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Current Perspective

History of the G Protein–Coupled Receptor (GPCR) Assays From Traditional to a State-of-the-Art Biosensor Assay

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Abstract. The G protein–coupled receptors (GPCRs) form the largest and the most versatile superfamily that share a seven-transmembrane-spanning architecture. GPCR-signaling is involved in vision, taste, olfaction, sympathetic/parasympathetic nervous functions, metabolism, and immune regulation, indicating that GPCRs are extremely important therapeutic targets for various diseases. Cellular dielectric spectroscopy (CDS) is a novel technology that employs a label-free, real-time and cell-based assay approach for the comprehensive pharmacological evaluation of cells that exogenously or endogenously express GPCRs. Among the biosensors that use CDS technology, the CellKey™ system not only detects the activation of GPCRs but also distinguishes between signals through different subtypes of the Gα protein (Gs, Gi/o, and Gq). In this review, we discuss the traditional assays and then introduce the principles by which the CellKey™ system evaluates GPCR activation, followed by a perspective on the advantages and future prospects of this system.

Keywords: G protein–coupled receptor, cellular dielectric spectroscopy, electrical impedance–based biosensor, CellKey™ system, GPCR assay method

1. Introduction

The G protein–coupled receptors (GPCRs) are the largest and the most versatile superfamily among cell surface receptors (1). The human genome encodes more than 800 GPCRs, which account for approximately 2% of the human genome (2, 3). Members of the GPCR family share structural similarities including a seven-transmembrane-spanning domain linked by an N-terminal extracellular domain, C-terminal intracellular domain, three extracellular loops, and three intracellular loops (4). Even so, different GPCRs interact with different types of ligands, including photons, ions, biogenic amines, peptide and non-peptide neurotransmitters, hormones, growth factors, and lipids (3). Consequently, GPCR signaling is involved in vision, taste, olfaction, sympathetic and parasympathetic nervous functions, metabolism, and immune regulation, indicating that GPCRs are very important therapeutic targets for various diseases (4). Indeed, several researchers over the world have received the Nobel Prize for their research in the field of GPCRs (5) (Table 1). It is evident that such significant and novel discoveries of GPCRs will potentially facilitate the development of novel therapeutic drugs. In fact, several pharmaceutical companies have developed medicines that can modulate GPCR signaling pathways, which are estimated to be 30% in the market (1, 3).

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Invited article

Table 1. History of Nobel Prize winners in the field of GPCR research

Year	Recipients	Research
1967	Ragnar Arthur Granit George Wald Haldan Keffer Hartline	Studies concerning the physiological and chemical visual processes in the eye
1970	Bernard Katz Ulf Svante von Euler Julius Axelrod	Discoveries of the neurotransmitters which activate GPCRs, and the storage, release, and reuptake of neurotransmitters at the nerve terminals
1971	Earl Wilbur Sutherland Jr.	Discovery of cyclic adenosine monophosphate (cAMP) as the second messenger for GPCRs
1988	James Whyte Black Gertrude Belle Elion George Herbert Hitchings	Discovery of two clinical GPCR blockers (propranolol and cimetidine), which inhibit the action of β-adrenergic receptor and the H_2 histamine receptor, respectively
1994	Alfred Goodman Gilman Martin Rodbell	Studies of G protein–dependent signaling pathways
2000	Arvid Carlsson Paul Greengard Eric Richard Kandel	Discovery of the endogenous GPCR agonist dopamine
2004	Richard Axel Linda B. Buck	Discovery and functional analysis of G protein–coupled odorant receptors
2012	Robert Joseph Lefkowitz Brian Kent Kobilka	Structural studies of GPCRs to reveal the GPCR signal transduction from extracellular information into intracellular chemical messages

Multiple lines of evidence demonstrate that the agonist-bound GPCRs induce a conformational rearrangement of these receptors and subsequently activate the heterotrimeric G proteins, composed of α-, β-, and γ-subunits (3, 4). The conformational change of the ligand-bound GPCRs catalyzes the exchange of guanidine diphosphate (GDP) for guanidine triphosphate (GTP) on the α-subunit (Gα) of the G proteins, thereby dissociating Gα from the dimeric β- and γ-subunits (G$\beta\gamma$) (4). Based on the amino acid sequence homologies of their Gα subunit, heterotrimeric G proteins are classified into four families: $G\alpha_s$ (Gs), $G\alpha_i$ (Gi), $G\alpha_q$ (Gq), and $G\alpha_{12}$ (G12) (6). In addition, the functions of $G\alpha_o$ (Go) proteins are similar to those of Gi, so that both the Gi and Go proteins belong to the Gi/o category of the G-protein families (4). Upon activation of GPCRs, each Gα subunit stimulates and integrates a distinct signaling pathway. Despite extensive studies, a comprehensive characterization of the G protein–coupling profile of GPCRs remains incomplete: for example, some GPCRs can couple only to a single type of G protein (e.g., Gs or Gq), whereas many GPCRs couple to a broader range of G protein families such as Gs/Gi, Gi/Gq, or Gq/G12 (6).

In addition to the classical roles of GPCRs, the concept of homodimeric or heterodimeric GPCRs has gained acceptance in the last few decades (3, 7). GPCRs exist in a dynamic equilibrium between a monomeric and dimeric state, and their heterodimerization affects pharmacological and cellular signaling responses differently compared to when only the receptor subtypes are individually expressed (7). These novel findings were impossible to discover without the development of sophisticated assay for GPCRs (3). In this review, we provide a historical account of the representative GPCRs assay and then introduce a novel, recently developed "biosensor-based cellular assay".

2. Traditional assays for the GPCRs

The classical GPCR assays are classified into two categories, ligand-binding assay and functional assay. In the late 1960s and early 1970s, GPCR research began with the development of assays that used radioisotopes (1). Subsequently, several assays were developed and improved with time (3). This review discusses some of these frequently used assays.

2.1. Ligand-binding assay

GPCRs are activated when the agonist binds to its receptor. The ligand-binding assay is used to assess whether a target compound binds to a cell surface receptor. In the classical method, the binding capacity of the target compound that competes with the radiolabeled ligand for a common receptor is measured (1, 3). Recently, radiolabeled ligands have been replaced with fluorescent ligands in this competition binding assay (3).

2.2. Functional assay

The functional assays for GPCRs are based on the detection of receptor activation and signaling. Among the Gα protein subtypes, several assays for Gs-, Gq-, and Gi/o-protein–coupled receptors are well known.

2.2.1. Assay for the activation of Gs protein–coupled receptors

Gs protein activates adenylyl cyclase (AC) that causes an increase in the intracellular concentration of cyclic adenosine 3′,5′-monophosphate (cAMP) produced from adenosine 5′-triphosphate (ATP), thereby activating protein kinase A (PKA) (6) (Fig. 1). Thus, the levels of cytosolic cAMP is routinely determined as a measure of the activation of the Gs protein–coupled receptors (1, 3). This assay was developed in the 1970s and mostly utilizes the enzyme immunoassay (EIA) using whole cell or tissue lysates (1, 3). Recent technological advances have facilitated the real-time detection of changes in the intracellular cAMP level without the cell lysis step (8, 9).

2.2.2. Assay for the activation of Gq protein–coupled receptors

Gq proteins activate phospholipase C (PLC), which catalyzes the production of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2) (3, 6) (Fig. 1). DAG activates protein kinase C (PKC), whereas IP_3-bound IP_3 receptors in the endoplasmic reticulum (ER) increase the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) by releasing Ca^{2+} from the ER into the cytoplasm (6). In Gq protein–coupled receptors, measurement of the increase in $[Ca^{2+}]_i$ is usually performed. Since the 1980s, changes in $[Ca^{2+}]_i$ in living cells can be detected using fluorescent calcium indicator dyes such as Fura-2 acetoxymethyl ester (AM), Fluo-3 AM, and Fluo-4 AM (their binding to intracellular Ca^{2+} increases the intensity of fluorescence) (1, 10).

Besides the fluorescent probes, genetically encoded Ca^{2+} sensors (GECSs) are also used for measuring $[Ca^{2+}]_i$ (11, 12). GECSs are divided into two classes: luminescent and fluorescent sensors. These are transfected into target cells and tissues and are suitable for the measurement of $[Ca^{2+}]_i$ for a relatively longer duration (11, 13). In our laboratory, G-CaMP, a fluorescent sensor that is a protein complex composed of mutated green-fluorescent protein (GFP), calmodulin (CaM), and Ca^{2+}/CaM-binding peptide, was used for the visualization of changes in $[Ca^{2+}]_i$ (14). G-CaMP (G-CaMP1) was originally established by J. Nakai et al. in 2001 (15). A series of G-CaMPs were developed to improve Ca^{2+}-binding affinity, fluorescence sensitivity, and to express some particular regions such as just under the plasma members (11 – 13, 15, 16). Different types of G-CaMPs (G-CaMP1-8) have been developed and are used in different experimental conditions depending on the kinds of cells/tissues or fluorescence sensitivity for detecting $[Ca^{2+}]_i$ levels in the cells/tissues (11 – 13, 15, 16).

2.2.3. Assay for the activation of Gi/o protein–coupled receptors

Gi/o proteins inhibit the activity of AC and decrease the intracellular cAMP levels (6) (Fig. 1). The activity of Gi/o protein–coupled receptor can be evaluated by the inhibition of cAMP production, which was previously activated by an AC activator or Gs protein-activating receptors (17). In this method, the activity of Gi/o protein–coupled receptor cannot be measured directly. To circumvent this problem, the GTPγS binding assay is commonly used for detecting the activity of Gi/o protein–coupled receptor (18). As shown in Fig. 1, the activation of Gα proteins including Gi/o protein is induced by the exchange of GDP for GTP, indicating that the binding of GTP to Gα protein is a key step in the activation of GPCRs (18). The GTPγS binding assay was developed in the 1980s (19) and has been widely used in the drug screening process (3). This assay requires preparation of the plasma membrane fraction from GPCR-expressing cells or tissues as well as labeled GTP analogues such as GTPγS ([^{35}S]-GTPγS or Eu-GTPγS) that cannot be readily hydrolyzed (17, 18).

3. A novel assay for GPCRs using cellular dielectric spectroscopy

Cellular dielectric spectroscopy (CDS) is a novel technology that utilizes a label-free, real-time and cell-based assay approach and enables comprehensive pharmacological evaluation of cells that exogenously or endogenously express receptors (20, 21). This technology is universal and allows the measurement of multiple types of receptors, including GPCRs, tyrosine kinase receptors, and nuclear receptors on the same platform without any modification of the cells (20, 21). CDS is classified into two types, an optical-based biosensor and an electrical impedance–based biosensor (3). The CellKey^{TM} system (MDS Sciex, Ontario, Canada), one of the electrical impedance–based biosensors, is specifically tailored to GPCR detection and produces response profiles that are consistent across a wide range of assay conditions and can distinguish signals between Gs, Gi/o, and Gq (20).

3.1. Principles of the CellKey^{TM} system

The CellKey^{TM} assay detects the changes in impedance (ΔZ) of a cell layer, defined by the ratio of the voltage

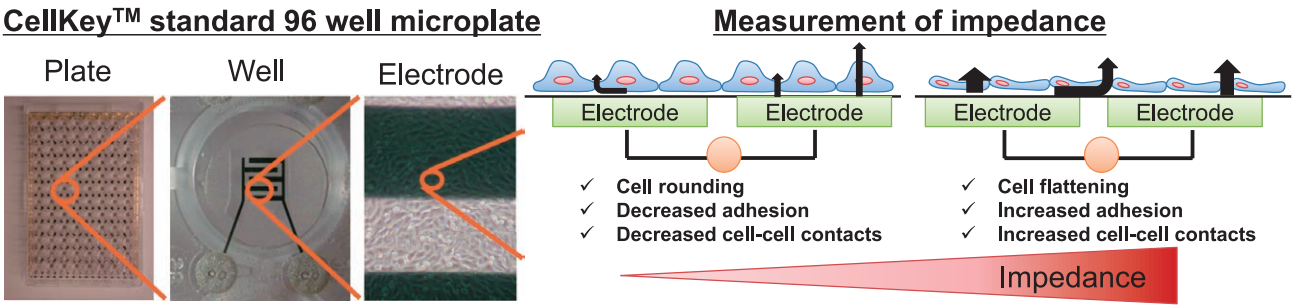

Fig. 1. Photographs of the standard CellKey™ 96-well microplate containing electrodes at the bottom of the wells and a schematic drawing of the CellKey™ system.

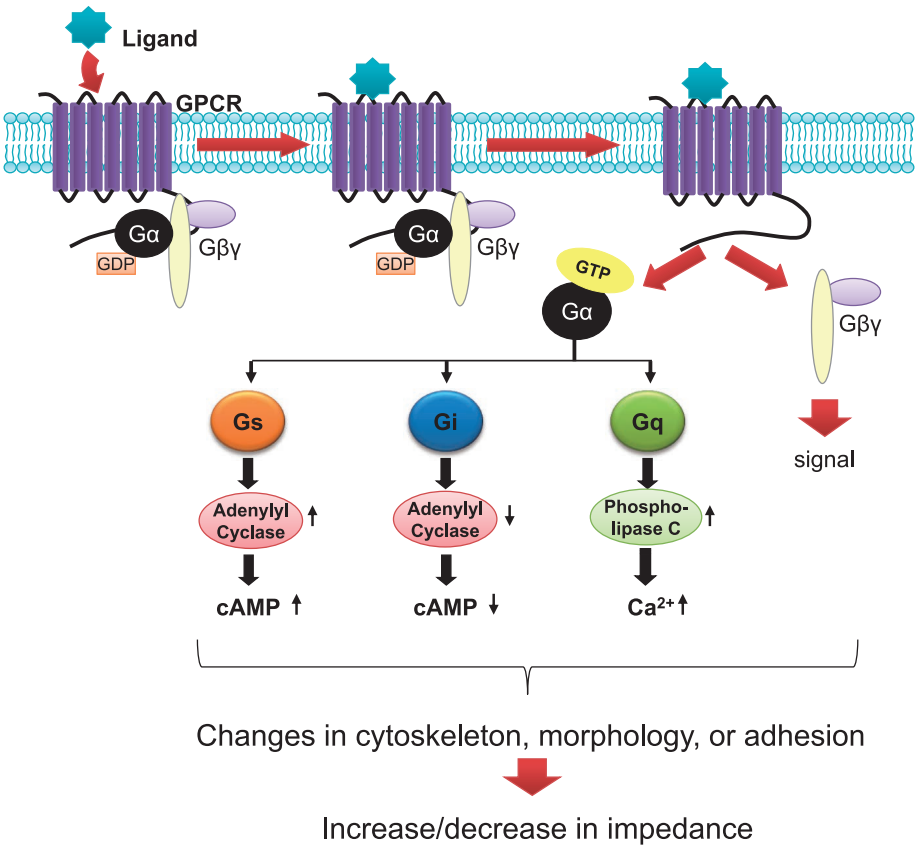

Fig. 2. Schematic representation of cell signaling induced by the activation of GPCR and the resulting changes in impedance.

(applied by the CellKey™ instrument) to the current (measured by the CellKey™ instrument), as described by Ohm's law (Z = V/I). Cells are seeded onto a standard CellKey™ 96-well microplate that contains electrodes at the bottom of the wells (Fig. 2). Small voltages are applied to these electrodes every 2 or 10 s and the impedance changes according to the changes in cell adherence, cell shape and volume, cell-to-cell interaction, and other cellular alterations (Figs. 1 and 2). Many reports indicated that cell signaling induced by GPCR activation can induce changes in morphology, and there are well-established links between Gs, Gi, and Gq signaling pathways (21 – 24). The activation of GPCR, therefore, changes the impedance, which can be interpreted as the differences of GPCR activation.

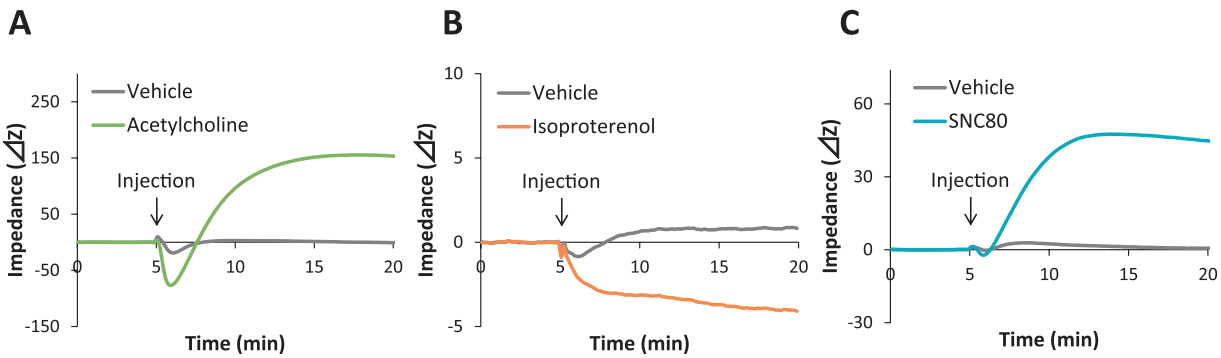

Fig. 3. Changes of impedance induced by GPCR activation. A) Effect of 10 μM acetylcholine on the changes in impedance (ΔZ) in human embryonic kidney 293 (HEK293) cells. B) Effect of 100 nM isoproterenol on ΔZ in rat C6 glioma cells. C) Effect of 10 nM SNC80 on ΔZ in δ-opioid receptor (DOR)-expressing HEK293 cells. The cells were plated onto a standard CellKey™ 96-well microplate and incubated in culture medium at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 for 24 h. After washing with CellKey™ buffer composed of Hanks' balanced salt solution (1.3 mM $CaCl_2 \cdot 2H_2O$, 0.81 mM $MgSO_4$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 136.9 mM NaCl, 0.34 mM Na_2HPO_4, and 5.6 mM D-glucose) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.1% bovine serum albumin (BSA), the cells were treated with the vehicle or each of the agonists at 28°C. Impedance between the electrodes was measured every 10 s.

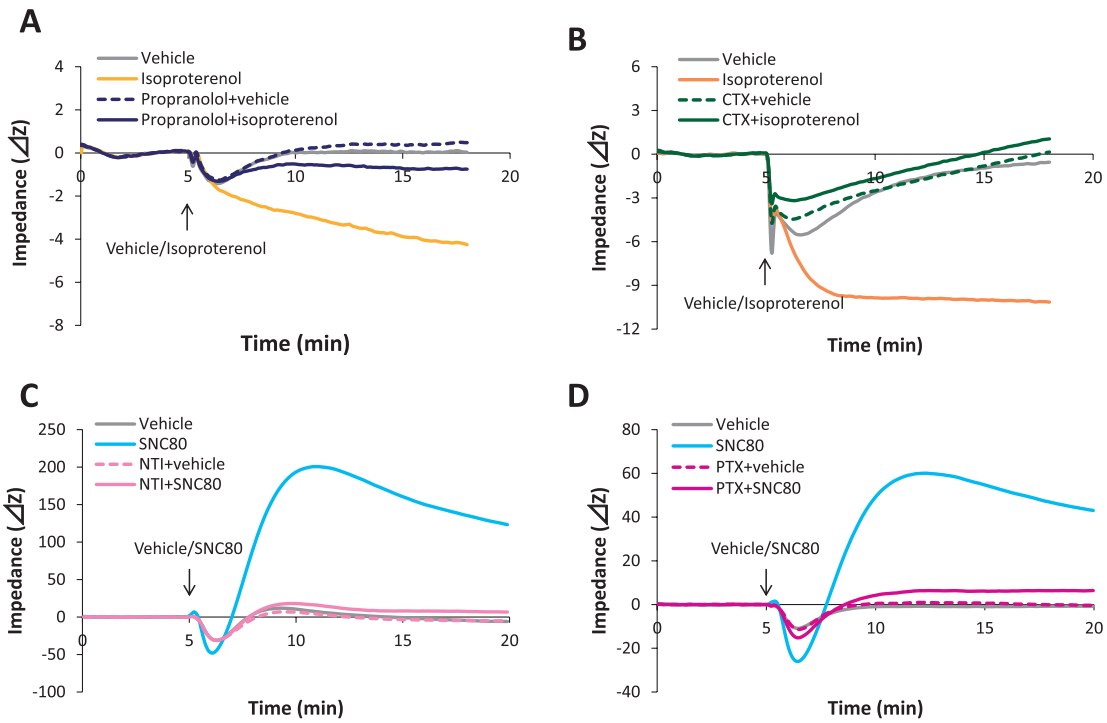

Fig. 4. Effects of antagonists or G protein inhibitors on the changes of impedance (ΔZ) induced by the GPCR activation. A) Effect of propranolol on the isoproterenol-evoked increase in ΔZ in rat C6 glioma cells. Rat C6 glioma cells were pretreated with 10 μM of propranolol for 30 min and subsequently treated with 100 nM of isoproterenol for 13 min. B) Effect of cholera toxin (CTX) on the increase in ΔZ induced by isoproterenol in rat C6 glioma cells. Rat C6 glioma cells were pretreated with 100 ng/mL of CTX for 30 min and subsequently treated with 100 nM of isoproterenol for 13 min. C) Effect of naltrindole (NTI) on the SNC80-evoked increase in ΔZ in δ-opioid receptor (DOR)-expressing HEK293 cells. DOR-expressing HEK293 cells were pretreated with 1 μM of NTI for 30 min and subsequently treated with 1 μM of SNC80 for 15 min. D) Effect of pertussis toxin (PTX) on the increase in ΔZ induced by SNC80 in DOR-expressing HEK293 cells. DOR-expressing HEK293 cells were pretreated with 100 ng/mL of PTX for 30 min and subsequently treated with 1 μM of SNC80 for 15 min.

3.2. Evaluation of GPCR activation using the CellKey™ system

Figure 3 illustrates the different patterns of ΔZ caused by each ligand for specific types of GPCRs (Gq, Gs, and Gi/o). Among the 5 muscarinic acetylcholine receptors (M1 – M5), M1, M3, and M5 acetylcholine receptors couple with the Gq protein, whereas M2 and M4 acetylcholine receptors coupled with the Gi/o protein (25, 26). In human embryonic kidney 293 (HEK293) cells, M3 is endogenously expressed at a low level (25, 26). We therefore stimulated HEK293 cells with the vehicle or acetylcholine (10 μM). Compared to the vehicle, acetylcholine induced a rapid decrease followed by a gradual increase in ΔZ (Fig. 3A). In addition, we used the β-adrenergic receptor as the Gs protein–coupled receptor. In rat C6 glioma cells that endogenously express both β1- and β2-adrenergic receptors (27), treatment with isoproterenol (100 nM), an agonist of the β-adrenergic receptor, induced a decrease in ΔZ (Fig. 3B). This decrease was significantly suppressed by pretereatment with propranolol, an inhibitor of the β-adrenergic receptor (Fig. 4A) and with cholera toxin (CTX), an inhibitor of the activated Gs protein (Fig. 4B). These data suggest that isoproterenol induces a decrease in ΔZ via activation of β-adrenergic receptor and Gs protein. Figure 3C shows that SNC80 (10 nM), a selective δ-opioid receptor (DOR) agonist, induces an increase in ΔZ in HEK293 cells that exogenously express DOR. The increase in ΔZ evoked by SNC80 was suppressed by naltrindole (NTI) and pertussis toxin (PTX), inhibitors of DOR and Gi/o protein activation, respectively (Fig. 4: C and D). These data suggest that SNC80 activates Gi/o protein through DOR, thereby inducing an increase in ΔZ.

Figure 3 also demonstrates that the CellKey™ system can detect the activation of endogenous GPCRs, whose expression levels are lower than those of exogenous GPCRs. Moreover, Scott and Peters (21) reported that the CellKey™ system is highly sensitive compared to traditional assays, such as the GTPγS binding assay, cAMP assay, and Ca^{2+} imaging assay. Taken together, these results affirm that the CellKey™ system can be used to generate pharmacologically high-quality data.

3.3. Advantages of the CellKey™ assay system

Compared to the classical assays, the CellKey™ system has the following advantages. First, this system can detect signaling induced by various combinations of Gα subunits simultaneously, whereas traditional assays detect only single Gα subunits. While some GPCRs can couple only to a single type of G protein (e.g., Gs or Gi/o), many GPCRs often couple to a broader range of G-protein families such as Gs/Gi or Gi/Gq (6). In HEK293 cells expressing μ-opioid receptor (MOR), ΔZ induced by morphine, an MOR agonist, were inhibited not only by PTX (an inhibitor of Gi/o protein activation) but also by Y-27632 [an inhibitor of Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK)], which is activated by G12 protein activation] (data not shown). Thus, the CellKey™ system is a simple assay in which a single assay platform can detect multiple types of GPCR signaling. In addition, the CellKey™ system does not require labeled ligands and is designed with usage of 96- or 384-well plates. Thus, simplified assay design, minimal artifacts, and shortened assay time, contribute to higher throughput of the CellKey™ system.

Second, the CellKey™ system is able to distinguish between each of the three major G protein–dependent pathways (Gs, Gq, and Gi/o) with different kinetic ΔZ. This characteristic can be attributed only to the CellKey™ system among all the CDS-based instruments (20, 21).

Third, the CellKey™ assay is particularly superior for the activation of Gi/o protein–coupled receptors compared to traditional assays, such as GTPγS binding or cytosolic cAMP assays, among the three major types of G protein–dependent signaling (Gs, Gq, and Gi/o) (17). The GTPγS binding assay requires radioactive substances that are a safety concern. In the cAMP assay, stimulation of AC with forskolin is required to measure the decrease in cytosolic cAMP induced by the activation of Gi/o protein–coupled receptor, indicating that we evaluated a reversal effects of Gi/o protein–coupled receptor agonist against increase in cytosolic cAMP induced by forskolin. This assay cannot actually detect the activity of the Gi/o protein–coupled receptor. On the other hand, the CellKey™ assay can detect the direct activation of the Gi/o protein–coupled receptors using a non-labeled, whole-cell, and real-time approach.

Lastly, the CellKey™ system has similar or higher sensitivity than a traditional system using label-based assays such as the GTPγS, Ca^{2+} flux, and cAMP assays. They demonstrated that EC_{50} values between CellKey™ and Ca^{2+} or cAMP were almost the same level. In Gi-coupled receptor, however, the EC_{50} value in the CellKey™ system was three- to ten-fold more potent than that in the GTPγS assay (19).

3.4. Future Prospects of the CellKey™ system

In this review, we have introduced the basic features of the CellKey™ system. However, the features of the CellKey™ system are not limited to either a simple operation or a high-throughput analysis. We are now developing novel assays for making new discoveries.

3.4.1. Evaluation of G protein–independent signaling pathways

Recent studies have indicated that there are G protein–independent signaling pathways, one of which is related to *β*-arrestin, a cytosolic signal transducing protein (3). *β*-Arrestin binds to ligand-activated GPCRs, which are phosphorylated by G protein–coupled receptor kinases (GRKs) to induce desensitization. The receptor–*β*-arrestin complex then loses the ability to couple with the G protein, suggesting that GRKs and *β*-arrestin play an important role in GPCR desensitization by preventing the coupling with G protein (3, 28, 29). In addition, *β*-arrestin facilitates agonist-induced internalization via clathrin-coated pits (28, 29). *β*-Arrestin–mediated internalization is required for the phosphorylated receptors to restore the responsiveness of GPCR-mediated signaling (28). Thus, *β*-arrestin has two roles, desensitization and resensitization. The acidic conditions in the endosomes induce conformational changes in the receptor and enhance dephosphorylation by G protein–coupled receptor phosphatases (GRP) (28). The dephosphorylated receptor then returns to the cell surface and is ready for responding to a new stimulus. This is termed "recycling of the receptor" (28).

Many GPCRs are known to undergo *β*-arrestin–mediated receptor internalization via an actin cytoskeleton–dependent process (21, 30). CDS biosensors containing CellKey™ system can measure changes in the actin cytoskeleton (20, 21), suggesting that this system detects *β*-arrestin–mediated receptor internalization or *β*-arrestin–dependent cell signaling. Consequently, we are trying to establish assays for the evaluation of *β*-arrestin–dependent signaling using so-called "biased" ligands, which are defined as agonists that induce G protein- or *β*-arrestin–biased signaling (29).

3.4.2. Evaluation of GPCR heterodimerization

In the last few decades, the concept of GPCRs forming homodimers and heterodimers with other GPCRs has gradually gained acceptance. GPCRs exist in a dynamic equilibrium between a monomeric and dimeric state (3, 7). GPCR heterodimerization is known to alter ligand binding, cellular signaling responses, and internalization in comparison to GPCR monomers (7). The greatest challenge involved in conducting studies on GPCR heterodimers is the lack of appropriate assays for detecting the functional alteration of GPCRs. Consequently, high-throughput analysis of GPCR heterodimers has never been accomplished (7). The CellKey™ system detects the activation of GPCRs as whole-cell responses, which are mediated by various cell signaling modulators. Thus, it is possible that the CellKey™ system may enable the detection of the GPCR heterodimer–dependent signaling in a high-throughput mode.

4. Conclusions

This review discusses traditional assays for the detection of GPCRs coupled to Gs, Gq, and Gi/o proteins, as well as the state-of-the-art CellKey™ system. The CellKey™ system can measure the activity of GPCRs in one platform with the same or even higher sensitivity as the traditional assays. In addition, this system employs a non-label, whole-cell, real-time, and high-throughput approach, indicating that it is simpler than the traditional assays. The CellKey™ system is expected to contribute to novel insights in GPCR studies, such as the establishment and validation of concepts of biased ligands or heterodimer theory, which may facilitate the rational design of novel therapeutic drugs. However, it is necessary for developing this assay further to determine how impedance induced by the activation of each GPCR changes or which types of cell signaling are important for changes in impedance. We are actively working to overcome the challenging issues. We expect that establishment of new assays and technology using the CellKey™ system will lead to innovative drug development.

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