Analytical Biochemistry 392 (2009) 103–109

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Cell-based β_2-adrenergic receptor–ligand binding assay using synthesized europium-labeled ligands and time-resolved fluorescence

Eija Martikkala [a], Mirva Lehmusto [a], Minna Lilja [b], Anita Rozwandowicz-Jansen [a], Jenni Lunden [c], Takenori Tomohiro [d], Pekka Hänninen [a], Ulla Petäjä-Repo [b], Harri Härmä [a,*]

[a] Laboratory of Biophysics, University of Turku, FI-20520 Turku, Finland
[b] Department of Anatomy and Cell Biology, Institute of Biomedicine, University of Oulu, FI-90014 Oulu, Finland
[c] Wallac Oy, PerkinElmer Life and Analytical Sciences, FI-20520 Turku, Finland
[d] Laboratory of Biorecognition Chemistry, University of Toyama, Toyama 930-0194, Japan

ARTICLE INFO

Article history:
Received 27 December 2008
Available online 21 May 2009

Keywords:
β_2-Adrenergic receptor
GPCR
G protein-coupled receptor
Time-resolved fluorescence
Cell-based assay
Ligand synthesis

ABSTRACT

High-sensitivity, high-throughput, and user-friendly lanthanide-based assays for receptor–ligand interactions provide an attractive alternative to the traditional radioligand displacement assays. In this study, three small-molecule pindolol ligand derivatives were synthesized and their binding properties were tested in a radioligand displacement assay. The ligand derivatives were further labeled with fluorescent europium(III) chelate for β_2-adrenergic receptor–ligand binding assay. The europium-labeled pindolol ligands having no spacer (C0) or a 12-carbon spacer (C12) arm bound to the human β_2-adrenergic receptors overexpressed in human embryonic kidney HEK293$_i$ cells. Europium ligand with a 6-carbon spacer arm (C6) showed no binding. Competitive binding assays were developed with the functional labeled ligands. The IC_{50} values for β_2-adrenergic antagonist propranolol were 60 and 37 nM, the Z' values were 0.51 and 0.77, and the signal-to-background ratios were 5.5 and 16.0 for C0 and C12, respectively. This study shows that functional time-resolved fluorescent assays can be constructed using fluorescent lanthanide chelates conjugated to small-molecule ligands.

G protein-coupled receptors (GPCRs)[1] represent one of the largest gene families in the human genome [1,2]. These polytopic membrane proteins regulate a wide range of cellular processes in response to external stimuli such as neurotransmitters and hormones. Nowadays, approximately 60% of drug targets in the pharmaceutical industry are GPCRs [3] and there is an ever-increasing need for more efficient techniques for screening new drug candidates.

Ligand binding studies play a significant role in GPCR drug discovery processes. Millions of drug candidates can be produced with combinatorial chemical methods, and the ability to screen these in a rapid, inexpensive, and sensitive manner is crucial. Many ligand binding assays have been developed, and the usefulness of these assays is dependent on appropriate labels. An ideal label should not affect the binding properties of the ligand, should be easy to use and manufacture, and should provide a sufficient signal-to-noise ratio. The most extensively used labels are those with radioactive isotopes such as ^{125}I and ^{3}H. The advantage of these labels is that they cause an insignificant change in the binding properties of the ligand. On the other hand, they have problems related to production, delivery, and disposal of radioactive materials [4]. Fluorescent labels provide an attractive alternative over the radiolabels because they are more rapid, stable, and safe to use. However, the high fluorescence background originating from reagents, plastics, optics, and biological specimens limits the use of such labels. The high fluorescence background of conventional short-lived fluorophores can be overcome by lanthanide chemistry and time-resolved fluorescence detection [5]. Using temporal resolution in detection and lanthanide chelates with a millisecond lifetime, large Stokes' shift, and sharp emission peak, orders of magnitude improvement in detection sensitivity compared with conventional fluorophores can be achieved.

A number of lanthanide-based ligand binding assays have been developed for GPCRs. These include assays for CXCR1 and CXCR2 chemokine receptors [6], neurotensin receptor [7], melanin-con-

* Corresponding author. Fax: +358 2 3337060.
E-mail address: harri.harma@utu.fi (H. Härmä).

[1] *Abbreviations used:* GPCR, G protein-coupled receptor; β_2AR, β_2-adrenergic receptor; Eu, europium; HEK, human embryonic kidney; hβ_2AR, human β_2AR; BSA, bovine serum albumin; ICI-118,551, (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; CGP-20712, 1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride; ESI–TOF, electrospray ionization time-of-flight; NMR, nuclear magnetic resonance; cDNA, complementary DNA; RT, room temperature; MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; TFA, trifluoroacetic acid; DMF, dimethylformamide; TEA, triethylamine; TEAAc, triethylammonium acetate; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.05.022

centrating hormone receptors 1 and 2 [8,9], melanocortin-4 receptor [10], δ-opioid receptor [11], and RXFP2 insulin-like peptide-3 receptor [12]. Importantly, all of these assays involve lanthanide chelate derivatization of peptide ligands. No derivatization of small nonpeptide GPCR ligands has been reported to date. Here we describe a heterogeneous ligand binding assay for the β_2-adrenergic receptor (β_2AR), a biogenic amine receptor that is one of the most extensively studied GPCRs. The β_2AR is expressed, for example, in the airway smooth muscle tissue and is an important drug target in the treatment of asthma [13]. The assay relies on the detection of fluorescent europium (Eu)-labeled pindolol on intact stably transfected human embryonic kidney (HEK) 293_i cells overexpressing the human β_2AR (hβ_2AR).

Materials and methods

Materials

The reagents and analytical-grade solvents used for compound synthesis were purchased from Fluka (Buchs, Switzerland), Sigma–Aldrich (St. Louis, MO, USA), TCI (Zwijndrecht, Belgium), Riedel–Haen (Seelze, Belgium), Lab-Scan (Gliwice, Poland), and J.T. Baker (Deventer, Netherlands) and were used without further purification. Eu(III)-chelate-7d ({2,2′,2″,2‴-{[4-[(4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl]-bis(methylenenitrilo)}tetrakis(acetato)} europium(III)) and DELFIA enhancement solution were obtained from Wallac Oy, PerkinElmer Life and Analytical Sciences (Turku, Finland). Thin-layer chromatography plates (Si 60 F254) and silica gel 60 for adsorption chromatography were purchased from Merck (Whitehouse Station, NJ, USA). Bovine serum albumin (BSA), Dulbecco's modified Eagle's medium, fetal calf serum, penicillin, streptomycin, isoproterenol, ICI-118,551 ((±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)-amino]-2-butanol), and pindolol were obtained from Sigma–Aldrich. C12 low Fluor maxi microtiter wells were obtained from Nunc (Wiesbaden, Germany). pOG44 plasmid, Lipofectamine 2000 transfection reagent, and tetracycline were obtained from Invitrogen (Carlsbad, CA, USA), and blasticidin S, zeozin, and hygromycin B were obtained from Invivogen (San Diego, CA, USA). Protease inhibitor mix tablets were obtained from Roche (Indianapolis, IN, USA), and CGP-20712 (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride) were purchased from Tocris (Ellisville, MO, USA). Propranolol was obtained either from Acros Organics (Geel, Belgium) or Tocris.

Instrumentation

Mass spectra were measured using an electrospray ionization time-of-flight (ESI–TOF) MS Mariner System 5272 (Applied Biosystems, Foster City, CA, USA) and ^{1}H nuclear magnetic resonance (NMR) spectra using a Bruker AVANCE DRX 500 spectrometer (Bruker, Karlsruhe, Germany). Time-resolved fluorescence emission spectra and lifetime of the Eu(III) chelate and the Eu(III)-labeled C12–pindolol ligand were monitored with a Cary Eclipse spectrophotometer (Varian, Palo Alto, CA, USA) using excitation wavelength of 323 nm and emission wavelength of 614 nm with 1 ms decay time and 0.1 ms delay time. In microtiter plate assays, time-resolved fluorescence emission signals were measured at 615 nm using 340 nm excitation wavelength, 400 μs delay time, and 400 μs decay time with a Victor^2 1420 multilabel counter (Wallac Oy). An MWR-96T harvester was used for membrane harvesting (Brandel, Gaithersburg, MD, USA). Radioactivity was measured using a Microbeta TriLux scintillation counter (Wallac Oy).

DNA constructs, transfection, and cell culture

The DNA construct encoding the hβ_2AR with a cleavable influenza hemagglutinin signal peptide, N-terminal *Myc* tag, and C-terminal *Flag* tag was created as described previously [14] by cloning receptor complementary DNA (cDNA) into the modified pcDNA5/FRT/TO (pFT–SMMF) vector [15].

The hβ_2AR was expressed in stably transfected tetracycline-inducible $HEK293_i$ cells [16]. The stable cell line was prepared by cotransfecting cells with the receptor construct and pOG44 plasmid using Lipofectamine 2000 transfection reagent under blasticidin S (4 μg/ml) and hygromycin B (400 μg/ml) selection. Clones were isolated, expanded, and tested for zeozin sensitivity and β-galactosidase activity, and the tetracycline-inducible expression was verified by radioligand binding and Western blot analysis. The clone selected was sensitive to zeozin but lacked β-galactosidase activity and had high hβ_2AR expression. Cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%), penicillin (100 U/ml), streptomycin (0.1 g/L), blasticidin S (1 or 4 μg/ml), and hygromycin B (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C.

Synthesis of Eu(III) chelate ligand

Compound **1** was synthesized from 4-hydroxyindole and epichlorohydrin following a known protocol [17]. The product was purified by silica gel chromatography using methanol/dichloromethane (1:40, v/v) as eluent. Compound **2** was synthesized from 1,2-diamino-2-methylpropane and di-*tert*-butyldicarbonate (Scheme 1).

*tert-Butyl N-(2-amino-2-methylpropyl)carbamate (**2**)*

1,2-Diamino-2-methylpropane (1.0 g, 11.3 mmol) and NaOH (0.5 g, 12.5 mmol) were dissolved in dioxane/water (1:1, v/v, 40 ml), and the reaction mixture was cooled down in an icewater bath. di-*tert*-Butyldicarbonate (2.5 g, 11.5 mmol) was dissolved in dioxane (10 ml) and added dropwise to the reaction mixture. The reaction mixture was stirred at 0 °C for 3.5 h and at room temperature (RT) for 2.5 h. The solvents were removed under reduced pressure, and the product was extracted from water with dichloromethane (three times). Organic fractions were combined, washed with saturated NaCl, and dried with Na_2SO_4. Organic fraction was evaporated to dryness, yielding 1.9 g (89%) of the compound **2**. ^{1}H NMR (500 mHz, δ ppm): 5.18 (1H, s [broad], –NH–), 3.11 (2H, d, $-CH_2-$), 1.45 (9H, s, $-CH_3$), 1.25–1.19 (2 × s, 6H, $-CH_3$). Mass spectrometry (matrix-assisted laser desorption/ionization) [MS (MALDI)]: found 189 (M+1), calculated 189 (M+1).

*tert-Butyl 2-(3-(1H-indol-4-yloxy)-2-hydroxypropylamino)-2-methylpropylcarbamate (**3**)*

A mixture of compound **1** (0.5 g, 2.6 mmol) and compound **2** (0.5 g, 2.6 mmol) in acetonitrile (5 ml) was heated to reflux for 48 h. After cooling, the reaction mixture was evaporated to dryness under reduced pressure and the crude product was purified by silica gel chromatography using MeOH/CH_2Cl_2 (1:10, v/v) as eluent. Fractions containing the product were combined and evaporated to dryness, yielding 490 mg (50%) of the pure product. ^{1}H NMR (500 mHz, δ ppm): 8.21 (1H, Ar–NH), 7.13–7.03 (3H, m, Ar–H), 6.66 (1H, s [broad], Ar–H), 6.53 (1H, d, Ar–H), 4.93 (1H, s [broad], –OH), 4.17 (3H, m, –CH–, $-CH_2-$), 3.08 (2H, m, $-CH_2-$), 2.86 (2H, m, $-CH_2-$), 1.44 (9H, s, CH_3), 1.10 (6H, s, CH_3). MS (MALDI): found 378 (M+1), calculated 378 (M+1).