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András Visegrády, András Boros, Zsolt Némethy, Béla Kiss and György M. Keseru

J Biomol Screen 2007; 12; 1068
DOI: 10.1177/1087057107309035

Application of the BD ACT*One*™ Technology for the High-Throughput Screening of G_s-Coupled Receptor Antagonists

ANDRÁS VISEGRÁDY,[1] ANDRÁS BOROS,[2] ZSOLT NÉMETHY,[2] BÉLA KISS,[2] and GYÖRGY M. KESERŰ [1]

A novel technology for monitoring the changes of 3,′5′-adenosine cyclic monophosphate (cAMP) in live cells suitable for drug screening relies on the use of cyclic nucleotide-gated channels as biosensors coexpressed with the appropriate target receptor. The technique (termed BD ACT*One*™) offers measurement of cAMP-dependent calcium influx or membrane depolarization with conventional fluorescent methods both in kinetic and in endpoint modes, optimal for high-throughput and subsequent compound screening. The utility of the technique is reported here based on assay development and high-throughput screening for small-molecule antagonists of the peptide parathyroid hormone 2 receptor (PTH2R). The dual-signaling properties of the receptor were retained in the recombinant system, and the observed pharmacological profile corresponded to data from radiolabeled cAMP determination. The membrane-potential-based high-throughput assay produced reproducible actives and led to the identification of several chemical scaffolds with potential utility as PTH2R ligands. (*Journal of Biomolecular Screening* 2007:1068-1073)

Key words: cyclic nucleotide-gated channel, high-throughput screening, cAMP, G-protein-coupled receptor, peptide parathyroid hormone 2 receptor

INTRODUCTION

G-PROTEIN-COUPLED RECEPTORS (GPCRs) represent a major group of potential therapeutical targets with more than 30% of currently marketed prescription drugs targeting GPCRs.[1] Modern drug discovery programs targeting GPCRs require simple and robust in vitro methods for screening effectors of these proteins. Even though methods for receptors coupling predominantly to G-proteins containing G_q-class Gα subunits (i.e., fluorescent or luminescent determination of intracellular calcium concentration) are well established,[2] a general screening method for the large group of physiologicaly relevant receptors coupling to G_s or G_i subunit-containing G-proteins is still lacking.

G_s and G_i G-proteins regulate the production of the intracellular signaling molecule 3,′5′-adenosine cyclic monophosphate (cAMP). Current technologies for cAMP detection rely either on competitive antibody-based determination of cAMP level from cell lysates or on cAMP-driven gene transcription measurements (reviewed in Williams[3] and Warrior et al.[4]). Alternative ways to monitor receptor activation are offered by utilization of promiscuous or engineered chimeric Gα subunits, driving signaling towards calcium mobilization[5] or the generally applicable yet lower throughput and instrumentation-intensive β-arrestin-translocation-based assays.[4] Although the above methods have been widely validated and applied for screening purposes, they do not offer simple, kinetic measurements in live cells, a feature that might be beneficial in the assay development and hit validation phases of the discovery program.

BD ACT*One*™ is a high-throughput G-protein-coupled receptor screening technology that can measure the intracellular changes of the secondary messenger cyclic AMP in living cells, in real time (see Mannion et al.[6] and further description at www.atto.com/products/actone). It is performed using cell lines that exogenously express a proprietary modified cyclic nucleotide-gated (CNG) ion channel. The CNG channel is colocalized with adenylate cyclase at the plasma membrane[7] and opens when the cAMP level near the plasma membrane increases, resulting in ion flux and cell membrane depolarization. The influx of cations through the CNG channel can be quantified using fluorescent ion indicators or membrane-potential dyes.[8]

Availability of this new technology prompted us to evaluate the BD ACT*One*™ system for the screening for small-molecule antagonists of the parathyroid hormone 2 receptor (PTH2R),[9] a receptor belonging to the family of secretin-like G-protein-coupled receptors with a potential to activate a dual-messenger cascade.[10] Although the biological roles of PTH2R are not yet established,

[1]Lead Discovery Unit, [2]Pharmacological and Drug Safety Research, Gedeon Richter Plc, Budapest, Hungary.

Received Apr 23, 2007, and in revised form Aug 28, 2007. Accepted for publication Aug 30, 3007.

Journal of Biomolecular Screening 12(8); 2007
DOI: 10.1177/1087057107309035

regarding its anatomical localization, the role of neuropeptides in spinal circuits, and the results of physiological studies, it may be involved in the regulation of pain sensation.[9]

METHODS

Materials

Dulbecco's modified Eagle medium, heat-inactivated fetal bovine serum, fluo-4 acetoxymethyl ester (fluo-4 AM), and Pluronic F-127 were obtained from Invitrogen (Paisley, UK); G418 sulfate was from Merck (Darmstadt, Germany); bovine tuberoinfundibular peptide of 39 residues (TIP39) was from Bachem (Bubendorf, Switzerland); and TIP[7-39] was from Phoenix Pharmaceuticals (Burlingame, CA). The BD ACT*One*™ fluorescent membrane-potential assay kit was from BD Bioscience (Erembodegem, Belgium). [^{3}H]cAMP was obtained from Amersham Biosciences (GE Healthcare, Chalfont St. Giles, UK). Other reagents, if not stated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO).

TIP39 and TIP[7-39] were dissolved in distilled water, stored in aliquots at –80 °C, and allowed for less than 3 freeze-thaw cycles. When used, they were diluted in DMSO and finally in assay buffer.

Cell culture methods

The HEK293-CNG-PTH2R cell line produced by BD Bioscience (Erembodegem, Belgium) is based on the BD ACT*One*™ parental cell line stably expressing a mutated CNG gene in HEK293H cells. The PTH2R gene in the retroviral vector pBabe was introduced by retroviral infection from the supernatant of transfected phoenix-ampho cells followed by selection of puromycin-resistant clones of virus-infected cells by measuring membrane-potential changes triggered by receptor activation.

Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 1 µg/mL puromycin, and 250 µg/mL G418 at 37 °C, 5% CO_2. Cells were harvested by gentle trypsinization and seeded aseptically into poly-D-lysine-coated 384-well cell culture plates (BD Biocoat) at a density of 10,000 cells per well and cultured overnight at 37 °C. For direct cAMP measurements, cells were cultured in poly-D-lysine-coated 96-well plates (Corning, Corning, NY).

Fluorescent measurement of intracellular Ca^{2+} concentration

Cells were loaded with 2 µM fluo-4 AM supplemented with 0.04% Pluronic F-127 and 2 mM probenecid in medium for 60 min at 37 °C, washed twice with a HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-based assay buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 20 mM D-glucose, 10 mM HEPES, pH 7.4), followed by antagonist/vehicle addition. Plates were then transfered into a Fluoroskan Ascent (Thermo Fisher Scientific, Waltham, MA) fluorescence microplate reader, where fluorescence was read with 485-nm excitation and 538-nm emission band pass filters. First, baseline fluorescence was read for 20 s, followed by agonist addition. Fluoresence was then continuously monitored at 10-s intervals for 5 min. For measurements in the absence of extracellular calcium, $CaCl_2$ in the assay buffer was replaced with 10 µM ethylene glycol-bis(beta-aminoethyl ether)-N,N,N,′N′-tetra acetic acid (EGTA).

Fluorescent measurement of membrane potential

Fluorescent measurement of membrane potential was carried out with the BD ACT*One*™ Membrane Potential Dye Kit. Membrane Potential Dye was diluted 8-fold with Dye Dilution buffer and was added to cells without medium removal. Cells were then incubated at 37 °C for 75 min. Plates were subsequently treated with antagonists/test compounds and incubated for a further 10 min prior to determination of baseline fluorescence with a POLARstar Optima multimode microplate reader (BMG Labtech, Offenburg, Germany) with 544-nm excitation and 590-nm emission filters. Following baseline measurement, cells were treated with agonist solution and incubated for an additional 20 min, followed by a second fluorescence read.

Direct measurement of intracellular cAMP level

On the day of the experiment, the cell culture medium was removed, and cells were preincubated in 60 µL Hank's Balanced Salt Solution (HBSS) for 20 min at 37 °C. Cells were subjected to test compounds in 60 µL HBSS complemented with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 100 µM) for 20 min, at 37 °C. After the addition of 20 µL 1 M perchloric acid to terminate the reaction, plates were frozen (for overnight, at –20 °C) and thawed, and 50 µL ice-cold KOH (0.5 M) was added to neutralize the samples for 30 min at 4 °C. Plates were then centrifuged with 700 *g* at 4 °C for 10 min; 50 µL of the supernatant was incubated together with 0.15 pmol/well [^{3}H]cAMP in 50 µL distilled water and 25 µg cAMP-binding protein (in 200 µL 50 mM Tris, pH 7.4) at 4 °C for 130 min, then filtrated onto GF/B filters. Radioactivity of the samples was determined by a TopCount NXT (PerkinElmer, Waltham, MA). cAMP content was expressed in pmol/well.

Data handling

Data both from intracellular calcium and membrane-potential measurements were expressed as change in fluorescence normalized to baseline fluorescence ($\Delta F/F_0$). Data were analyzed in Excel (Microsoft Corp., Redmond, WA) and fitted in Origin 7.5 (OriginLab Corp., Northampton, MA). Data demonstrated are representatives of 2 to 6 independent measurements and, where appropriate, are expressed as mean ± standard deviation from triplicates.

Z′ parameter, a measure of assay quality in high-throughput screening (HTS) applications, was calculated according to Zhang et al.[11]

Realization of the HTS

The HTS campaign was performed on a fully automated robotic assay station built around a CRS Catalyst 5 robotic arm on linear track (Thermo Fisher Scientific) and a Twister II cylindric robotic arm (Caliper Life Sciences, Hopkington, MA) as reported elsewhere.[12]

Cell plates initially loaded into an automated incubator (Cytomat 2C, Thermo Fisher Scientific) were delidded, loaded with membrane-potential dye, and returned for a 75-min incubation. Meanwhile, the next compound plate stored in a plate carousel was picked and had its barcode read, and it was placed into a Multimek 96 automated pipettor (Beckman Coulter, Fullerton, CA). Following dye incubation, the cell plate also was transferred into Multimek, and the compound transfer took place. The cell plate was then incubated for 10 min at 37 °C and was subsequently placed into a POLARstar Optima plate reader for baseline measurement. It was transferred to a Multidrop Micro dispenser for agonist addition, followed by a second fluorescence read 15 min after stimulation.

Data were stored in a dedicated Oracle database and were further evaluated by an in-house Web interface designed for HTS data sets. To insert the raw and calculated data into the Oracle database, we have used an in-house developed Tcl/Oratcl program. To assess and eliminate the effect of patterning within plates, both normalization with vehicle-treated plates and the median-polishing algorithm[13] were implemented into data analysis.

RESULTS AND DISCUSSION

Characterization of PTH2R signaling in the CNG cell line

The HEK293-CNG-PTH2R cell line was first characterized by means of intracellular Ca^{2+} measurements for functionality of the ACT*One*™ system. Application of the PTH2R receptor agonist TIP39 as well as the adenylyl cyclase activator forskolin resulted in a robust elevation of intracellular calcium (**Fig. 1A**), indicating an intact signaling complex. Responses to forskolin and low TIP39 levels were monophasic, with a steadily elevating calcium concentration leveling off after 4 to 5 min, whereas calcium responses at high TIP39 concentrations were biphasic, with a rapid increase in intracellular calcium within 60 s of agonist application superimposed onto the slow elevation (**Fig. 1A**).

To identify the origin of the receptor-specific calcium responses, measurements were performed both in the presence of the phospholipase C inhibitor U73122 and in the absence of extracellular calcium. As demonstrated in **Figure 1B**, U73122

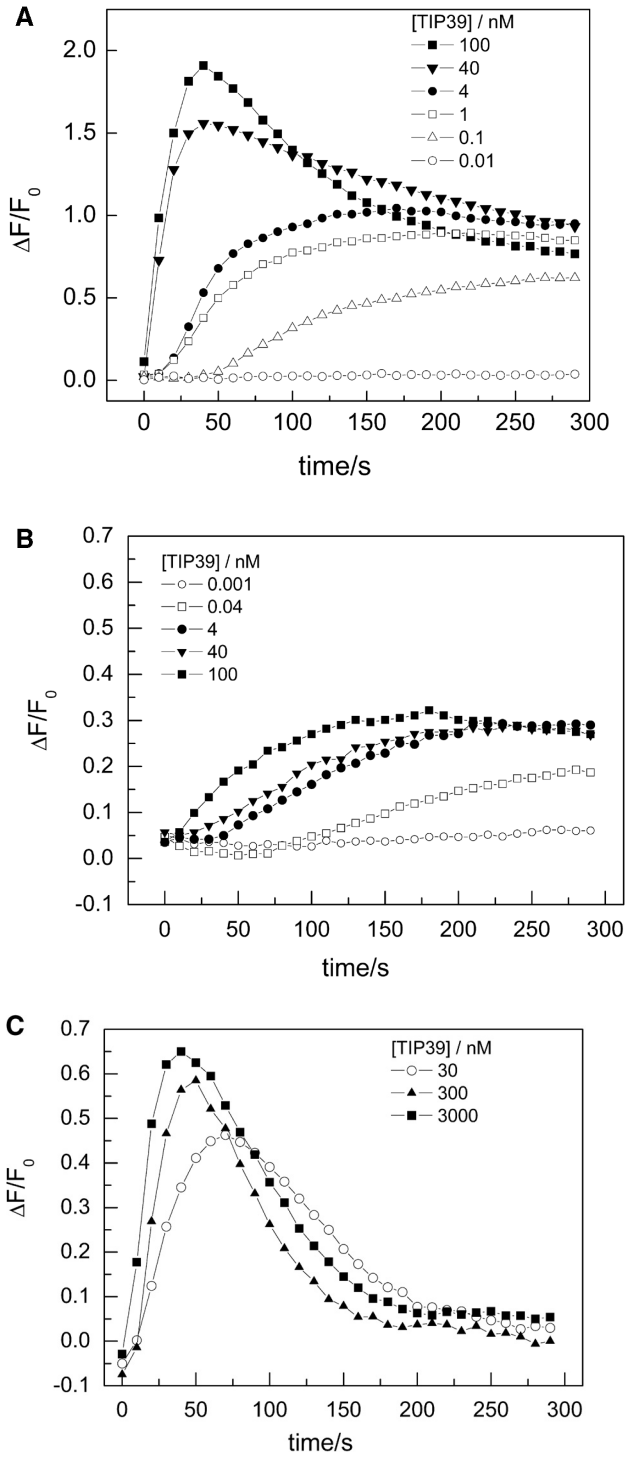


FIG. 1. (**A**) Calcium-dependent fluorescent responses to TIP39 in HEK-CNG-PTH2R cells and (**B**) the effect of 5 μM U73122 or (**C**) the absence of extracellular calcium and the presence 10 μM EGTA upon calcium responses, respectively. TIP39 concentrations are indicated in the legends.

abolished the rapid calcium transients with a slight inhibitory effect on slow calcium signals, whereas removal of extracellular calcium led to complete elimination of the late phase of the calcium response (**Fig. 1C**). As PTH2R belongs to the family of class B GPCRs, the biphasic receptor-mediated calcium response probably reflects the dual signaling of the receptor, with the rapid phase originating from inositol 1,4,5-trisphosphate-dependent internal calcium release and the slower component arising through cAMP-mediated and CNG channel-mediated calcium influx from the extracellular side. As our studies focused onto cAMP signaling, in further experiments, the cAMP-dependent late phase of the calcium response was evaluated, and response amplitudes determined from the fluorescence levels reached 260 to 300 s after stimulation.

Pharmacological characterization of the PTH2 receptor included the determination of agonist potency of TIP39 as well as demonstration of the antagonism of the most potent, though nonselective, PTH2R antagonist: The truncated peptide TIP [7-39].[14] EC_{50} values determined for TIP39 ranged from 12 to 112 pM, with a mean ± SD of 59 ± 39 pM ($n = 7$), whereas inhibition by TIP[7-39] in the low micromolar concentration range was also confirmed. Also reinforcing that calcium responses monitored changes in cAMP level, the phosphodiesterase inhibitor IBMX, a compound that should augment the accumulation of intracellular cAMP, shifted EC_{50} to even lower values (data not shown).

To confirm that CNG-mediated calcium influx corresponds to changes in the rate of cAMP production, a CNG channel-independent assay technology for the determination of adenylyl cylase (AC) activity was used. The AC activity (cAMP formation) was assayed by a protein-binding method.[15] In our experiments, basal cAMP production of HEK-PTH2R cells was 0.6 ± 0.1 pmol/well. cAMP production was stimulated by TIP39 in a concentration-dependent manner with an EC_{50} value of 35 pM, and TIP[7-39] concentration dependently inhibited the TIP39-induced cAMP production with an IC_{50} value of 200 nM (**Fig. 2**), indicating similar agonist and somewhat higher antagonist potency compared to the CNG channel-dependent method. Forskolin (10 μM) also resulted in a robust cAMP accumulation (37.1 ± 1.6 pmol/well).

After pharmacological confirmation of the physiological functioning of the receptor, a decision about readout had to be made for the high-throughput screen. As calcium measurements required washing of the cells and no response was obtained with the single no-wash calcium kit commercially available at the time, measurements were undertaken with a no-wash membrane potential assay kit. This assay produced more robust signals as opposed to calcium measurements without the need for media removal (**Fig. 3A**). Although concentration-response curves obtained with the membrane-potential assay were generally steeper than with calcium measurement (mean power of logistic sigmoidal fit was 3.4 for membrane-potential assays vs. 1.1 of calcium measurements), EC_{50} values were in the similar range (42-141 pM, mean ± SD of

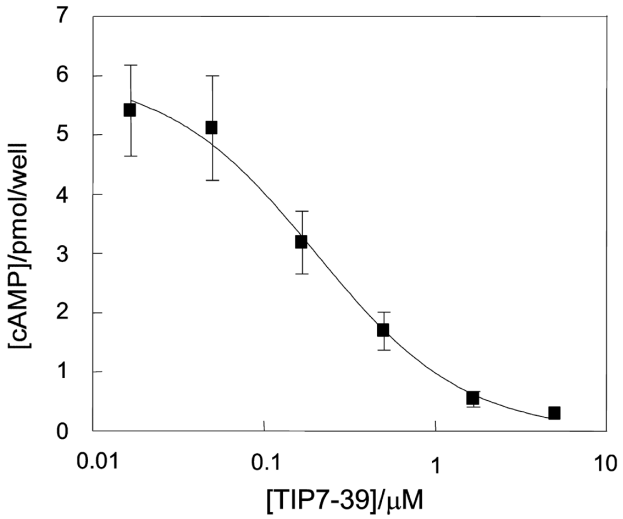


FIG. 2. Concentration-dependent inhibition of TIP39-induced 3,′5′-adenosine cyclic monophosphate (cAMP) accumulation by TIP[7-39] as determined by radiolabeled cAMP-displacement.

82 ± 31 pM, $n = 21$). The antagonist TIP[7-39] also inhibited membrane-potential changes in a dose-dependent manner with apparent potency dependent on agonist stimulus strength (**Fig. 3B**) and K_i values ranging from 1.0 to 1.8 μM. Based on a similar pharmacological profile as the calcium response and robust data with a simple assay protocol, the membrane-potential assay was chosen for the realization of the HTS.

Assay development

The performance of the functional assay was optimized for various assay parameters, including cell density, plate type, incubation times, solvent tolerance, solution stability, and automated liquid-handling settings. At an overnight culture, data quality was optimal at 10,000 cells seeded per well, and the assay tolerated incubation times between 60 and 120 min at 37 °C for loading. Confirming vendor information, we found the membrane-potential signal to be stable as early as 15 min after stimulation and, as opposed to calcium responses, to remain steady for minutes, facilitating robotic adaptation of the assay.

As for DMSO tolerance, a gradual decrease both in signal window and in agonist potency was observed with increasing DMSO concentration in the reaction mixture (data not shown). As both preincubation (i.e., test compound-related) DMSO load and DMSO content at agonist preparation (i.e., sufficient DMSO for proper dissolution of TIP39) affected assay performance, a balance for assay quality had to be achieved. Based on the obligate DMSO content originating from the chemical stocks of our chemical collection, experiments were performed at 1% total DMSO, where the assay window was still acceptable ($\Delta F/F_0$ values

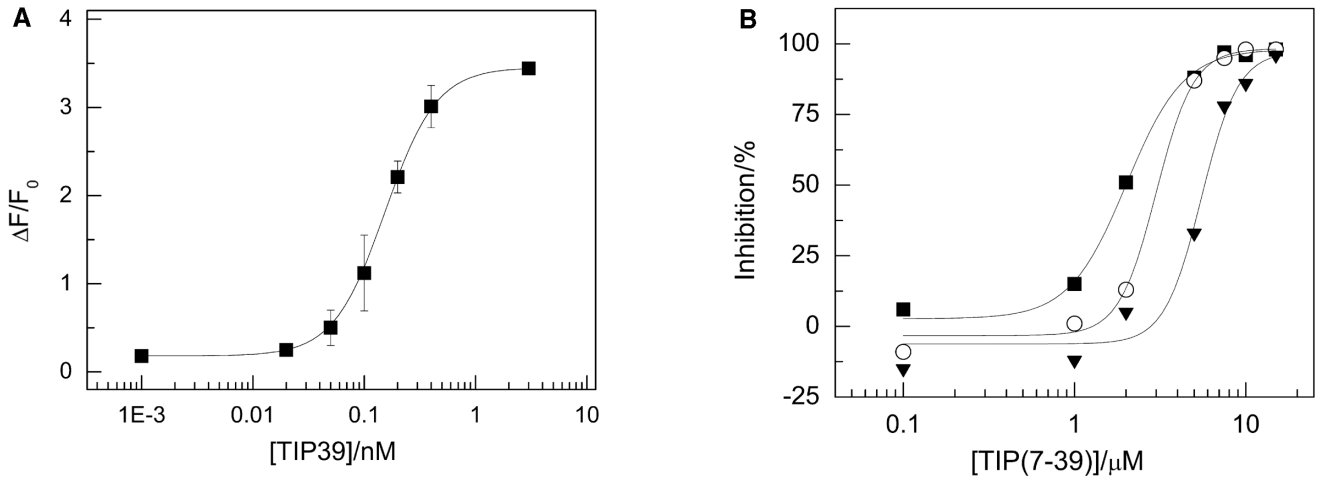


FIG. 3. (**A**) Concentration-response relationship of membrane-potential signals for TIP39 in and (**B**) concentration-dependent inhibition by TIP[7-39] of membrane-potential responses evoked by 2-fold (*filled triangles*) and 1.5-fold (*open circles*) concentration of EC_{50}, as well as EC_{50} concentration (*filled squares*) of TIP39 in HEK-CNG-PTH2R cells.

ranging between 3 and 5)—although significantly less than achieved with 0% to 0.25% DMSO (data not shown; see Tang et al.[8])—but also enabled a satisfactory compound concentration. Another difficulty was encountered at the automated dispensing of the 39-membered peptide ligand agonist. Despite extensive priming of the Multidrop Micro dispenser, a significant amount of TIP39 was found to adhere to the instrument tubing that could not be prevented with bovine serum albumin addition to solutions. Following assessment of this loss and considering the steep concentration-response curves obtained with membrane-potential readout, the 5-fold concentration of the daily determined EC_{50} value was chosen as a nominal agonist concentration that resulted in an effective concentration of approximately 2- to 2.5-fold EC_{50}.

High-throughput screening

HTS was run on a preselected library of approximately 80,000 compounds from our corporate compound collection on 384-well plates. All compounds were evaluated on plates with Z′ above 0.4. Average Z′ was 0.60 ± 0.10, and standard deviation of raw inhibition for all compounds was 13.4%, acceptable for a cell-based HTS assay.[13] To minimize position-related biasing of data, selection criteria of mean + 3× standard deviation were set for raw as well as median-polished and normalized data sets, respectively (inhibition of 41%-46%). Compounds exceeding any of these criteria, 949 in total (hit rate ca. 1.20%), were selected for confirmation by remeasuring their activity in triplicates.

Interestingly, the confirmation rate was found to depend on "selection strength." Of the 566 compounds identified as hits in all 3 data sets, 184 (32.5%) were successfully confirmed, whereas only 28 (10.4%) compounds were found active upon retesting the 267 compounds identified solely upon a single inhibition value. Multiple selection criteria might therefore increase selection prediction value and might thus lead to the enrichment of actives in low-threshold hit lists.

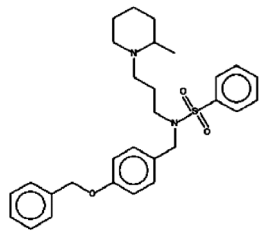
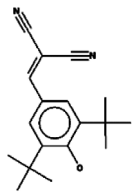
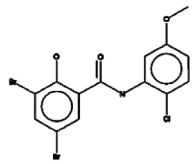
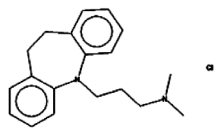
Table 1 shows representatives of confirmed hit series identified in the PTH2R HTS. Specificity determination and further characterization of hits are in progress.

CONCLUSION

Simple, reliable, and physiologically relevant probes of GPCR function are lacking for G_s and G_i protein-coupled receptors. The BD ACT*One*™ technology offers a method to investigate changes in cAMP levels in living cells with minimal intervention into signaling pathways by the use of conventional fluorescent Ca^{2+} or membrane-potential dyes. The utility of this screening technique was assessed in a cell line expressing PTH2R. Measurement of intracellular calcium indicated that the recombinant cell line retained the dual-signaling property of the class B GPCR PTH2R and theoretically even enabled the simultaneous monitoring of 2 signal transduction pathways.

The sensitivity of the assay was similar to direct cAMP measurements, and the cell line was stable in the development and screening period. The technique proved to be easy to implement for automated screening in a homogeneous format and for standard instrumentation. Based on the results of an HTS based on the ACT*One*™ system, data quality, hit rate, and hit confirmation rate (reproducibility) were acceptable by HTS standards, and several chemical series were identified as hits. Although a tendency of interference at the level CNG channel was observed in several hit compounds, these hit series can be easily eliminated with a direct cAMP counterscreen.

Table 1. Representatives of Several Different Chemical Structures Identified in the Parathyroid Hormone 2 Receptor (PPH2R) High-Throughput Screening Together with Confirmed Inhibition at 5 μM

Structure	*Activity ± SD (Inhibition %)*
	100.1 ± 18.0
	70.4 ± 9.8
	70.1 ± 5.7
	65.3 ± 7.3

ACKNOWLEDGMENTS

We acknowledge Dr. László Molnár, Attila Bielik, and Krisztina Recska for their valuable help in data handling and assay realization; Dr. Andrea Baki for providing additional comparative data; and Dr. Xiao Li (BD Bioscience) for providing details about assay technology.

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Address correspondence to:
András Visegrády, PhD
Lead Discovery Unit
Gedeon Richter Plc.
H-1103 Budapest
Gyömrői Str. 19-21, Hungary

E-mail: visegrady@richter.hu