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Full-length article

Development of a universal high-throughput calcium assay for G-protein-coupled receptors with promiscuous G-protein Gα15/16[1]

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Key words

G-protein-coupled receptors; G-protein; Gα15/16; high-throughput screening; calcium assay; GTPγS binding

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Abstract

Aim: To develop a universal high-throughput screening assay based on Gα15/16-mediated calcium mobilization for the identification of novel modulators of G-protein-coupled receptors (GPCR). **Methods:** In the present study, CHO-K1 or HEK293 cells were co-transfected with plasmids encoding promiscuous G-protein Gα15/16 and various receptors originally coupled to Gαs, Gαi, or Gαq pathways. Intracellular calcium change was monitored with fluorescent dye Fluo-4. **Results:** We found out for all the receptors tested, Gα15/16 could shift the receptors' coupling to the calcium mobilization pathway, and the EC_{50} values of the ligands generated with this method were comparable with reported values that were obtained using traditional methods. This assay was validated and optimized with the δ-opioid receptor, which originally coupled to Gαi and was recently found to play important roles in neurodegenerative and autoimmune diseases. A large-scale screening of 48 000 compounds was performed based on this system. Several new modulators were identified and confirmed with the traditional GTPγS binding assay. **Conclusion:** This cell-based calcium assay was proved to be robust and easy to automate, and could be used as a universal method in searching for GPCR modulators.

Introduction

G-protein-coupled receptors (GPCR) constitute one of the largest and most versatile families of cell surface receptors[1]. GPCR recognize and respond to a variety of extracellular stimulants and endogenous ligands, including light, odors, taste substances, hormones, chemotactic factors, and neurotransmitters[2]. Due to the variety of physiological and pathological functions regulated by GPCR, they were considered the most promising drug targets in the pharmaceutical industry. It is estimated that over 50% of the marketed drugs are modulators of GPCR functions[3,4]. With the completion of the Human Genome Project, approximately 1000 genes encoding GPCR were identified, but only about 200 have known ligands and functions[5]. Searching for ligands of the orphan GPCR and better modulators of known receptors will provide new opportunities in future drug discovery.

GPCR are 7 transmembrane proteins with the amino terminal and carboxy terminal located in the extracellular and intracellular spaces, respectively[6]. After ligand stimulation, GPCR undergo conformational change and activate the intracellular G-proteins, which are composed of α, β, and γ-subunits, and then initiate signaling to the cell interior[7]. Based upon the structure and downstream signaling cascade, the Gα subunit is mainly divided into 4 families: Gαs, Gαi/o, Gαq, and Gα12[8]. GPCR coupling to Gαs (including Gαs[s], Gαs[l], Gαs[xl], Gαs[xxl], and Gαolf) activate adenylate cyclase, which catalyses cAMP production. Contrarily, Gαi/o (including Gαt[r], Gαt[c], Gαgust, Gαi1, Gαi2, Gαi3, Gαo1, Gαo2, Gαo3, and Gαz) inhibit cAMP production. GPCR coupling to Gαq (including Gαq, Gα11, Gα14, and Gα15/16) activate phospholipase Cβ, which catalyzes the generation of IP_3 and calcium release from intracellular store[9,10]. Gα12 (including Gα12 and Gα13) is believed to be related to the activation of Ras, Raf, and ERK pathway[11].

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Based on the signal transduction cascade of GPCR, several assay techniques for GPCR ligand screening, such as radioligand binding, [^{35}S]-GTPγS binding, reporter gene, cAMP detection, and calcium mobilization are commonly used. Radiometric techniques not only require an advanced laboratory, but also generate environment pollution and impair people's health. So non-radiometric assays, especially cell-based functional assays, played more important roles in primary screening[12]. However, these assays can only be applied for selected Gα subtypes. For example, cAMP assay can only be used for Gαs and Gαi/o-coupled GPCR, and calcium mobilization only for Gαq-coupled receptors. These assays require well-characterized signaling pathway of the receptors, so they would be difficult to apply to orphan GPCR.

It is therefore apparent that a universal high-throughput screening (HTS) approach for GPCR ligand screening would be valuable. Previous studies have demonstrated that most receptors promiscuously couple to several Gα subtypes, but because one of the G-proteins occupied the dominant status, it is hard to detect signals induced by other Gα subtypes[13]. Overexpression of certain Gα subunits can shift the original coupling pathway of GPCR to the new one[13,14]. In the present paper, we tested the coupling of the promiscuous G-protein Gα15/16 (mouse/human orthologs, respectively[15]) with various receptors that originally coupled to the Gαs, Gαi, or Gαq pathways. We found out for all the receptors tested, Gα15/16 shifted the receptors coupling to the calcium mobilization pathway, and intracellular calcium change could be easily detected with a Fluo-4 fluorescent indicator. Ligand efficacy measured by this method was comparable with the value obtained using traditional methods. This assay was validated with the δ-opioid receptor (DOR), which originally coupled to Gαi and may play important roles in pain, neurodegenerative, and autoimmune diseases[16–18]. A large-scale screening of 48 000 compounds was performed based on this system. Several new modulators (including both agonists and antagonists) were identified and confirmed with the traditional [^{35}S]-GTPγS binding assay. This cell-based calcium assay was proved to be robust and easy to automate, and could be used as a universal method for the search of GPCR modulators.

Materials and methods

Reagents Mammalian expression vectors encoding cannabinoid receptors 1 and 2 (CB1 and CB2), α1a adrenergic receptor (α1aAR), α2b adrenergic receptor (α2bAR), dopamine receptor 5 (DRD5), and Gα15/16 were purchased from UMR cDNA Resource Center (Rolla, MO, USA). Plasmids encoding chemokine receptors CCR5, CXCR4, δ-opioid receptor, and β2 adrenergic receptor (β2AR) were kindly provided by Dr Gang Pei from Shanghai Institutes for Biological Sciences (Shanghai, China). Fluo-4 AM was purchased from Invitrogen (Carlsbad, CA, USA). FlashBlue GPCR scintillation beads and [^{35}S]-GTPγS were products of PerkinElmer (Boston, MA, USA). SDF-1 was purchased from GL Biochem (Shanghai, China). Sulfinpyrazone, RANTES, DPDPE, isoproterenol, phenylephrine, dopamine, noradrenalin, TIPP-ψ, naltrindole, [*D*-Ala^2]-deltorphin II and DADLE were purchased from Sigma–Aldrich (St Louis, MO, USA). Other reagents and solvents used in the experiments were of analytical grade.

Cell transfection CHO-K1 or HEK293 cells were obtained from ATCC (Manassas, VA, USA) and maintained in F12 nutritional medium or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 mg/L penicillin, and 100 mg/L streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. For transient transfection, approximately 1×10^6 cells were mixed with 2 μg plasmids in 200 μL transfection buffer, and electroporation was carried out with a Scientz-2C electroporation apparatus (Scientz Biotech, Ningbo, China). The experiments were carried out 24 h after transfection. For stable cell line generation, the transfected cells were seeded into 10 cm dishes, and proper antibiotics (500 μg/mL G418 and/or 20 μg/mL blasticidin) were added to the culture medium the next day. The selection medium was changed every 3 d until colonies were formed. The single colony was picked up, expanded, and tested for the expression of transfected genes.

Calcium mobilization assay CHO cells co-transfected with receptors and Gα15/16 were plated onto 96-well plates at a density of 30 000 cells/100 μL per well and incubated overnight. The cells were loaded with 2 μmol/L Fluo-4 AM in Hanks' balanced salt solution (HBSS; containing 5.4 mmol/L KCl, 0.3 mmol/L Na_2HPO_4, 0.4 mmol/L KH_2PO_4, 4.2 mmol/L $NaHCO_3$, 1.3 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 0.6 mmol/L $MgSO_4$, 137 mmol/L NaCl, 5.6 mmol/L *D*-glucose, and 250 μmol/L sulfinpyrazone, pH 7.4) at 37 °C for 50 min. After removal of the excess dye, the cells were rinsed with HBSS once. In the antagonist mode, 50 μL HBSS containing known antagonists (positive control), compounds of interest, or DMSO (negative control, final concentration 1%) were added. After incubation at room temperature for 10 min, 25 μL agonists were dispensed into the well with a FlexStation II micro-plate reader (Molecular Devices, Sunnyvale, CA, USA), and intracellular calcium change was recorded with an excitation wavelength of 485 nm and emission wavelength of 525 nm. In the agonist mode, 50 μL HBSS was added to the dye-

loaded cells, and 25 μL of known agonists (positive control), compounds of interest, or DMSO (negative control, final concentration 1%) were added with FlexStation II, and calcium change was measured.

[^{35}S]-GTPγS binding assay Cell membranes were isolated as previously described[19]. In brief, CHO/DOR cells were pelleted by centrifugation and resuspended in lysis buffer (5 mmol/L Tris-HCl, 5 mmol/L EDTA, and 5 mmol/L EGTA, pH 7.5), and then homogenized with a Dounce tissue grinder. The lysate was centrifuged at 1000×*g* for 10 min. After removal of the deposition, crude membranes were then pelleted by centrifugation at 12 000×*g* for 15 min at 4 °C. The membranes were resuspended in reaction buffer (20 mmol/L HEPES, 100 mmol/L NaCl, and 5 mmol/L $MgCl_2$, pH 7.4), and the protein concentration was determined using the Bradford method[20]. The exchange of [^{35}S]-GTPγS was measured using a scintillation proximity assay, as previously described[21]. For each assay point, 5 μg membrane was incubated in 100 μL reaction buffer for 3 h at 30 °C with 100 μg FlashBlue GPCR beads, 10 μmol/L GDP, 10 μg/mL saponin, 0.2 nmol/L [^{35}S]-GTPγS, and the indicated concentration of compounds. For non-specific basal binding measurement, 2 μmol/L GTPγS was added. Membrane-bound [^{35}S]-GTPγS was measured with a Microbeta scintillation counter (PerkinElmer, Waltham, MA, USA).

HTS campaign The compound library used for the screening of DOR modulators was comprised of 48 000 different compounds. A 10 compound pool/well mix was applied to the primary screening in the antagonistic mode, with an average final concentration of 4.4 μmol/L for each compound. This matrix system maximized the advantage of HTS and allowed duplicate screening of each compound[22]. In each 96-well plate, 8 wells were used as positive controls (100 nmol/L TIPP-ψ in 1% DMSO) and another set of 8 wells as negative controls (1% DMSO). The inhibition rate of 100 nmol/L TIPP-ψ was normalized to 100%, and that of the negative control was 0. The inhibition rate of each compound was calculated with the following equation:

Inhibition %=(Calcium peak value $_{compound}$–calcium peak value $_{1\%DMSO}$)/(calcium peak value $_{TIPP\text{-}\psi}$ –calcium peak value $_{1\%DMSO}$)×100%. The samples showing more than 70% inhibition were considered "hits" in the primary screening.

Data analysis Data were analyzed with GraphPad Prism software (GraphPad, San Diego, CA, USA). Non-linear regression analyses were performed to generate dose-response curves and calculate EC_{50} or IC_{50} values. Linear regression was used to analyze data reproducibility. Two-tailed Student's *t*-test was applied to analyze differences. The Z' factor was calculated by the following equation:

$Z'=1-(3SD_+ + 3SD_-)/|Ave_+ - Ave_-|$, where SD_+ is the standard deviation of the positive control, SD_- is the standard deviation of the negative control, Ave_+ is the mean value of the positive control, and Ave_- is the mean value of the negative control.

Results

Gα15/16 can couple to various GPCR and mediate calcium response In the present study, we first tested whether promiscuous G-protein Gα15/16 could couple to different types of GPCR and mediate calcium response upon stimulation. Five Gαi/o-coupled (DOR, CB1, CB2, CCR5, and CXCR4), 3 Gαs-coupled (α2bAR, β2AR, and DRD5), and 1 Gαq-coupled (α1aAR) receptors were co-expressed in CHO-K1 or HEK293 cells with Gα15/16, and calcium assay was carried out as described earlier. Representative kinetic and dose-response curves are shown in Figure 1, and the EC_{50} of various ligands are summarized in Table 1. For most of the Gαi/o- and Gαs-coupled receptors, agonist stimulation caused little or no change in the intracellular calcium concentration, and the calcium assay could not be used to measure the EC_{50} value of ligands (Figure 1; Table 1). When co-expressed with Gα15/16, all receptors produced a significant calcium-elevating effect after proper stimulation (Figure 1; Table 1). We also found that overexpression of Gα15/16 made little difference in the calcium response generated by Gαq-coupled receptor α1aAR (Figure 1E,1F). One of the receptor α2bAR mainly coupled to Gαs, was also reported to induce calcium response by coupling to plasma membrane calcium channels [23]. Overexpression of Gα15/16 with α2bAR increased the calcium assay's sensitivity, as indicated in the reduction of EC_{50} value of noradrenaline (Table 1). For all the receptors tested, the sensitivity of Gα15/16-mediated calcium assay was comparable with or sometimes more sensitive than the traditional cAMP or [^{35}S]-GTPγS assays (Table 1). We also found cells stably transfected with DOR and Gα15/16 gave higher and longer-sustained calcium signals compared to transient transfected cells (supplement Figure 1S). This was likely due to the higher expression level of the receptor and Gα15/16 protein in stably transfected cells (supplement Figure 1S, 2S and Table 1S). For further characterization of this calcium assay, stable cell lines were used.

Agonist and antagonist mode of the calcium assay DOR was chosen as a model receptor to test the applicability of this calcium assay. DOR is a Gαi/o-coupled receptor that plays important roles in various diseases, but lacks straightforward functional HTS assays. We tested a

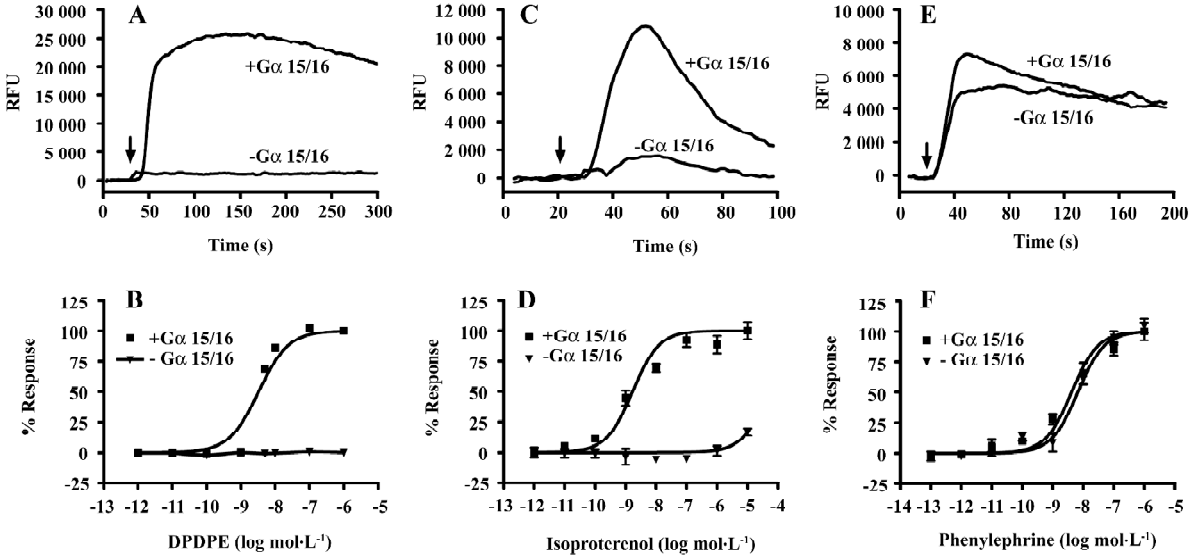

Figure 1. Kinetic and dose-response curves of calcium mobilization assay of representative GPCR. CHO or HEK293 cells were transfected with DOR (A,B), β2AR (C,D), or α1aAR (E,F) together with Gα15/16 or control plasmids. Proper ligands (5 nmol/L DPDPE for DOR, 10 nmol/L isoproterenol for β2AR, and 10 nmol/L phenylephrine for α1aAR) were added at the time points indicated by arrows, and representative kinetic curves of calcium response are presented (A,B,C). B, D, and F represent dose-response curves of proper ligands on their corresponding receptors. $n \geq 3$. Data are mean±SEM. RFU: relative fluorescent unit.

Table 1. Comparison of EC_{50} values of various ligands obtained by calcium assay and previously reported methods.

Receptor	G-protein	Agonist tested	EC_{50}/calcium assay (95% CI, nmol/L)		Reported EC_{50} (nmol/L) / Functional assay used
			–Gα15/16	+Gα15/16	
CB1	Gαi/o	CP55940	UD	2.8 (2.2–3.5)	2.6±1.0/cAMP assay[33]
CB2	Gαi/o	CP55940	UD	8.7 (5.7–13.1)	2.9±1.4/cAMP assay[33]
CCR5	Gαi/o	RANTES	UD	3.8 (3.3–4.5)	~1/IP release assay[34]
CXCR4	Gαi/o	SDF-1	UD	22.1 (19.7–24.8)	~10/[^{35}S]-GTPγS binding assay[35]
DOR	Gαi/o	DPDPE	UD	3.3 (2.4–4.4)	~4.6/cAMP assay[36]
α2bAR	Gαs	Noradrenaline	33.5 (18.0–62.7)	7.0 (4.2–11.6)	35.6±16.9/calcium assay[37]
DRD5	Gαs	Dopamine	UD	23.8 (12.5–45.4)	364±91/cAMP assay[38]
β2AR	Gαs	Isoproterenol	UD	1.7 (1.0–2.7)	2.4±0.2/AC activity assay[39]
α1aAR	Gαq	Phenylephrine	6.6 (4.5–9.8)	4.1 (2.6–6.5)	530±60/ [^{3}H]-IP1 accumulation assay[40]

AC, adenylyl cyclase; CI, confidence interval; IP, inositol phosphate; IP1, inositol monophosphate; UD, undetectable.

group of known DOR ligands (including 3 agonists: DPDPE, deltorphin II, and DADLE, and 2 antagonists: TIPP-ψ and naltrindole) on cells that stably express DOR and Gα15/16 with 2 different setups. In the antagonist testing mode (Figure 2A), test compounds were pre-incubated with the cells for 15 min; then calcium assay was initiated by the addition of agonist DPDPE. In this setup, antagonists showed blocking effects as anticipated, and agonists also blocked the DPDPE-induced calcium response due to receptor desensitization during the pre-incubation period[24,25]. In the agonist-testing mode (Figure 2A), calcium assay was initiated by the direct addition of test compounds. All agonists showed a robust calcium-elevating effect, and antagonists did not cause any changes. So in the later HTS campaign, all compounds were tested in the antagonist mode in the primary screening to reveal any compounds that might block (antagonist) or desensitize (agonist) the receptor. The agonist mode was used in the secondary screening to distin-

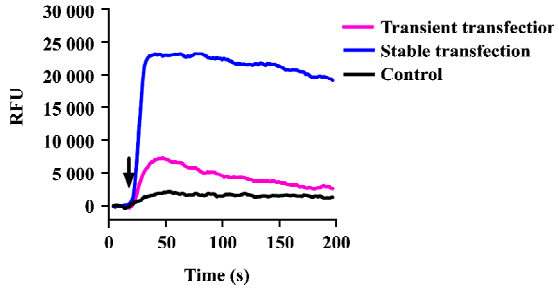

Figure 1S. Calcium signals in cells transiently or stably transfected with DOR and Gα15/16. Compared to the weak and transient calcium signal in transient transfected cells, the signal of stable cell line was much higher and sustained longer. Sham transfected CHO cells were used as control.

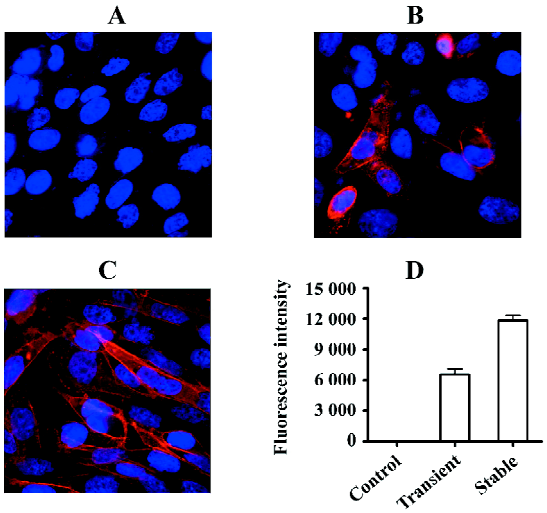

Figure 2S. Receptor expression level in cells transiently or stable transfected with DOR and Gα15/16. Fluorescent conjugated anti-HA antibody was used to detect HA-tagged DOR. (A), (B) and (C) are representative images of sham transfected, transient transfected and stably transfected CHO cells. (D) The statistical analysis of whole cell fluorescent intensity by ArrayScan® 4.0 HCS Reader (Cellomics, PA). More than 3 000 cells from 10 randomly selected fields were used for the statistical analysis.

guish agonists from antagonists.

The EC_{50} values of known agonists were generated with the agonist mode of the calcium assay and the IC_{50} of known antagonists with the antagonist mode. These values were compared with those obtained with the traditional [^{35}S]-GTPγS binding assay (Table 2), and both assays showed similar sensitivity.

Optimization and performance of the HTS assay Various experimental conditions were tested to optimize the assay for HTS. We found that cell density did not affect the EC_{50} value of the agonist DPDPE, but the signal to background (S/B) ratio of the calcium response reached a plateau at a cell density of 30 000/well. The solvent used for compounds, DMSO, did not affect the S/B ratio at concentrations up to 1%, and hardly interfered with the dose-response curves at concentrations up to 2% (Figure 3C,3D). The final assay conditions for HTS were determined as follows: the cell density was 30 000/well, the final concentration of DMSO was 1%, and the DPDPE concentration was 10 nmol/L (approximately EC_{80}).

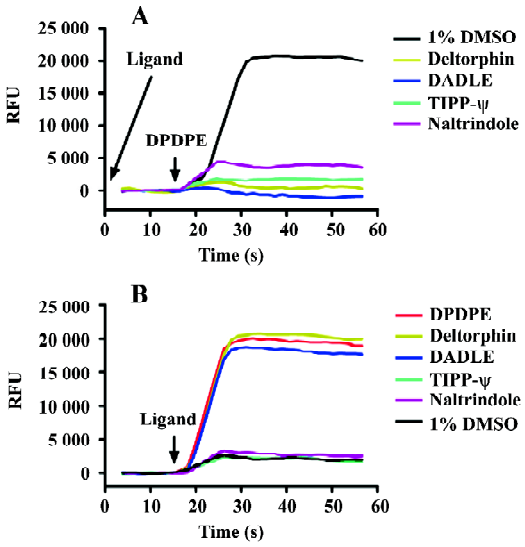

Figure 2. Antagonist and agonist mode of calcium mobilization assay demonstrated on DOR. (A) in the antagonist mode, CHO-K1 cells stably expressing DOR and Gα15/16 were loaded with Fluo-4 AM and pre-incubated with various ligands (including 2 agonists: deltorphin and DADLE, and 2 antagonists: TIPP-ψ and naltrindole, all at 100 nmol/L, 1% DMSO as control) for 15 min. Then the DOR agonist DPDPE (10 nmol/L) was added, and the kinetic curves of calcium response were recorded. Due to receptor desensitization, pre-incubated agonists also showed blocking effects. (B) in the agonist mode, the cells were loaded with dye, and calcium assay was carried out by the direct addition of ligands. Agonists displayed a calcium-elevating effect, and antagonists did not induce any response.

The Z' value is a metric used to assess the robustness of an assay for screening and is the normalized 3 standard deviation window between the negative controls and positive controls[26]. As shown in Figure 4A, the Z' value for the assay was 0.64, and the S/B ratio was 18.86, indicating that the system was adequately optimized for HTS. Furthermore, to investigate reproducibility between duplicate plates, the corresponding wells from 2 different 96-well plates were treated with the same concentration of TIPP-ψ and then 10 nmol/L DPDPE. The data from the corresponding wells of different plates were investigated with liner regression analysis[27]. The correlation coefficient was 0.95, showing a high

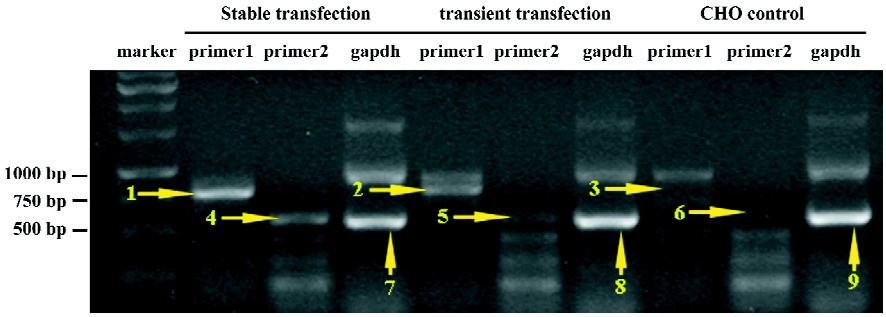

Figure 3S. RT-PCR results of cells transiently or stable transfected with DOR and Gα15/16. RT-PCR was applied to detect the mRNA level of Gα15/16. Arrow 1 and 2 indicated the expected products from primer pair 1. Arrow 4 and 5 indicated the expected products from primer pair 2. The stable transfected cells showed higher transcription level of Gα15/16. Arrow 3 and 6 pointed out that Gα15/16 was not expressed in control cells. The transcription levels of GAPDH were similar in all cells (arrow 7, 8 and 9). Sequences of primer pairs were listed in Table 1S.

Table 1S. List of primers used for RT-PCR.

Primer	Sense	Antisense	Product length
Primer pair 1 Gα15/16	5'-GGTGCCTGACGGAGGATGAG-3'	5'-TGAGAAAGAGGATGACGGAT-3'	793 bp
Primer pair 2 Gα15/16	5'-CTACTATGAGCGTCGGCGGG-3'	5'-GGCTGAAGAGGCGTCGGGAT-3'	588 bp
GAPDH	5'-ATCTTCTTGTGCAGTGCCAGCC-3'	5'-GGTCATGAGCCCTTCCACAATG-3'	560 bp

Table 2. Comparison of EC_{50} or IC_{50} values of known DOR ligands generated with Gα15/16-mediated calcium assay and traditional [^{35}S]-GTPγS binding assay.

Ligands tested	Nature of ligand	Calcium assay		[^{35}S]-GTPγS binding assay	
		EC_{50} (nmol/L, 95% CI)	IC_{50} (nmol/L, 95% CI, DPDPE=10 nmol/L)	EC_{50} (nmol/L, 95% CI)	IC_{50} (nmol/L, 95% CI, DPDPE=10 nmol/L)
DPDPE	Agonist	4.0 (2.5–6.7)	NA	1.3 (0.4–3.7)	NA
[D-Ala^2]-deltorphin II	Agonist	2.2 (1.3–3.5)	NA	7.2 (4.0–12.9)	NA
DADLE	Agonist	2.1 (1.1–4.2)	NA	4.2 (2.4–7.2)	NA
TIPP-ψ	Antagonist	NA	1.0 (0.5–2.4)	NA	4.7 (2.3–9.6)
Naltrindole	Antagonist	NA	2.7 (1.2–5.7)	NA	4.6 (1.0–21.4)

NA: not applicable.

degree of reproducibility between duplicate sample plates.

Results of HTS campaign Of the 48 000 compounds initially screened, 273 hits (0.57%) showing greater than 70% inhibition on 10 nmol/L DPDPE-induced calcium response were discovered (Figure 5A). Secondary screening (single compound per well) was done to further confirm the hits (Figure 5B). Finally, 8 compounds displaying consistent inhibitory effects from the secondary screening were picked out and tested on other GPCR (CCR5 and CXCR4; data not shown) for receptor specificity. Four compounds with relatively high receptor specificity for DOR were further tested to distinguish their agonist or antagonist nature (Figure 5C, 5D). Three of the compounds (TZ-02, TZ-03, and TZ-04) showed moderate to weak agonist properties, as they were found to induce calcium response in DOR- and Gα15/16 co-expressing cells. One compound TZ-01 showed pure antagonist property. The activities of these compounds were further validated with [^{35}S]-GTPγS binding assay (Table 3). Compounds TZ-02 and TZ-04 were proven to be agonists, and compound TZ-01 was an antagonist. Compound TZ-03

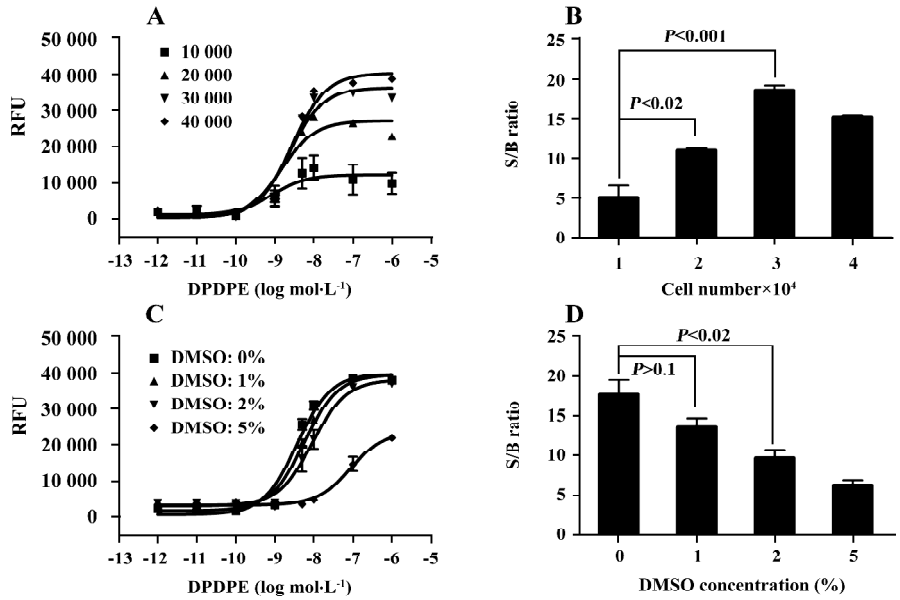

Figure 3. HTS assay optimization. (A) with different cell densities, the EC_{50} values of DOR agonist DPDPE obtained from the calcium assay were almost the same (0.8–2.8 nmol/L), but the S/B ratio was significantly higher with increased cell density (B). (C,D) at concentrations up to 2%, DMSO did not affect dose-response curves; and at concentrations up to 1%, did not affect the S/B ratio. $n \geq 3$. Data presented are mean±SEM.

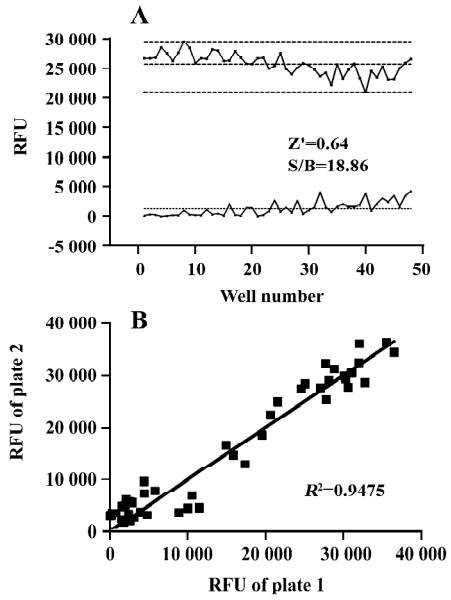

Figure 4. Assay performance. (A) Z' factor determination. At the optimized conditions, 48 replicates of positive and negative signals were studied. Dashed lines indicate mean±3 SD of 48 data points. Z' value for the assay was 0.64, and the S/B ratio was 18.86, indicating that the system was adequately optimized for HTS. (B) reproducibility. Corresponding wells from 2 different 96-well plates were stimulated with same concentration of DPDPE. Reproducibility of data from duplicate plates was investigated with a linear regression analysis. Correlation coefficient was 0.95, showing a high degree of reproducibility between duplicate sample plates.

was a partial agonist/antagonist. Its weak agonist activity can only be detected in the sensitive calcium assay, but not in the [^{35}S]-GTPγS binding assay due to a limited assay window. The strong antagonist property of TZ-03 was confirmed by both assays.

Discussion

Considerable effort has been directed towards the development of HTS platforms for the GPCR because these cell surface receptors represent important drug targets[28]. Detection methods have moved a long way from membrane-based radioligand binding assay towards cell-based functional assays. Most functional assays rely on the detection of the changes of different downstream effectors induced by receptor activation. Due to the versatility of GPCR-induced intracellular changes, it is sometimes difficult to handle and compare results from different assay systems. Meanwhile, with the cloning of more and more orphan GPCR, their implications as potential drug targets require vigorous validation. Little knowledge exists today regarding their native ligands and coupling mechanisms, and this makes HTS assay development extremely difficult. Thus, a universal HTS approach for GPCR ligand screening would be highly valuable.

Calcium mobilization assay with fluorescent dyes is a highly sensitive and easy-to-handle method that has been

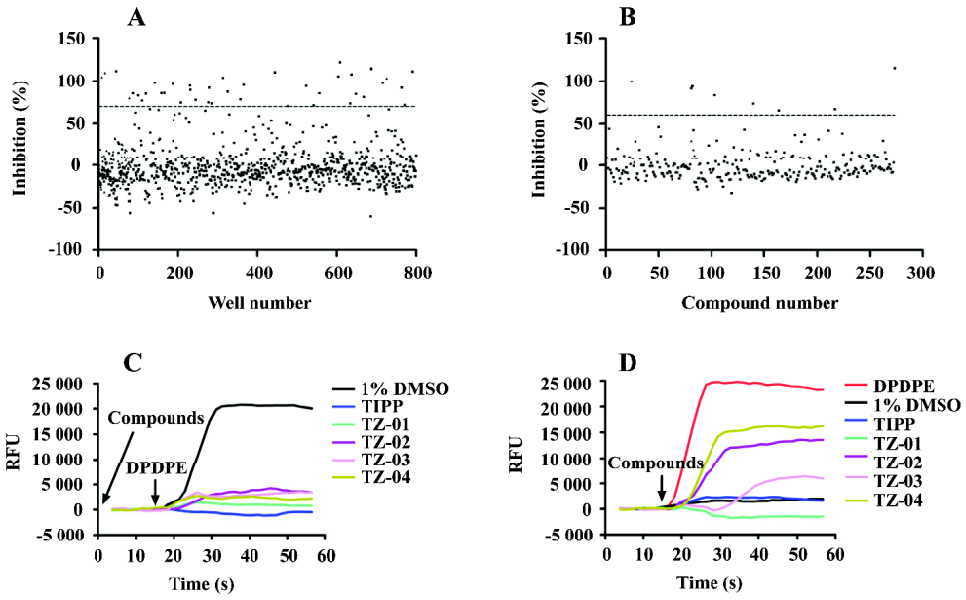

Figure 5. HTS of 48 000 compounds. (A) representative result of primary screening of 800 wells. (B) compounds with ≥70% inhibition rate in the primary screening were further tested in a single compound/well setup. Four compounds with novel structure from secondary screening were picked out and further tested in a triplet setup to identify their agonist or antagonist nature. (C) in the antagonist testing mode, all compounds showed a consistent blocking effect of DPDPE. (D) in the agonist testing mode, 3 compounds (TZ-02, TZ-03, and TZ-04) showed moderate to weak agonist activity, and 1 compound TZ-01 showed pure antagonist property.

Table 3. Validation of newly-discovered DOR modulators with [^{35}S]-GTPγS binding assay.

Compounds	Nature of compounds	Calcium assay		[^{35}S]-GTPγS binding assay	
		EC_{50} (95% CI, μmol/L)	IC_{50} (95% CI, μmol/L) (DPDPE=10 nmol/L)	EC_{50} (95% CI, μmol/L)	IC_{50} (95% CI, μmol/L) (DPDPE=10 nmol/L)
TZ-01	Antagonist	NA	46.6 (16.4–132.2)	NA	40.0 (18.9–84.7)
TZ-02	Agonist	3.5 (2.2–5.6)	NA	1.8 (1.1–3.1)	NA
TZ-03	Partial agonist /antagonist	0.8 (0.3–2.2)	0.015 (0.009–0.024)	UD	0.0002 (0.00003–0.0016)
TZ-04	Agonist	0.6 (0.4–1.1)	NA	1.1 (0.6–2.0)	NA

widely applied to study ligand or voltage-gated ion channels and GPCR coupled to the Gαq-protein[29]. It is critical to provide the receptors with a universal and efficient calcium signal transducer if this method is to be used to search modulators for various GPCR. It has been reported that the Gαq-protein with the last 5 amino acids exchanged with Gαs- or Gαi-proteins (designated as Gqs5 and Gqi5[30]) can couple to GPCR that originally coupled to Gαs or Gαi, and induce calcium mobilization upon stimulation. In the present study, we tested the versatility of another Gαq subfamily protein Gα15/16.

A panel of 9 GPCR that originally coupled to different types of G-proteins was studied. These included 5 Gαi/o-coupled (DOR, CB1, CB2, CCR5, and CXCR4), 3 Gαs-coupled (α2bAR, β2AR, and DRD5) and 1 Gαq-coupled (α1aAR) receptors. With the exception of α1aAR, which originally coupled to Gαq, and α2bAR, which was reported to modulate plasma membrane calcium channels[23], other receptors could not elicit measurable calcium responses upon stimulation when they were expressed alone in CHO-K1 or HEK293 cells. After co-expression with Gα15/16, all receptors were coupled to the calcium mobilization pathway, and the EC_{50} values of the ligands measured with this assay were in close agreement or more sensitive than other reported methods.

We further characterize the Gα15/16-mediated calcium assay on DOR. DOR has been heavily studied in the past for its roles in pain and drug addiction[31,32]. It has been a focus of attention again recently due to its involvement in neurode-

generative and autoimmune diseases[16,18]. Traditional HTS methods for searching DOR ligands include radioligand binding, [^{35}S]-GTPγS binding, and cAMP assay. Both [^{35}S]-GTPγS binding and cAMP assay can only be used to search agonists or antagonists in a single HTS run. Radioligand binding is the only way to find both agonists and antagonists simultaneously, even though the separation of agonists from antagonists needs a secondary functional assay. We found the calcium assay to be very efficient in detecting both agonists and antagonists if the testing compounds were pre-incubated with the cells before the addition of agonist DPDPE. In this experimental setup, agonists can also block the DPDPE-induced calcium response due to receptor desensitization during the pre-incubation period. Thus, any compounds that reduce the DPDPE-elicited calcium signal could be a potential DOR modulator. Later on, the agonist or antagonist nature of the compound can be simply distinguished by direct application of the compound to the cells to see whether it can induce calcium change or not.

Various assay parameters were optimized to improve the assay window and stability. The Z' factor is a useful tool for evaluating bioassay qualities[26]. In general, a Z' value above 0.5 suggests that an assay is robust enough for HTS. The calcium mobilization system described herein displayed a Z' value of 0.64, which indicated that the assay was of a high-quality nature. This assay was applied to a large-scale screening of a compound library consisting of 48 000 synthetic compounds. Four compounds with novel structures and relatively high receptor specificity were sorted out and further validated with a traditional [^{35}S]-GTPγS binding assay. Two of these compounds were found to be agonists and 1 to be antagonist. The other was a partial agonist/antagonist that displayed very weak agonist, but strong antagonist activity.

In summary, a universal, cell-based, Gα15/16-mediated calcium assay was developed and validated for the identification of compounds that modulate DOR activity. Its application may be expanded to other GPCR and even orphan receptors.

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