2-aminophenylethanol | 10 | 5 | 8 |
| XV | ARC-239 | 14 | 1 | 14 |
| XVII | Guanfacine | 9 | 13 | 7 |
| XVIII | Guanabenz | 3 | 9 | 3 |
| XIX | Detomidine | 8 | 7 | 6 |

** We have hypothesized that the compound which gives the maximum rise in the SPR signal for each receptor subtype binds most tightly to it.

fully expressed all three subtypes of $\alpha_2$-adrenergic receptor and two $\alpha_{2B}$-AR mutants in the baculovirus Sf9 system. We have also, for the first time, been able to demonstrate ligand binding to the detergent-solubilized state of $\alpha_2$-adrenergic receptor. This study is a step forward in the application of SPR methods to GPCRs based on earlier reports of application of the same to rhodopsin [21]. G protein binding has also been demonstrated to human δ-opioid receptor with a similar technique using plasmon-waveguide resonance (PWR) [22].

The expression levels for the recombinant receptors were relatively good, varying between 11 and 64 pmol/mg of membrane protein (Table 2), which corresponds to approximately 0.05–0.3% of the total membrane protein. Though this level of expression is two to three fold lower than observed for SFV expression system [2], it is still a good starting point for scale up and production because it is cheaper and easier than SFV expression.

Immunoblot analysis reveals that for the constructs 5D, 5D-His and 6D, the receptor is expressed mainly in the intact form, unlike $\alpha_{2B}$-AR-WT (Fig. 2). The large 3rd intracellular loop is the site for cleavage; the lower band (Fig. 2B) corresponds to cleavage in the 3rd intracellular loop, leading to the retention of the green fluorescence in the membrane even though full length $\alpha_{2B}$-AR-eGFP is not detected. The upper band corresponds to $\alpha_{2B}$-AR without the eGFP tag (see Results). In addition, when we delete a large part of the loop, the expressed receptor is more stable and there is mainly a single band (Fig. 2C). The equilibrium dissociation values for ligand binding for the deletion mutants are similar, confirming that the deletion construct behaves almost like wild type (Table 2). The deletion constructs, 5D and 6D, would thus be the best candidates for purification and crystallization. Indeed, we have shown that 5D, 5D-His and 6D have native binding and activity profile when expressed at near-native levels in human cells [14].

We have also, for the first time, been able to express and purify a His-tagged $\alpha_2$-AR. Our previous efforts in using N-terminal $His_6$-tags (Sen et al., unpublished data) have been unsuccessful in baculovirus, SFV/CHO and yeast cells. It is therefore very encouraging that the 5D-$His_6$ construct was expressed at 52 pmol/mg and could be purified using a Ni-affinity column. The expression and purification of the N-terminal His tagged receptor may not work properly since positive charge is retained on the exofacial N-termini due to the presence of the histidine residues, which is opposite to that of the membrane potential.

Radioligand binding data from the three subtypes as well as from the deletion-mutant constructs show that the equilibrium dissociation constant values (Table 2) agree with values that we and others have earlier reported [2].

We observe similar results as earlier [2] with the accumulation of the receptors in the intracellular compartments as detected by fluorescence microscopy (Fig. 3). One plausible explanation could be the over-expression of the receptor, leading to saturation of the biosynthetic protein transport machinery. Others have suggested [19] that these could also correspond both to receptors on their exocytic pathway towards the plasma membrane, and to the receptors in their endocytic pathway, upon reaching the PM. The co-expression of target protein with chaperones including ER-resident BiP, calreticulin or ER-membrane bound calnexin has improved the secretion pathway of the receptors, without leading to unproductive folding pathways [11].

We demonstrate here for the first time SPR-based detection of ligand binding to $\alpha_2$-ARs. SPR has a number of advantages over other assay techniques. First, the receptor molecules are homogeneous in preparation, both in the membrane-bound and detergent-solubilized states, and are distributed evenly upon binding to the antibody surface. Second, excess receptor that is not bound upon

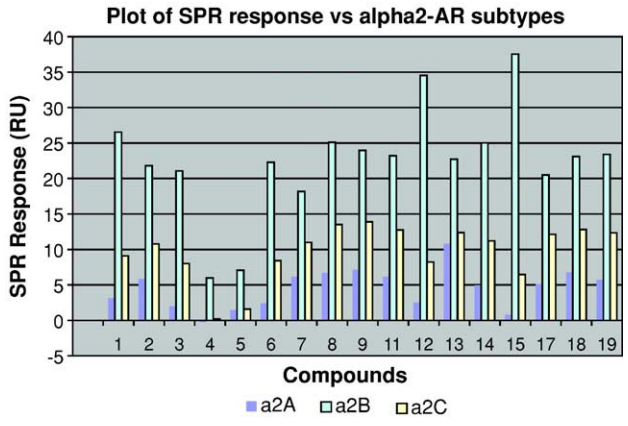


Fig. 5. Drug screening with Biacore SPR. The relative response (RU units) obtained from the various small molecule ligands upon binding to the three subtypes of $\alpha_2$-ARs is shown. The different affinities of binding to the different subtypes illustrate the substrate specificity. The compound numbers refer to the compounds described in Table 4.

injection is easily washed off during buffer injection. Third, the amount of receptor captured on the sensor chip surface can be controlled by varying the amount injected and is reproducible within the limits of experimental fluctuations. In addition, using antibody–antigen capture for immobilization of the receptor was clearly superior to covalent immobilization for SPR. Antibodies allow the capture of high density functional receptor on the sensor surface, the maximum density being determined by the amount of antibody immobilized. Perhaps, most importantly, conditions employed for stripping the receptor from the antibody surface are milder (pH shift to 2.5) than the harsh chip regeneration conditions. The chip can therefore be reused more often, reducing cost. One possible concern was that the antibody–receptor interaction would interfere with the ligand binding, but this was not the case. For $\alpha_{2A}$-AR and $\alpha_{2C}$-AR, the N-terminal-HA tag was sufficiently distant from the ligand binding pocket, even though it is on the same (extracellular) face of the receptor. For $\alpha_{2B}$-AR, the 7G1 monoclonal antibody recognizes IL3 and is thus on the opposite face of the receptor.

SPR worked not only with receptor in whole membrane fraction, but also with detergent-solubilized $\alpha_2$-ARs. Furthermore, the $K_d$ values obtained from the whole membranes and detergent-solubilized receptors correlated well with each other and with literature values (Table 3). This is the first demonstration that, contrary to our earlier results [20], the conformation of $\alpha_2$-AR is not significantly altered by solubilization. It also explains why the addition of 100 μM phentolamine protects detergent-solubilized $\alpha_{2B}$-AR against proteolysis, as phentolamine would be able to bind to the receptor. In our other assay systems [1], ligand binding to the detergent-solubilized receptor could not be detected due to the inherent nature of the assays. For harvester-based assays, the size of the detergent-solubilized receptor was too small to be retained by the glass-fiber filter and, in case of the Scintiplate assay system, the detergent itself interfered in the assay giving rise to a huge background signal. The SPR system can be utilized to optimize GPCR solubilization conditions, unlike the other assay techniques. It is thus a far superior system. In addition, the rapidity of analysis will allow the screening of large numbers of compounds for the generation of hits in the pharmaceutical industry.

The biosensor analysis avoids any kind of labels (fluorescent tags, radioisotopes or reporters) that may interfere with the biological interaction. Consequently, work associated with purifying and labelling material is greatly reduced and there are no label disposal costs. The process can be used to follow every step during solubilization and purification, unlike harvester-based or Scintiplate systems. Moreover, as the system is based on a non-invasive technology, there is no interference from light adsorption or scattering. Comprehensive real-time kinetic characterization of potential lead compounds can be performed with this analysis, thus making Biacore SPR suitable for drug discovery applications too.

In conclusion, we describe here a baculovirus-based expression system of $\alpha_2$-ARs and demonstrate for the first time the binding of small molecule ligands to detergent-solubilized $\alpha_2$-ARs. This system should be applicable to other membrane receptor systems and for studies on detergent-solubilized receptors.

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