Monitoring Activation at G-Protein Coupled Receptors with Functional Screening Assay Systems

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Introduction

G-protein coupled receptors (GPCRs) possibly represent the most important class of proteins for drug discovery. Up to 40% of clinically marketed drugs are active at this receptor family. However, these drugs exhibit their activity at less than 10% of known GPCRs. Thus, a major challenge for the drug development industry is to associate the many orphan GPCRs with disease to potentially identify novel pharmaceutical agents of the future. Receptors in this group act by facilitating the binding of guanosine triphosphate (GTP) to specific G-proteins. The binding of GTP to the α subunit can be used to develop a functional assay for G-protein activation (GTPγS, see below) [(1)]. GTP binding activates the G-protein, such that it, in turn, can regulate the activity of specific effectors (Table 1).

G- Protein Subunit Regulation	Effectors / Signalling Pathways
αs	Adenylate cyclase (cAMP) ↑
αi	Adenylate cyclase (cAMP) ↓
αo	Ca^{2+} ↓
αq	Phospholipase C (IP_3) ↑
α13	Na^+/H^+ exchange ↑
αt	cGMP-phosphodiesterase (vision) ↑
αolf	Adenylate cyclase (cAMP) ↑
$\beta\gamma$	K^+ channels
$\beta\gamma$	Adenylate cyclase (cAMP) ↑or ↓
$\beta\gamma$	Phospholipase C (IP_3)

Table 1. The relationship of G-Protein Coupled Receptors and Signalling Pathways

Here, we describe a range of functional screening assay systems to assist with the identification of new lead compounds and orphan GPCRs. Agonist stimulation initiates a cascade of signals that involve the activation of the heterotrimeric G-proteins, consisting of α, β, and γ subunits. In order to study GPCR activation, a variety of functional biochemical and cellular assay methodologies are typically used. Examples of functional assay systems for monitoring GPCR activation include the intracellular measurement of the GPCR effector targets, cAMP, cGMP and D-*myo*-inositol 1,4,5-trisphosphate (IP_3). A number of homogeneous assay methodologies such as Scintillation Proximity Assay (SPA), Fluorescence Polarization (FP) and Enzyme Fragment Complementation (EFC) have been successfully used for the measurement of these agents. Furthermore, ligand–induced stimulation of GPCRs results in the exchange of GDP for GTP, and this event can be monitored by the binding of [^{35}S]GTPγS. Representative data is presented here illustrative of the use of the above techniques in cellular systems.

Methods

Several, rapid, selective and highly sensitive methods for measuring activation at GPCRs have been described.

Technologies include:

1. Radioimmunoassay (RIA) for cAMP and cGMP using a high specific activity adenosine or guanosine 3', 5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I] iodotyrosine methyl ester together with a second antibody bound to magnetic polymer particles.
2. Enzyme immunoassay (EIA) for cAMP and cGMP, involving the linking of succinyl cAMP or cGMP to horseradish peroxidase (HRP), and combining this with stable second antibody-coated microtitre plates [(2)].
3. Homogeneous (non-separation) RIA for cAMP and cGMP using scintillation proximity assay (SPA) technology [(3)]. A recent development has involved the use of SPA imaging beads specifically designed for ultra high-throughput drug screening on the LEADseeker™ MultiModality Imaging System. (See Figures 2, 3a & 4).
4. Homogeneous fluorescence polarization assays (FP) for cAMP and IP_3 [(4)]. (See Figures 3b & 6).
5. Homogeneous enzyme fragment complementation (EFC) immunoassay (HitHunter™) technology for cAMP [(5)]. (See Figures 1 & 5).

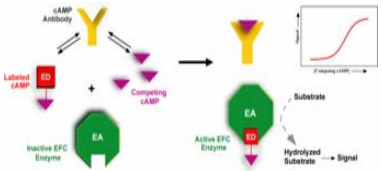

Figure 1. EFC-based HitHunter cAMP Technology.

6. A SPA system, replacing the GTP with the non-hydrolyzable analogue, guanosine 5'-[γ-^{35}S]-thiotriphosphate ([^{35}S] GTPγS), allowing the measurement of the exchange process in the presence of added GDP. With this approach, the SPA bead is composed of a matrix whose outer surface has been modified by a coating of wheat-germ agglutinin (WGA) to enable receptors to bind. In this case, there is no need in this SPA format, unlike that in conventional filter based systems, to separate the receptor-G-protein-[^{35}S]GTPγS complex from the free [^{35}S]GTPγS. The SPA system will detect only radioactivity that is associated with the bead. (See Figure 7).

Because of the few steps involved, homogeneous assay technologies are best-suited for high-throughput drug screening. Assay performance in drug-screening assays is monitored by the use of Z′, as described by Zhang *et al*. [(6)]. Z' values exceeding 0.5 are acceptable for a screen, those values approaching 1.0 are ideal for drug-screening applications.

Results

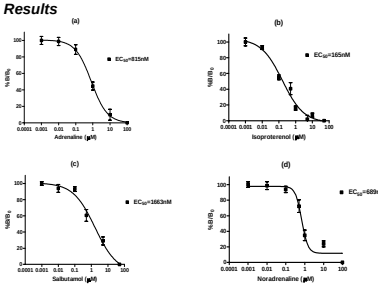

Figure 2. Agonist-induced cAMP generation from A431 cells (a) adrenaline, (b) isoproterenol, (c) salbutamol, (d) noradrenaline. Values are means ± 1 SEM. cAMP levels were measured using SPA imaging beads on LEADseeker.

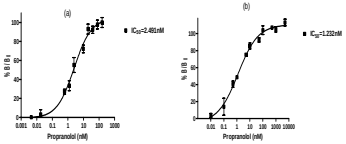

Figure 3. Effect of propranolol on isoproterenol-induced cAMP generation. Values are means ± 1 SEM. cAMP levels were measured with (a) SPA imaging beads on LEADseeker, and, (b) cAMP FP on a modified Tecan Ultra™.

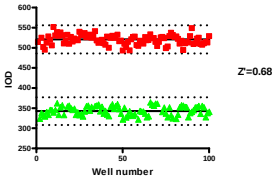

Figure 4. Z' values calculated from forskolin-stimulated CHO cells. cAMP levels were measured with SPA imaging beads on LEADseeker.

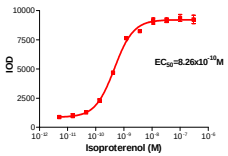

Figure 5. Agonist-stimulation of CHO-K1 cells expressing β2-adrenergic receptor using cAMP XS (HitHunter) EFC assay on LEADseeker. Results are means ± 1 SEM (n=3).

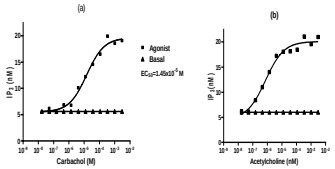

Figure 6. Agonist stimulation of CHO-M1 cells expressing muscarinic receptors. IP_3 was measured using FP HitHunter assay. (a) Carbachol and (b) acetylcholine. Graphs show results from single experiments.

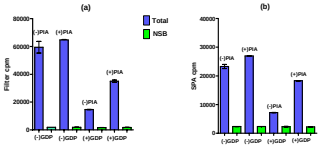

Figure 7. Binding of [^{35}S]GTPγS (377pM) to cloned rat adenosine A1 receptor membranes. (a) Receptor (19.25μg) was pre-incubated with adenosine deaminase for 0.5h at 25°C, and used directly in the assay. Assays used 5μM GDP and 10μM(-)-N^6-(2-phenylisoproyl-adenosine) (PIA) at 25°C for 1.5h, followed by harvesting on to GF filter mats. Counting, after drying was in a BetaPlate™ counter using Meltilex™ solid scintillant. Non-specific binding (NSB) was determined in the presence of 10mM GTPγS. (b) SPA was performed essentially as before, except that 1.5mg WGA-coated SPA beads was added to the assay mix, followed by counting in a MicroBeta™ scintillation counter after centrifugation.

Discussion

GPCRs are one of the largest families in the human genome. This group of receptors is likely to remain the most tractable and effective set of targets for therapeutic drug design. Thus, screens for ligands that interact with GPCRs are integral for basic research and the drug-discovery process. Although there is an ongoing debate about the relative benefits of ligand-binding screens versus functional assays, downstream monitoring does appear to offer real advantages over simple ligand-binding screens. GE Healthcare Bio-Sciences have introduced a number of functional assay systems for compound screening at GPCRs, including assay approaches for cAMP, cGMP, IP_3 and GTP binding. These methods are all well-suited for screening at GPCRs, often enabling the direct measurement of the drug target in homogeneous, radioactive, or non-radioactive miniaturized formats, frequently without inconvenient, time-consuming extraction.

References

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