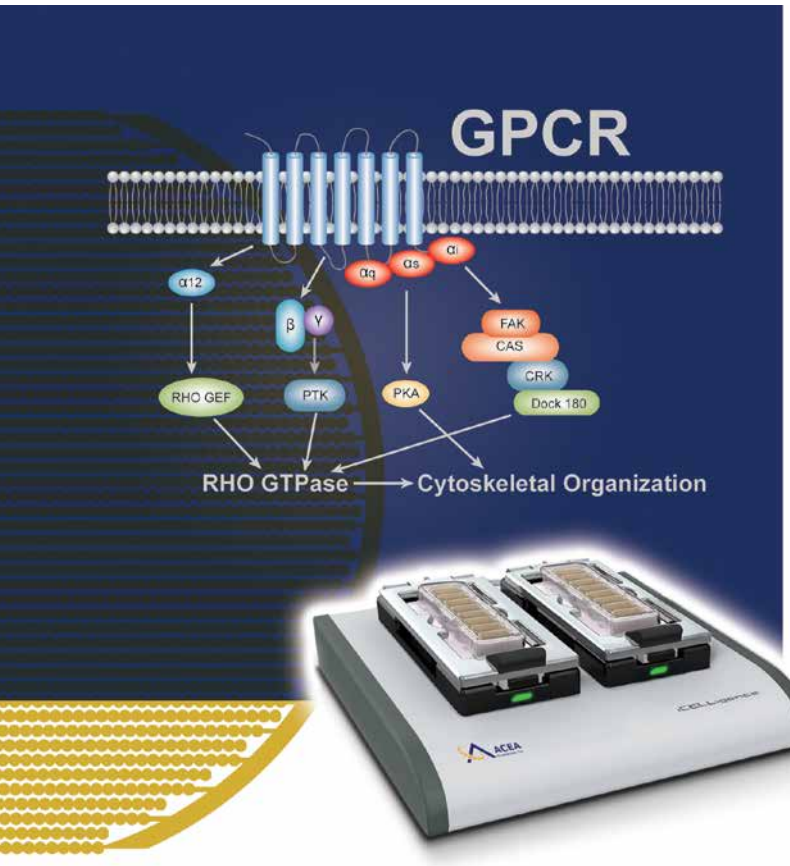
iCELLigence System Application Note No. 3 December 2012

Dynamic Monitoring of G-Protein Coupled Receptor Activation in Living Cells



Introduction

The seven-transmembrane, or G-protein coupled receptor (GPCR) family of proteins is perhaps the largest class of disease-relevant signal transduction molecules. Non-olfactory GPCRs comprise the bulk of targets for small molecule drugs in clinical research. With well over 300 members in the human genome, including many without known ligands (orphans), these receptors continue to be an active area for basic research. Indeed, investigation into the functioning of GPCRs is continuing to reveal a richness and complexity that underpins diverse aspects of biology and may be exploited in developing novel treatments for many different diseases (1).

The iCELLigence System is a unique, impedance-based label free cell based assay system that allows real-time monitoring of GPCR function. The system is composed of a station that fits inside a standard tissue culture incubator and a Control Unit which runs the software and operates the station. At the core of the system are microtiter plates (E-Plate L8 devices) that have integrated microelectrode sensors in the bottom of the wells. Adherent cells cultured on the surface of the sensors exert an insulating effect on the electronic and ionic exchange between cell culture media and the microelectrodes when a small electrical field is applied to the system. This effect is measured as a change in electrical impedance, and is expressed as a "Cell Index" value reflecting the cell number, morphology, and degree of attachment to the electrodes. The impedance readout provides a very precise and sensitive measurement of subtle changes in cytoskeletal structure and cellular morphology such as those induced by GPCR activation, as well as revealing the overall biological status of the cells including changes in cell adhesion and number (2-4).

Introduction

All of the major second messenger pathways through which GPCRs signal (cyclic AMP/protein kinase A, calcium/phospholipase C, beta-arrestin/MAPK activation, Rho family GTPases) can result in morphological changes of a magnitude detectable by cellular impedance readouts (4). Label-free assays have demonstrated great utility in the study of GPCR function in a cellular context, and provide many advantages over standard assays, with the potential to reveal more novel biology in the future (2). While recombinant cell lines are often productively assayed on the system, a key advantage is that many GPCRs may be assayed in the endogenous context, in disease-relevant cellular models. This contrasts with the requirement for many assays that the receptor is overexpressed in an engineered cell line derived from biologically irrelevant sources such as Chinese hamster ovary (CHO) cells. Endogenous assays preserve the natural signaling modalities of the receptor in terms of the stoichiometry of interactions with regulatory molecules, including potential receptor heterodimer formation, as well as downstream signal transducers.

Another key advantage of the use of label-free assays is the unbiased nature in which receptor function is assayed; since all major coupling classes may induce changes in cell behavior detected by the impedance readout, the net effect of all biologically relevant signals may be assayed simultaneously. An exciting avenue of GPCR research involves the elucidation of the balance of these downstream signals engaged by different ligands acting on the same receptor. This phenomenon, variously termed pleiotropic signaling, functional selectivity, or ligand-biased signaling, is readily detectable using the impedance readout and may prove to be useful for the development of more selective drugs with fewer undesired side effects (3).

In addition, the unbiased nature of the impedance readout has the potential to open up investigation of GPCRs where downstream signaling is poorly characterized or unknown, such as for orphans of GPCRs with non-canonical functionality. Finally, the non-invasive nature of the impedance readout allows for monitoring of cell responses over the entirety of the assay, potentially including multiple treatments. Partial or full antagonists and agonists, as well as allosteric modulators and receptor desensitization effects, may be distinguished in the same assay by continuous monitoring of cell responses to a first treatment, followed by a second (or even additional treatments).

Figure 1. The iCELLigence System. The station houses two E-Plate L8 devices and transfers data via Wi-Fi to the iCELLigence Control Unit for analysis. The E-Plate L8 devices contain 8 wells each with an electrode grid area at the bottom for impedance-based measurements and a view area for microscopic analysis. The Control Unit of the iCELLigence System controls the experimental setup, displays the data in real time, and serves as a platform to perform data analysis.

Materials and Methods

Cell Culture

Cell lines were obtained from ATCC. HeLa (#CCL-2), U2OS (#HTB-96), SH-SY5Y (#CRL-2266), CHO-K1 (#CCL-61), human vascular endothelial cells (HUVEC; # PCS-100-010) and mixed renal primary epithelial cells (MREC; # PCS-400-012) were cultured according to ATCC recommendations.

Reagents

All reagents were purchased from Tocris (Minneapolis, MN) with the exception of histamine, purchased from Sigma (St. Louis, MO).

Measurement of Cell-Electrode Impedance

Detailed experimental procedures have been described previously (2). Briefly, 150 µL of selective medium was added to wells of the E-Plate L8 to obtain background readings, then 300 µL of cell suspension containing the 24,000 HeLa cells was added. The E-Plates containing the cells were incubated at room temperature for 30 minutes, then placed on the device station (in the 37°C CO_2 incubator). Impedance was continuously recorded and converted to a Cell Index (CI). The cells were allowed to attach and spread, typically for 20-24 hours, to reach a stable baseline before agonists were added. Buffer exchange was conducted and growth media was replaced with HBSS buffer containing 0.1% BSA and 20 mM HEPES. After ~30 minutes equilibrium on the station to establish the baseline CI, 25-50 µL of a stock solution of agonist or antagonist was then gently added to the well and recording was resumed. For antagonist assays, cells were monitored for 20 minutes, then agonist was added. Cells were monitored for 30 minutes after agonist addition. Unless otherwise indicated, results were expressed as a normalized CI, which is derived from the ratio of CIs before and after addition of the compounds. For concentration-dependent studies, maximal response to a given concentration of the compound was used to plot a concentration-dependent curve. EC50 was calculated by GraphPad Prism (San Diego, CA).

Results and Discussion

Dynamic Monitoring of Endogenously Expressed GPCRs in Living Cells

Dynamic monitoring of endogenously expressed histamine receptors in living cells was conducted using HeLa cells. Cells were seeded on the E-Plate L8 and stimulated the next day with increasing concentrations of histamine. As shown in **Figure 2A** and **2B**, histamine stimulation of HeLa cells leads to a dose-dependent and transient increase in Cell Index (CI) (**Figure 2A**). The peak normalized CI value was plotted versus the log concentration of the agonist to generate a dose-response curve, and an EC50 value of 5.4 µM was obtained (**Figure 2B**).

Histamine acts through members of the Gαq coupled histamine receptor family. Stimulation of HeLa cells with various ligands to numerous other GPCRs encompassing all the major coupling classes revealed different response profiles, some involving increases and some decreases in CI. Many of these receptors may be assayed in the endogenous context in HeLa cells using the impedance readout (**Figure 3**), and many additional receptors may be assessed in other cell types (**Table 1**).

Antagonist Assay on Endogenously Expressed GPCRs in Living Cells

Many therapeutic agents targeting GPCRs act as antagonists. The α-adrenergic receptors play diverse biological roles including vasoconstriction, and antagonists are used in the clinic to treat several diseases, including hypertension. Antagonist assays were conducted on endogenously expressed α-adrenergic 2A receptors, which are Gαi/o coupled. HeLa cells were seeded on the E-Plate L8 and treated the next day with increasing concentrations of the α-2A antagonist rauwolscine. After 20 minutes incubation, the selective α-2A agonist UK14,304 was added and the response monitored in real time. UK14304 stimulation of HeLa cells leads to an increase in Cell Index (CI), which is blocked by rauwolscine pretreatment in a dose-dependent manner (**Figure 4A**). The peak normalized CI value was plotted versus the log concentration of the antagonist to generate a dose-response curve, and an IC50 value of 38.5 nM was obtained (**Figure 4B**).

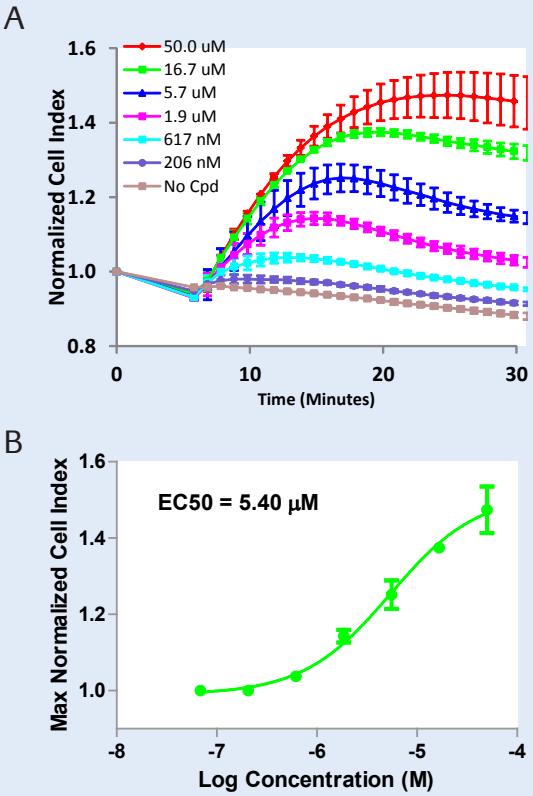


Figure 2. Pharmacological study of endogenous histamine GPCR function. HeLa cells were seeded at 24,000 cells per well on E-Plates, treated the next day with increasing concentrations of histamine, and the cell response was monitored every 30 seconds for 30 minutes (A). Plotting the peak cell index responses versus the corresponding log concentrations allows calculation of the EC50 of histamine (B).

Receptor	HeLa	CHO-K1	U2OS	SH-SY5Y	MREC	HUVEC
Histamine	X		X	X	X	
Acetylcholine	X			X		
Adrenergic	X		X	X	X	X
Dopamine	X		X	X		
Prostaglandin	X	X		X	X	X
CXC Chemokine	X					X
Cannabinoid	X			X	X	
Endothelin	X					
Lysophospholipid	X	X	X		X	X
Purinergic	X	X			X	
Free Fatty Acid	X	X	X	X	X	
Serotonin		X				
Calcitonin		X				

Table 1 Endogenous GPCR responses. "X"indicates at least one receptor family member exhibited a robust impedance response (> 3 standard deviations from control).

The development of more biologically relevant assay systems is expected to help improve our understanding of GPCR function, and label-free systems such as iCELLigence offer the opportunity to gain novel insights. Here we have demonstrated the basic functionality of iCELLigence for measurement of endogenous GPCR agonist and antagonist responses. These assays are sensitive and precise, and may be performed on disease-relevant cells (including primary cells) as well as recombinant receptor overexpressing cell lines. Label-free cell-based assays such as the iCELLigence System have the potential to elucidate a complete picture of the cellular response to GPCR signaling. For additional information on how impedance technology can be utilized for assessment of GPCR signaling, please see the reference list provided below (1-4).

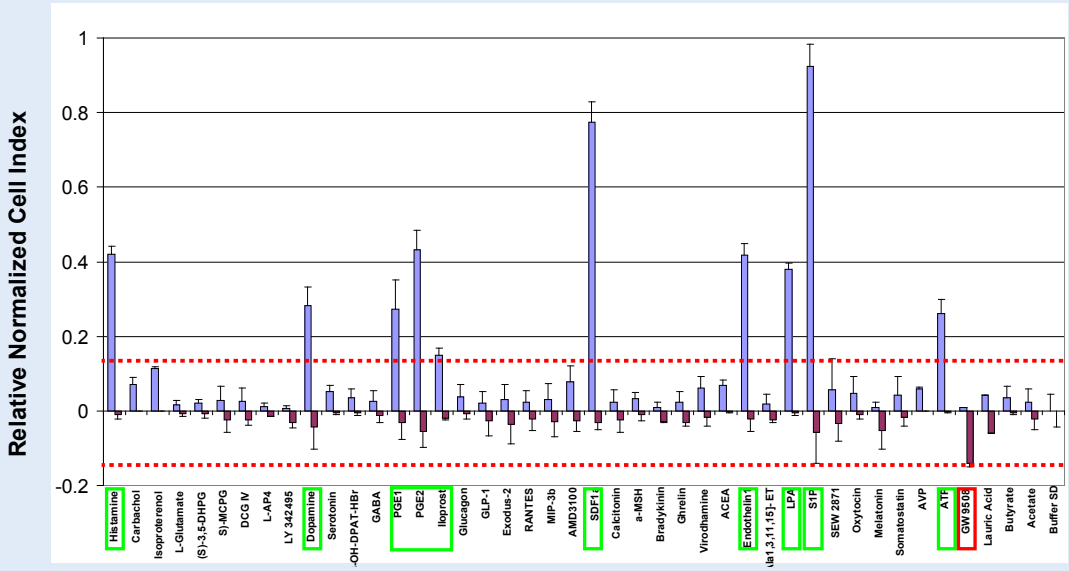
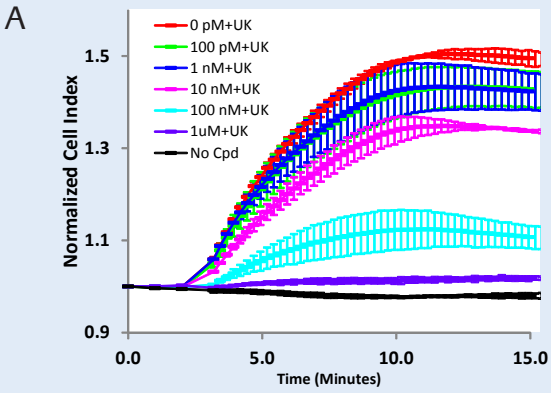


Figure 3. Endogenous GPCR responses. HeLa cells were assessed for responses to a panel of GPCR modulators. Maximal and minimal Cell Index values were determined for each well relative to buffer treated controls. Replicate values were plotted as the mean value and error bars represent one standard deviation. The red dotted line represents the value of three standard deviations relative to the control wells. Compounds that clearly produced an increase (green line) or decrease (red line) in Cell Index values are highlighted by solid boxes.



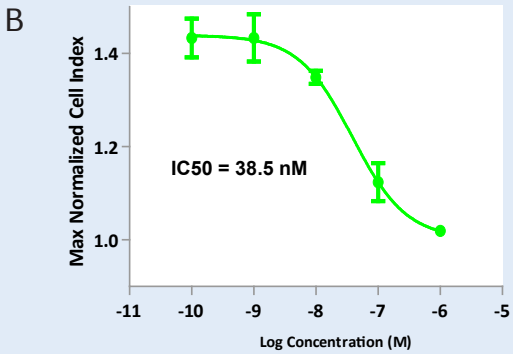


Figure 4. GPCR antagonist assay. Pharmacological study of antagonists to endogenous α-adrenergic receptor GPCR function. HeLa cells were seeded at 24,000 cells per well on E-Plates and treated the next day with increasing concentrations of the α-2A antagonist rauwolscine. After 20 minutes incubation, the selective α-2A agonist UK14,304 was added and the cell response was monitored every 30 seconds for 30 minutes (A). Plotting the peak cell index response versus the corresponding concentration allows calculation of the IC50 of rauwolscine (B).

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Anal Chem. **78**:35-43.

Ordering Information

Product	Cat. No.	Pack Size
RTCA iCELLigence System Bundle	**00380601000**	**1 Instrument and 1 Control Unit**
RTCA iCELLigence Instrument	00380600970	1 Instrument
RTCA iCELLigence Control Unit	00380601020	1 Instrument
E-Plate L8	00300600840 00300600850	1 × 6 Plates 6 × 6 Plates
E-Plate Insert 16	06465382001	1 x 6 Devices (6 16-Well Inserts)

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