Cell-Based High-Throughput Screening Assay System for Monitoring G Protein-Coupled Receptor Activation Using β-Galactosidase Enzyme Complementation Technology

YU-XIN YAN, DEBORAH M. BOLDT-HOULE, BONNIE P. TILLOTSON, MELISSA A. GEE,
BRIAN J. D'EON, XIAO-JIA CHANG, CORINNE E. M. OLESEN, and MICHELLE A. J. PALMER

ABSTRACT

A novel cell-based functional assay to directly monitor G protein-coupled receptor (GPCR) activation in a high-throughput format, based on a common GPCR regulation mechanism, the interaction between β-arrestin and ligand-activated GPCR, is described. A protein-protein interaction technology, the InteraX™ system, uses a pair of inactive β-galactosidase (β-gal) deletion mutants as fusion partners to the protein targets of interest. To monitor GPCR activation, stable cell lines expressing both GPCR- and β-arrestin-β-gal fusion proteins are generated. Following ligand stimulation, β-arrestin binds to the activated GPCR, and this interaction drives functional complementation of the β-gal mutant fragments. GPCR activation is measured directly by quantitating restored β-gal activity. The authors have validated this assay system with two functionally divergent GPCRs: the β2-adrenergic amine receptor and the CXCR2 chemokine-binding receptor. Both receptors are activated or blocked with known agonists and antagonists in a dose-dependent manner. The β2-adrenergic receptor cell line was screened with the LOPAC™ compound library to identify both agonists and antagonists, validating this system for high-throughput screening performance in a 96-well microplate format. Hit specificity was confirmed by quantitating the level of cAMP. This assay system has also been performed in a high-density (384-well) microplate format. This system provides a specific, sensitive, and robust methodology for studying and screening GPCR-mediated signaling pathways.

INTRODUCTION

GPROTEIN-COUPLED RECEPTORS (GPCRs) mediate a wide variety of biological processes, including phototransduction, neurotransmission, chemoattraction, cardiac output, feeding behavior, pain modulation, and olfactory and gustatory sensory. We have developed a novel cell-based protein-protein interaction technology to directly monitor GPCR activation. This system is based on β-galactosidase (β-gal) enzyme complementation and comprises 2 inactive β-galactosidase deletion mutants as fusion partners to the protein pairs of interest. Physical interaction of the 2 target proteins drives complementation of the β-gal fusion fragments, resulting in measurable β-gal enzymatic activity.[1-4]

We used a GPCR and β-arrestin as protein partners to monitor GPCR activation through β-gal complementation. β-Arrestins are ubiquitously expressed in all cell types and have also been shown to mediate the internalization and resensitization of many known GPCRs.[5-8] β-Arrestins bind with high affinity to the agonist-activated GPCR carboxyl-terminal tail. This high-affinity binding requires a G protein-coupled receptor kinase (GRK) to phosphorylate the GPCR,[9-12] uncoupling the receptor from heterotrimeric G proteins, which results in receptor desensitization,[9,10,13-17] a common mechanism among functionally diverse GPCRs.[5,9,16,18] A GPCR coding sequence is fused to one β-gal mutant fragment (Δα), and β-arrestin is fused to another β-gal fragment (Δω). In cells stably expressing these fusions, following ligand stimulation, β-arrestin binds to the activated GPCR and drives complementation of the β-gal fragments. β-gal activity is then measured directly in the culture plate with a chemiluminescent β-galactosidase assay. The interaction between an agonist-stimulated GPCR and β-arrestin has been demonstrated by numerous studies. Translocation of a β-arrestin-GFP fusion protein to ligand-activated GPCRs at the cell surface[19,20] and bioluminescence resonance energy transfer between the β2-adrenergic receptor and β-arrestin[21] have both been used to validate the sensing of a GPCR-β-arrestin interaction to monitor GPCR signaling events.

The β2-adrenergic receptor (β2AR) is one of the most well-characterized members of the GPCR family and is an important

therapeutic target for several clinical conditions.[22] We constructed a β_2-adrenergic receptor cell line, which expresses the β_2-adrenergic receptor fused to the β-gal Δα fragment and β-arrestin 2 (βarr2) fused to the β-gal Δω fragment (β_2AR-Δα/βarr2-Δω). We have demonstrated dose-dependent β-gal activity in β_2AR-Δα/βarr2-Δω cells in response to agonist treatment, as well as inhibition with antagonist treatment. In addition, we have evaluated the CXCR2 chemokine receptor in this system with both agonists and antagonists. CXCR2 is a high-affinity receptor expressed primarily on neutrophils that responds to interleukin-8 (IL-8) and other CXC chemokines.[23,24]

To validate this technology for high-throughput screening (HTS) evaluation of GPCR agonist and antagonist identification, we screened the β_2AR-Δα/βarr2-Δω cell line against the Library of Pharmacologically Active Compounds (LOPAC™), a library collection of 640 compounds with known biological activities. We identified 5 out of 7 of the known β_2AR agonists with >50% maximum induction and all of 7 known β-adrenergic antagonists included in the LOPAC™ library. Assay performance criteria met the acceptable standards of a quantitative HTS assay with an average Z factor of > 0.5. To confirm the specificity of antagonists identified, we assayed cAMP production and evaluated compound toxicity. We also evaluated the β_2-adrenergic receptor cell line performance in a 384-well microplate format, requiring development of a suspension cell protocol. In addition, this system has been evaluated for high-throughput screening with a cell line containing EGFR fused to the β-galactosidase fragments. EGF-induced EGFR dimerization was validated in both 96-and 384-well screening formats, and favorably low false-inhibitor hit rates were obtained.[25]

This protein-protein interaction technology provides a robust, low false-positive cell-based assay system for GPCR activation and functional analysis of GPCR signaling pathways. In addition, the application of the InteraX™ system to GPCR signaling analysis meets the requirements of high-throughput screening.

MATERIALS AND METHODS

Cell line construction

The human β_2-adrenergic receptor, CXCR2 receptor, and β-arrestin 2 coding sequences were PCR-amplified and cloned into retroviral expression vectors containing the Δα or Δω β-galactosidase fragments, as described elsewhere.[1] To construct the β_2AR-Δα/βarr2-Δω and CXCR2-Δα/βarr2-Δω InteraX™ cell lines, we first introduced the retroviral GPCR-Δα expression vector into PT67 packaging cells (Clontech, Palo Alto, CA) with FuGENE™ 6 transfection agent (Roche, Indianapolis, IN). Two days later, media containing viral particles were used to infect C2C12 mouse myoblast cells (ATCC, Manassas, VA). Infected cells were selected with G418 (Life Technologies, Rockville, MD). Stable clones were obtained by limiting dilution, and expression was confirmed by β-gal ELISA, as described previously.[3] GPCR-Δα-expressing clones were infected with retrovirally packaged βarr2-Δω expression vector, as described above. Dual-infected pools were selected with G418 and hygromycin B and cloned by limiting dilution or by FACS for the

CXCR2 cell line using an anti-CXCR2 monoclonal antibody (R&D Systems, Minneapolis, MN) with the Alexa Fluor™ signal amplification kit (Molecular Probes, Eugene, OR). Cells coexpressing the GPCR and β-arrestin fusions were cultured in DMEM with 20% FBS, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 0.5 mg/ml hygromycin B, and 1 mg/ml G418 at 37 °C in 10% CO_2.

Induction/inhibition of β_2AR and CXCR2 receptors

β_2AR-Δα/βarr2-Δω cells were plated at 10,000 cells/well (96-well format) and cultured for 20 to 24 h. For agonist dose response, cells were then incubated with (–)isoproterenol (Sigma Chemical Co., St. Louis, MO) or procaterol (Sigma)/0.3 mM ascorbic acid in serum-free DMEM for 60 min at 37 °C. For antagonist dose response, cells were preincubated for 10 min in serum-free DMEM containing ICI-118,551 (Sigma) or propranolol (Sigma), followed by addition of 10 µM (–)isoproterenol/0.3 mM ascorbic acid for 60 min at 37 °C.

CXCR2-Δα/βarr2-Δω cells were plated at 10,000 to 20,000 cells/well (96-well format) and cultured for 20 to 24 h. For agonist dose response, cells were starved for 2 h in DMEM/0.1% FBS and then induced with IL-8 (Sigma) or GROα (Calbiochem, San Diego, CA) in DMEM/0.1% FBS for 2 h. For antagonist dose response, dilutions of antagonist SB225002 (Calbiochem) and 10 nM IL-8 were added sequentially in DMEM/0.1% FBS, and cells were then incubated for 2 h.

For all agonist and antagonist experiments, β-gal activity was then quantitated with the chemiluminescent Gal-Screen® reagent (Applied Biosystems, Foster City, CA). Gal-Screen® Buffer A (100 µl/well for 96-well format and 35 µl/well for 384-well format) was added, and plates were incubated at room temperature for 60 min. Light emission was measured with the TR717™ microplate luminometer (Applied Biosystems) for 1 s/well or imaged with the NorthStar™ HTS Workstation (Applied Biosystems) for 60 s/plate (96-well) and 120 s/plate (384-well). Data represent the average relative light units (RLU) of either duplicates or triplicates.

Screening automation

For screening, cell dispensing, media aspiration, compound addition, and assay reagent handling were performed with the Multidrop™ 384 microvolume dispenser (ThermoLabsystems, Franklin, MA), the El$_x$405™ Select plate washer (BIO-TEK Instrument, Winooski, VT), and the PlateMate™ automated pipettor (Matrix Technologies, Hudson, NH).

Agonist screen

β_2AR-Δα/βarr2-Δω cells were plated (10,000 cells/well) in 96-well plates and cultured for 24 h. Media were removed, and 90 µl of serum-free DMEM (phenol red-free)/0.3 mM ascorbic acid was added. LOPAC™ library compounds (Sigma) were diluted in serum-free DMEM (phenol red-free)/0.3 mM ascorbic acid and added (10 µl/well) at a final concentration of 2 µM and incubated at 37 °C for 60 min. β-gal activity was quantitated with the Gal-Screen® reagent. The performance of the agonist screen

was analyzed with the HTS Datadisplay 4.0 software (Applied Biosystems).

Antagonist screen

β₂AR-Δα/βarr2-Δω cells were plated (10,000 cells/well) in 96-well plates and cultured for 24 to 48 h. Media were removed, and 80 µl of serum-free DMEM (phenol red-free)/0.3 mM ascorbic acid was added. LOPAC™ compounds were diluted in serum-free DMEM (phenol red-free)/0.3 mM ascorbic acid and added (10 µl/well) at final concentrations of 10 µM or 2 µM and incubated for 10 min at room temperature. The β₂AR agonist, (–)isoproterenol (+)bitartrate salt, was then added (10 µl/well) at a final concentration of 10 µM and incubated at 37 °C for 60 min. β-gal activity was quantitated with the Gal-Screen® reagent. The performance of the antagonist screen was analyzed with the HTS Datadisplay 4.0 software (Applied Biosystems).

Measurement of cAMP

β₂AR-Δα/βarr2-Δω cells were plated (10,000 cells/well) in a 96-well plate for 24 h, and then medium was changed to serum-free DMEM (phenol red-free)/0.3 mM ascorbic acid/IBMX (Sigma). Compounds were incubated with cells for 15 min prior to measuring the cAMP level with the cAMP-Screen™ chemiluminescent immunoassay reagents (Applied Biosystems).[26] Light emission was measured with the TR717™ microplate luminometer (Applied Biosystems).

Toxicity assay

Cells (10,000 cells/well) were incubated with compounds for ~20 h. Then, the Alamar Blue™ reagent (10 µl/well, BIOSOURCE International, Camarillo, CA) was added and incubated for 4 h. Fluorescence was measured using an excitation wavelength of 550 nm, and emission (590 nm maximum) was measured at 595 nm with the HTS 7000 Bio Assay Reader (Perkin-Elmer, Shelton, CT).

HTS detection protocol for cells in suspension

β₂AR-Δα/βarr2-Δω cells were dissociated with 0.25% Trypsin/EDTA (Sigma) or with nonenzymatic solution (Sigma), washed with PBS, and suspended in serum-free DMEM (phenol red-free) medium containing 0.3 mM ascorbic acid. Cells were dispensed into a 96-well (90 µl/well) or a 384-well (30 µl/well) microplate with the Multidrop™ 384 microvolume dispenser (ThermoLabsystems). For both 96-well and 384-well plates, 10,000 to 20,000 cells/well were seeded, and compounds were then added immediately (10 µl/well for 96-well, 5 µl/well for 384-well) with the PlateMate™ automated pipettor (Matrix Technologies) and incubated at 37 °C for 60 min. β-gal activity was quantitated with the Gal-Screen® reagent as described. In brief, 100 µl (96-well) or 35 µl (384-well) of Gal-Screen® Buffer A was dispensed with the Multidrop™ dispenser (ThermoLabsystems) and incubated at 37 °C for 60 min. Light emission was measured either by the TR717™ luminometer (1 s/well for 96-well) (Applied Biosystems) or by the NorthStar™ HTS Workstation (120 s/plate for 384-well) (Applied Biosystems).
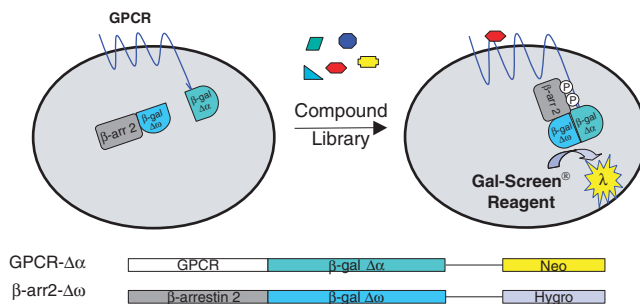

FIG. 1. The InteraX™-based G protein-coupled receptor (GPCR) assay system. The inactive β-gal deletion mutants, Δα and Δω, are fused to the carboxyl terminus of a GPCR and β-arrestin 2, respectively. Fusion proteins are stably expressed in cells following retroviral infection and clone selection. Interaction of ligand-activated GPCR with β-arrestin 2 drives complementation of the β-gal mutant fragments.

RESULTS AND DISCUSSION

Agonist and antagonist dose-dependent β-gal complementation

We have established 2 stable cell lines, β₂AR-Δα/βarr2-Δω and CXCR2-Δα/βarr2-Δω, to study GPCR activation with the InteraX™ assay system (illustrated in Figure 1). The 2 β-gal deletion mutants, Δα and Δω, are individually inactive and have a low affinity for each other. Following agonist stimulation, in cells coexpressing GPCR-Δα and βarr2-Δω fusion proteins, the complementation of β-galactosidase activity is a direct readout for ligand-induced GPCR activation via the β-arrestin interaction.

The association of the β₂-adrenergic receptor (β₂AR) with β-arrestin 2 is known to be agonist dependent.[9] Cells coexpressing both β₂AR-Δα and βarr2-Δω fusion proteins were analyzed for agonist-induced β-gal complementation to demonstrate the use of β-gal enzyme complementation to directly monitor ligand-induced GPCR activation and β-arrestin binding. As shown in Figure 2A, β₂AR-Δα/βarr2-Δω cells responded to two known agonists, isoproterenol and procaterol, with a dose-dependent increase in β-gal activity. The EC₅₀ obtained for isoproterenol is 27 nM, and the EC₅₀ for procaterol is 15 nM. The value obtained with isoproterenol is similar to that reported for isoproterenol-stimulated β₂AR sequestration in HEK-293 cells (11 nM) and adenylyl cyclase activity in Sf9 cells expressing β₂AR (23 nM),[27,28] while the EC₅₀ value for procaterol was not found in the literature.

Dose-dependent inhibition of the β-gal interaction readout was demonstrated with the β₂AR antagonists propranolol and ICI-118,551 in the presence of 10 µM isoproterenol (Fig. 2B). The IC₅₀ obtained is 140 nM for propanolol and 78 nM for ICI-118,551. These results demonstrate that β-gal complementation with the InteraX™ system can be used to monitor β₂-adrenergic receptor response to both agonists and antagonists in quantitative pharmacological studies.
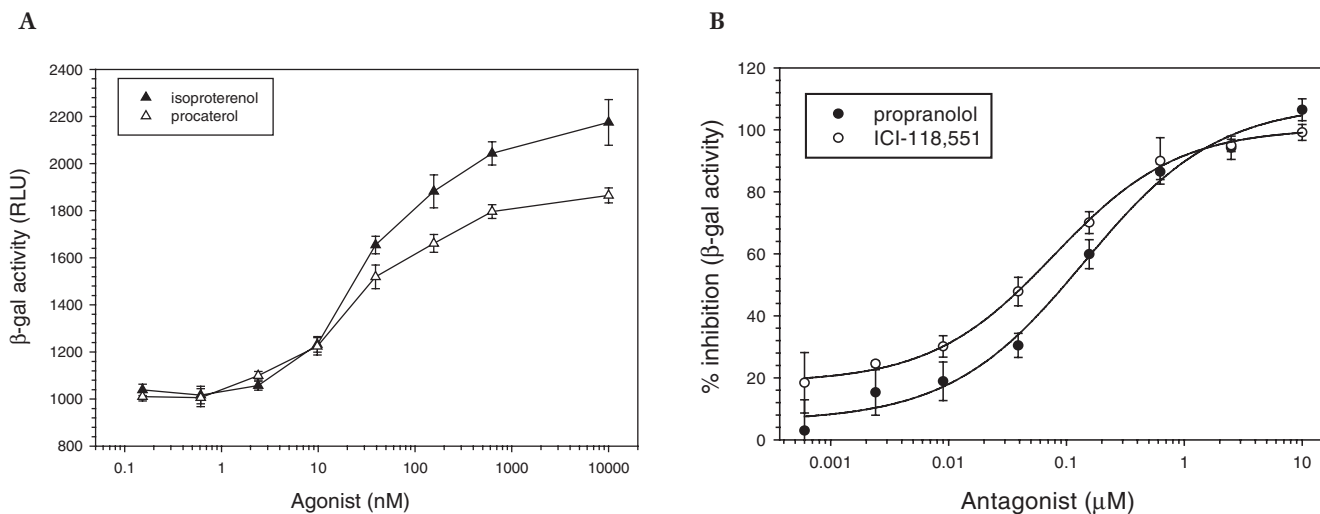
A

B

FIG. 2. Agonist and antagonist dose response with β_2AR-$\Delta\alpha$/$\beta arr2$-$\Delta\omega$ cells. (**A**) Agonist dose response with isoproterenol and procaterol. (**B**) Inhibition of isoproterenol-induced β-gal interaction readout. Dose-dependent inhibition of isoproterenol-stimulated β-gal activity is observed following pretreatment with propranolol and ICI-118,551. Data represent the average relative light units (RLU) of triplicates. Error bars indicate standard deviation.

Chemokine receptors form a distinct group of structurally related proteins within the GPCR superfamily. This assay method has been extended to the evaluation of the chemokine receptor CXCR2. Ligand-activated CXCR2 has been shown to interact with β-arrestin 1 by coimmunoprecipitation in HEK293 cells[29] and to rapidly desensitize and internalize on agonist stimulation.[30] Cells coexpressing CXCR2-$\Delta\alpha$ and $\beta arr2$-$\Delta\omega$ were analyzed for agonist-induced response (unpublished data), and EC_{50} values observed for IL-8 (1 nM) and GROα (3 nM) are very similar to those measured in human HEK293 cells expressing CXCR2 using a calcium flux response, 4 nM for IL-8 and 5 nM for GROα, respectively. Furthermore, this activation is specific, as macrophage inflammatory protein-1α, a CC chemokine specific for several CCRs, failed to induce activation of the CXCR2 receptor. To further confirm the specificity of this system as a functional readout for receptor activation and inhibition, we used a known CXCR2 antagonist, SB 225002,[31] to block agonist-induced CXCR2 interaction with β-arrestin 2. SB 225002 inhibited IL-8-induced CXCR2 binding to β-arrestin 2 in a dose-dependent manner with an IC_{50} value of 45 nM, similar to that reported.[31]

Agonist screening

To further validate this cell-based protein-protein interaction assay system, we performed a high-throughput screen for both agonist and antagonist activities with the β_2AR-$\Delta\alpha$/$\beta arr2$-$\Delta\omega$ cell line against the LOPAC™ compound library, which includes 640 known pharmacologically active compounds. For agonist screening, cells were incubated with compounds at 2 μM for 60 min in a serum-free medium containing 0.3 mM ascorbic acid. Compounds were tested in duplicate. A known agonist, isoproterenol, was used as the positive control, and the same media without isoproterenol were used as the untreated control. All wells were treated identically and contained 0.02% DMSO, which has been determined to have no adverse effect on the cells or the assay at that concentration (data not shown). The effect of each compound on the induction of β-gal complementation is shown in Figure 3. The agonist activities obtained from most of compounds fall below 34.8% β-gal induction, 3 times that of standard deviation, and are considered insignificant activities. Six compounds demonstrated significant induction (> 50%) of β-gal activity. Table 1 lists the 24 known adrenergic agonists included in the LOPAC™ library and the one additional compound with agonist activity. The agonist activity is expressed as the percentage of maximal β-gal induction by 2 μM isoproterenol. Of these 24 agonists, 7 are known β_2-, β-adrenergic and nonselective adrenergic agonists and would be expected to have agonist activity in this assay. Five out of 7 compounds were identified in the screening assay to have > 50% of maximum induction. The other 2 β- or β_2-adrenergic agonists showed slight to moderate activity. Compound N-153 (nylidrin), a β-adrenergic selective agonist, showed 12% of maximal induction, and the subtype-specific β_2-adrenergic agonist A-109 (salbutamol) showed 20% induction. These 2 compounds have been tested for dose-dependent β-gal complementation, and they demonstrated little or no activity on β-adrenergic receptor activation (data not shown). Compound C-147 also showed > 50% of maximum induction. This compound has been classified as an antagonist of the sigma receptor. However, neither the compound nor the sigma receptor has been reported to have any similarities to the structure of β-adrenergic agonists or to the receptor sequence. The overall results of the agonist screen indicate that this cell-based system has a low false-positive rate (1/640) for monitoring GPCR activation by sensing of the β-arrestin 2/activated-GPCR interaction. The low hit rate suggests that this cell-based functional assay system has high selectivity for agonist-induced activity.

TABLE 1. RESULTS OF THE AGONIST SCREEN WITH THE LOPAC™ LIBRARY

Compound ID	Position	Class of Action	Type of Receptor	Subtype Specificity	% Induction of β-gal Activity[a]	Comments[b]
E-104	RK002-A6	Agonist	Adrenergic	—	88.2	*
M-133	RK002-H7	Agonist	Adrenergic	—	56.5	*
I-102	RK002-B6	Agonist	Adrenergic	beta	85.8	*
I-104	RK002-C7	Agonist	Adrenergic	beta	82.9	*
I-103	RK002-C6	Agonist	Adrenergic	beta	71.1	*
N-153	RK002-F8	Agonist	Adrenergic	beta	11.9	*
A-109	RK002-A2	Agonist	Adrenergic	beta2	20.4	*
D-172	RK002-H5	Agonist	Adrenergic	beta1	4.7	
B-169	RK002-F4	Agonist	Adrenergic	beta3	2.4	
C-125	RK002-B5	Agonist/Antagonist	Adrenergic	beta	0	
N-113	RK002-C8	Agonist	Adrenergic	alpha, beta1	15	
B-001	RK002-F2	Agonist	Adrenergic	alpha2	0	
B-002	RK002-G2	Agonist	Adrenergic	alpha2	0	
B-012	RK002-D3	Agonist	Adrenergic	alpha	0	
B-161	RK002-D4	Agonist	Adrenergic	alpha2	0	
C-223	RK002-F2	Agonist	Adrenergic	alpha1A	0	
G-110	RK002-D6	Agonist	Adrenergic	alpha2	5.1	
I-114	RK002-D7	Agonist	Adrenergic	alpha2	2.8	
M-134	RK002-A8	Agonist	Adrenergic	alpha1	0	
O-101	RK002-H8	Agonist	Adrenergic	alpha	0	
O-110	RK002-A9	Agonist	Adrenergic	alpha2A	0	
P-133	RK002-A10	Agonist	Adrenergic	alpha1	5.3	
U-104	RK002-E11	Agonist	Adrenergic	alpha2	0.9	
X-101	RK002-F11	Agonist	Adrenergic	alpha2	2.4	
C-147	RK005-G4	Antagonist	Sigma	—	54.1	

[a]Complemented β-gal activity from 2μM (−)isoproterenol stimulation is used as 100% induction.
[b]The asterisks indicate that compounds are expected to have agonist activity.

Antagonist screening

For antagonist screening of the β_2AR-$\Delta\alpha$/$\beta arr2$-$\Delta\omega$ cells, compounds were tested in duplicate at both 2 μM and 10 μM. The performance of the antagonist screen was analyzed with the HTS Datadisplay 4.0 software. A total of 28 plates were assayed. After examining the data with 3 statistical measures, including percentage of coefficient of variation (%CV), Z factor, and signal to noise (S/N), 3 assay plates showed high %CV (7%-12%) and low Z factors (< 0.2) and were thus rejected.

The antagonist screen also demonstrates high specificity. Eight hits, including the 7 predicted hits, were obtained with > 50% inhibition of the isoproterenol-induced β-gal interaction readout at the 10 μM and 2 μM compound, while 8 other compounds only showed >50% inhibition at only 10 μM (see Table 2). Table 2 lists all of the known adrenergic antagonists included in the LOPAC™ library, as well as potential antagonist compounds showing > 50% inhibition. All of these compounds were tested for cytotoxicity with the fluorescent Alamar Blue™ assay, which measures metabolic activity by quantitation of the reduced redox indicator resulting from cell growth. The active compounds not known to target adrenergic receptors express varying degrees of toxicity, especially compounds C-198, C-129, and S-148 (data not shown).

To confirm specificity of initial hits, we repeated the receptor-arrestin interaction assay, and hits were validated with an addi-
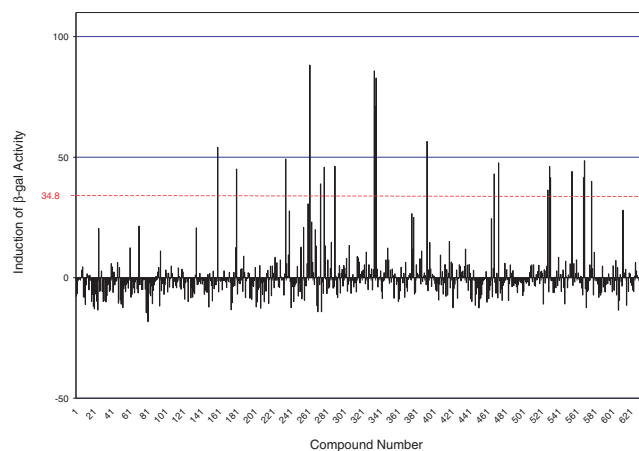

FIG. 3. LOPAC™ library agonist screening. The LOPAC™ library (640 compounds) was assayed in duplicate for each screen. Each bar represents the effect of each compound on the β-gal interaction readout. The β_2AR-$\Delta\alpha$/$\beta arr2$-$\Delta\omega$ cells were incubated with compounds at 2 μM at 37 °C for 60 min. The induction of β-gal activity is expressed as a percentage of isoproterenol induction (performed in the same plate).

TABLE 2.   RESULTS OF THE ANTAGONIST SCREEN WITH THE LOPAC™ LIBRARY

Compound ID	Position	Class of Action	Type of Receptor	Subtype Specificity	% Inhibition of β-gal Activity at 10 μM	% Inhibition of β-gal Activity at 2 μM	Comments
A-131	RK002-B2	Antagonist	Adrenergic	beta	75	94	
C-125	RK002-B5	Agonist/antagonist	Adrenergic	beta	81	92	
T-148	RK002-C11	Antagonist	Adrenergic	beta	94	89	
P-119	RK002-C9	Ligand	Adrenergic	beta	78	76	
B-012	RK002-B3	Agonist	Adrenergic	alpha	100	62	
I-127	RK002-E7	Antagonist	Adrenergic	beta2	87	76	
P-125	RK002-E9	Antagonist	Adrenergic	beta	98	81	
P-128	RK002-F9	Antagonist	Adrenergic	beta	84	85	
P-129	RK002-G9	Antagonist	Histamine	H1	56	0	
B-111	RK003-E3	Blocker	Ca^{2+} channel	—	50	23	
T-103	RK004-B11	Antagonist	Dopamine	D1/D2	55	0	
L-118	RK004-B8	Agonist/antagonist	Dopamine/serotonin	D2	71	42	
C-198	RK006-B4	Inhibitor	Enzyme	Protein kinase C	59	0	Toxic
C-129	RK007-A4	Inhibitor	Update	Serotonin	50	12	Slightly toxic
P-150	RK007-B8	Antagonist	Serotonin	5-HT1A	75	24	Structural isomer of P-119
S-148	RK008-D11	Inhibitor	Enzyme	ATPase (Mg^{2+} and Na^+/K^+)	100	0	Toxic

tional functional assay for inhibition of cAMP production, a well-characterized response in $β_2$-adrenergic receptor signaling. The specificity of 7 of the 16 initial hits was confirmed, with these compounds showing > 50% inhibition of the receptor-arrestin interaction readout and > 50% inhibition of cAMP production at both 10 μM and 2 μM. Figure 4 shows the correlation between the inhibition results of the receptor-arrestin interaction assay (retest data) and the secondary assay for cAMP production obtained at the 2 μM compound. Most compounds demonstrated a good correlation between inhibition of the receptor-arrestin interaction and inhibition of cAMP production.

Compound B-012, an α-adrenergic antagonist, showed inhibition of only the β-gal interaction readout (> 50%). There is little inhibition of cAMP production, indicating lack of effect on the G protein downstream signaling response. Compound S-148, an ATPase inhibitor, showed approximately 50% inhibition of the interaction readout but no inhibition of cAMP production, representing a nonspecific hit. The remaining 8 hits did not show inhibition of the receptor-arrestin interaction readout at 2 μM and did not significantly inhibit cAMP production. Two of these 8 compounds demonstrated significant toxicity (data not shown). To summarize, all compounds with known antagonist activity resulted in a hit, and only 1 compound (B-012) consistently showed a false-positive result. A low false-positive rate for antagonist screening (0.5%) has also been observed in an EGF receptor dimerization InteraX™ cell line containing EGFR fused to the β-gal fragments, screened against a random 1280-compound library, which compares very favorably to cell-based reporter gene assays that can typically result in false-positive rates of > 5%.[25] A low false-positive rate has also been observed in screens of other InteraX™ cell lines with the LOPAC™ library (unpublished data).
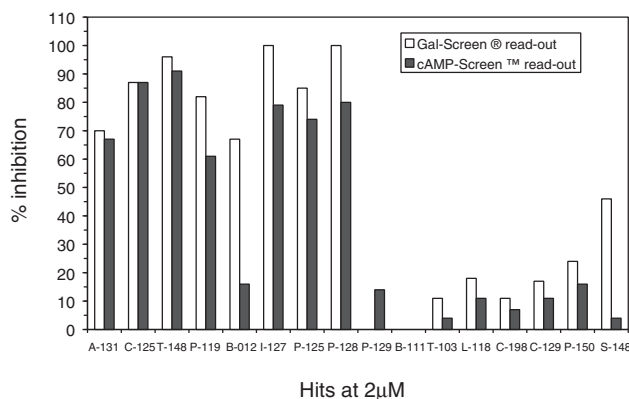

FIG. 4.   Confirmation and specificity of antagonist hits by cAMP quantitation. Sixteen hits obtained with > 50% inhibition of the receptor-arrestin interaction readout were tested for inhibition of cAMP production and rescreened with the receptor-arrestin interaction assay. The antagonist activity for either the interaction readout or cAMP production is expressed as a percentage of inhibition compared to the control level with isoproterenol stimulation.

In the agonist and antagonist screens, all compounds, in addition to positive and negative controls, were performed in duplicate. To demonstrate the repeatability of the assay system, we plotted the correlation between agonist replicates in Figure 5, using data from duplicates in separate sets of assay plates. The compounds are concentrated at the intersection of both axes (within 20% of zero), indicating inactive compounds, and values > 30% showed excellent reproducibility. Acceptance of assay plates was based on performance of S/N and Z' factor.[32] Although
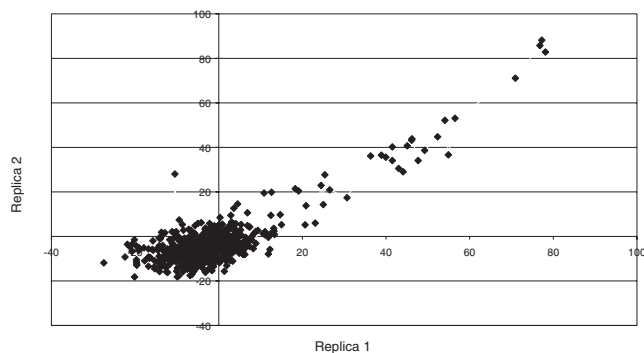
FIG. 5. Reproducibility of compounds in the G protein-coupled receptor (GPCR) assay system. Compounds were tested in duplicate for the agonist screen, and replica 1 is plotted versus replica 2. Each point represents the activity level of a different compound, expressed as a percentage of maximal induction of the receptor-arrestin interaction readout obtained with 2 µM isoproterenol. There is a good correlation between replicates for active compounds that show > 30% values, while most compounds are inactive, and accordingly, most of the data points are gathered around zero.
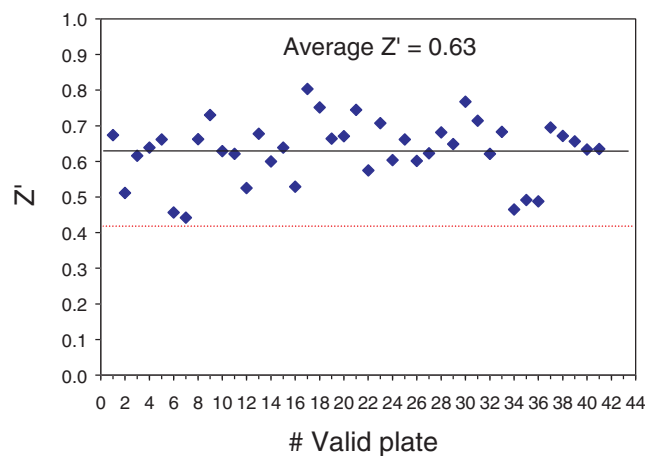

FIG. 6. Overall performance of both agonist and antagonist screens. Data from a total of 41 valid plates for agonist (16/16) and antagonist (25/28) screens are represented. The average signal to noise for all valid assay plates is 1.83 (data not shown), and the average Z' factor is 0.63.

average S/N was 1.8 for the screening run, the robustness of the assay is indicated by the fact that 41 of 44 screening plates had Z' values of > 0.4 (Fig. 6). Similar sensitivity is observed with other assay methods based on sensing of the GPCR/β-arrestin interaction, and thus the S/N achieved is likely limited by the biology of the particular receptor response or interaction. With other cell lines containing β-gal fusions for detection of protein-protein interaction, higher S/N is obtained.[3] Using a 50% activity cutoff for both agonists and antagonists, very few false positives were identified.

*Development of HTS protocol for cells
in suspension in 384-well microplate format*

A protocol was developed to enable direct addition of a cell suspension into a predispensed compound plate in a high-throughput screening application. A cell suspension was prepared from nearly confluent β_2AR-$\Delta\alpha$/βarr2-$\Delta\omega$ cells by dissociating the cells with either 0.25% trypsin/EDTA or a nonenzymatic dissociation solution immediately before assaying. Figure 7 shows a comparison of results obtained with both dissociation methods, using 20,000 cells/well in a 96-well plate. The EC_{50} values obtained following 1 h of incubation with isoproterenol with both suspension methods are very similar. The signal intensity obtained with trypsinized cells is lower than that from non-enzymatic-dissociated cells (Fig. 7), but the S/N of agonist response is the same with either method. The nonenzymatic method yielded greater assay variability due to incomplete dissociation of cells. In addition, isoproterenol dose-response results obtained with the cell suspension format were compared to those obtained with the standard format using adherent cells. β_2AR-$\Delta\alpha$/βarr2-$\Delta\omega$ cells were either grown 24 h (adherent cells, 10,000 cells/well) or plated immediately (suspension cells, 20,000 cells/well) prior to isoproterenol stimulation. The EC_{50} obtained with adherent cells (13 nM) is very simi-

lar to that obtained with suspension cells (16 nM) in a 96-well microplate format. The suspension cell protocol was also evaluated in a 384-well microplate format. Cells were dispensed at 10,000 to 20,000 cells/30 µl/well in a 384-well microplate with the Multidrop™ 384 dispenser. The receptor-arrestin interaction β-gal readout was measured with the NorthStar™ HTS Workstation. The EC_{50} for isoproterenol in the 384-well format is 11 nm, comparable to that obtained in 96-well format. To achieve a high S/N value, low coefficient of variation (CV), and an acceptable Z factor, several parameters were found to be important for the suspension cell protocol for the 384-well format. To obtain optimal S/N, the use of 90% to 95% confluent culture plates for preparation of the cell suspension is necessary. Cultures significantly below or above 90% to 95% confluence will not yield optimal results. Second, the use of automated liquid handling with high precision and accuracy that provides CVs within 5% to 6% is essential to achieve Z factors ≥ 0.4. These results were confirmed and repeatable over several independent 384-well format experiments.

CONCLUSION

We have demonstrated that fusions of a GPCR and β-arrestin to complementing fragments of β-galactosidase target proteins can be used as a sensor for monitoring GPCR signaling pathway activation in a cellular environment. The feasibility screen with the LOPAC™ library demonstrated that the InteraX™ GPCR assay system is able to identify agonists and antagonists for β_2AR. This assay method provides a suitable format for HTS automated screening with a simple lyse and read detection protocol using either adherent or suspension cells. The sensitivity, specificity, and ease of use of this GPCR assay system provide the means for discovering new therapeutic leads for known GPCRs and to potentially de-orphanize novel GPCRs.
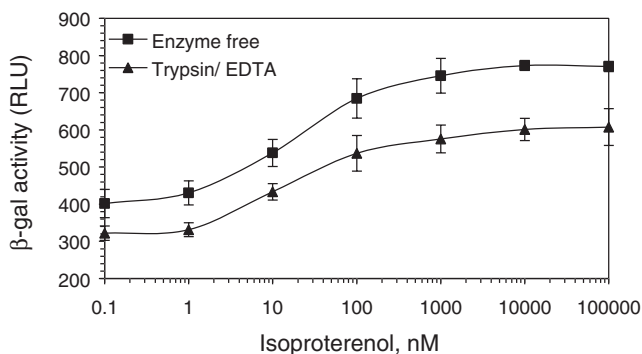
FIG. 7. Effect of trypsinization on agonist-induced response in β_2AR-$\Delta\alpha/\beta$arr2-$\Delta\omega$ cells. Cells were grown to 90% confluence, suspended with either 0.25% trypsin/EDTA or nonenzymatic cell dissociation solution, and seeded in 96-well plate at 20,000 cells/well and assayed immediately. The β-gal interaction readout was measured after cells were incubated with isoproterenol for 60 min.

ACKNOWLEDGMENTS

REFERENCES

1. Rossi F, Charlton CA, Blau HM: Monitoring protein-protein interactions in intact eukaryotic cells by β-galactosidase complementation. Proc Natl Acad Sci USA 1997;94:8405-8410.

2. Rossi FMV, Blakely BT, Blau HM: Interaction blues: protein interactions monitored in live mammalian cells by β-galactosidase complementation. Trends Cell Biol 2000;10:119-122.

3. Blakely BT, Rossi FMV, Tillotson B, Palmer M, Estelles A, Blau HM: Epidermal growth factor receptor dimerization monitored in live cells. Nature Biotech 2000;18:218-222.

4. Mohler WA, Blau HM: Gene expression and cell fusion analyzed by *lacZ* complementation in mammalian cells. Proc Natl Acad Sci USA 1996;93:12423-12427.

5. Mundell SJ, Loudon RB, Benovic JL: Characterization of G protein-coupled receptor regulation in antisense mRNA-expressing cells with reduced arrestin levels. Biochemistry 1999;38:8723-8732.

6. Ferguson SSG, Downey WE III, Colapietrok AM, et al: Role of β-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 1996;274:363-366.

7. Ferguson SSG: Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacological Reviews 2001;53:1-24.

8. Oakley RH, Laporte SA, Holt JA, et al: Association of β-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. J Biol Chem 1999;274:32248-32257.

9. Lohse MM, Benovic JL, Codina J, et al: β-arrestin: a protein that regulates β-adrenergic receptor function. Science 1990;248:1547-1550.

10. Lohse MM, Andexinger S, Pitcher J, et al: Receptor-specific desensitization with purified proteins: kinase dependence and receptor specificity of β-arrestin and arrestin in the β_2-adrenergic and rhodopsin systems. J Biol Chem 1992;267:85558-85564.

11. Gurevich VV, Benovic JL: Cell-free expression of visual arrestin: truncation mutagenesis identifies multiple domains involved in rhodopsin interaction. Biol Chem 1992;267:21919-21923.

12. Penn RB, Benovic JL: Regulation of G Protein-Coupled Receptors. In: Conn PM, editor. Handbook of physiology. Vol. 1. New York: Oxford University Press, 1998. p 125-164.

13. Krupnick JG, Gurevich VV, Benovic JL: Mechanisms of quenching of phototransduction: binding competition between arrestin and transducin for phosphorhodopsin. J Biol Chem 1997;272:18125-18131.

14. Kuhn H, Hall SW, Wilden U: Light-induced binding of 48-kDa protein to photoreceptor membranes is highly enhanced by phosphorylation of rhodopsin. FEBS Lett 1984;176:473-478.

15. Wilden U, Hall SW, Kuhn H: Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. Proc Natl Acad Sci USA 1986;83:1174-1178.

16. Attramadal H, Arriza JL, Aoki C, et al: β-Arrestin 2, a novel member of the arrestin/β-arrestin gene family. J Biol Chem 1992;267:17882-17890.

17. Pippig S, Andexinger S, Daniel K, et al: Over-expression of β-arrestin and β-adrenergic receptor kinase augment desensitization of β_2-adrenergic receptor. J Biol Chem 1993;268:3201-3208.

18. Sterne-Marr R, Gurevich VV, Goldsmith P, et al: Polypeptide variants of β-arrestin and arrestin3. J Biol Chem 1993;268:15640-15648.

19. Barak LS, Ferguson SSG, Zhang J, et al: A β-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. J Biol Chem 1997;272:27497-27500.

20. Zhang J, Barak LS, Anborgh PH, et al: Cellular trafficking of G protein-coupled receptor β-arrestin endocytic complexes. J Biol Chem 1999;274:10999-11006.

21. Angers S, Salahpour A, Joly E, et al: Detection of β_2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci USA 2000;97:3684-3689.

22. Hieble JP: Drugs targeting adrenergic receptors: does interaction with a specific subtype confer therapeutic advantage? Curr Opin Drug Disc Dev 2000;3:370-382.

23. Ahuja SK, Murphy PM: The CXC chemokines growth-regulated oncogene (GRO)α, GROβ, GROγ, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. J Biol Chem 1996;271:20545-20550.

24. Jones SA, Dewald B, Clark-Lewis I, et al: Chemokine antagonists that discriminate between interleukin-8 receptors. J Biol Chem 1997;26:16166-16169.

25. Graham DL, Bevan N, Lowe PN, et al: Application of β-galactosidase enzyme complementation technology as a high throughput screening format for inhibitors of the epidermal growth factor receptor. J Biomol Screen 2001;6:401-411.

26. Chiulli AC, Trompeter K, Palmer M: A novel high throughput chemiluminescent assay for the measurement of cellular cyclic adenosine monophosphate levels. J Biomol Screen 2000;5:239-247.

27. Ferguson SSG, Menard L, Barak LS, et al: Role of phosphorylation in agonist-promoted β_2-adrenergic receptor sequestration. J Biol Chem 1995;270:24782-24789.

28. Mouillac B, Caron M, Bonin H, et al: Agonist-modulated palmitoylation of the β_2-adrenergic receptor in Sf9 cells. J Biol Chem 1992;267:21733-21737.

29. Fan GH, Yang W, Wang XJ, et al: Identification of a motif in the carboxyl terminus of CXCR2 that is involved in adaptin 2 binding and receptor internalization. Biochemistry 2001;40:791-800.

30. Prado GN, Suzuki H, Wilkinson N, et al: Role of the C terminus of the interleukin 8 receptor in signal transduction and internalization. J Biol Chem 1996;271:19186-19190.

31. White JR, Lee JM, Young PR, et al: Identification of a potent, selective non-peptide CXCR2 antagonist that inhibits interleukin-8-induced neutrophil migration. J Biol Chem 1998;273:10095-10098.

32. Zhang JH, Chung TDY, Oldenburg KR: A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 1999;4:67-73.

Address reprint requests to:
Yu-xin Yan, Ph.D.
Applied Biosystems
47 Wiggins Avenue
Bedford, MA 01730

E-mail: yansy@appliedbiosystems.com