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

xCELLigence real-time cell analysis

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Introduction

G-protein-coupled receptors (GPCRs) represent the largest family of plasma membrane proteins involved in signal transduction across the plasma membrane. These receptors play a prominent role in regulating many physiological functions (such as sight, taste, smell), as well as fluid electrolyte balance, cardiovascular function, and neurotransmission.

Structurally, GPCRs share seven hydrophobic transmembrane domains, which are separated by three intracellular and three extracellular loops. Most GPCRs couple to and activate heterotrimeric G proteins and then stimulate second-messenger systems. The heterotrimeric G proteins consist of a Gα subunit and a Gβ/γ complex, both of which can activate downstream effectors. Depending on its components and corresponding second-message systems, the Gα subunit can be further divided into four subtypes: Gαs (Gs), Gαi/o (Gi/o), Gαq/11 (Gq11), and Gα12/13 (G12/13).

- Gs leads to stimulation of adenylate cyclase

- Gi/o inhibits adenylate cyclase

- Gq11 activates phospholipase C (PLC) and hence mobilization of intracellular calcium and activation of protein kinase C (PKC)

- G12/13 triggers Rho kinase activation

More than 50% of the current therapeutic agents on the market are targeted against GPCRs. The human genome sequencing project has identified approximately 720 genes that belong to the GPCR superfamily. Of these, 150 are orphan receptors whose ligands and functions are unknown. Discovering the functions of existing and emerging GPCRs will provide new insights into cell biology and disease processes, and offer hope for therapeutic intervention.

Cell-based assays monitoring functional activation of GPCRs are crucial to the drug discovery process. High-throughput functional assays, such as calcium measurements and reporter gene assays, have been used to screen chemical or peptide libraries for agonists and antagonists of the different GPCRs. The traditional means of studying and screening for GPCR function in cell-based assays all involve:

- Labeling the cells with radioactive precursors or fluorescent reagents

- Measuring single molecular entities such as inositol phosphate, calcium, or cAMP, depending on the pathways of interest

These assays also involve engineering cells to express certain reporter genes or proteins, such as promiscuous G-proteins. These assays are also invasive and involve destruction of the cells. While these manipulations may be convenient for screening purposes, the physiological relevance and interpretation of the results need to be examined and supported by other data. It is also often difficult to correlate the discrete intracellular events with the overall integrated cellular response after the receptors are activated.

The impedance-based xCELLigence system allows label-free, real-time monitoring of cellular processes such as cell growth, proliferation, adhesion, and morphology changes. The system is composed of a device station that

fits inside the cell culture incubator, an electronic analyzer, and a computer that runs the software and operates the entire system. At the core of the system are electronic plates (E-Plates) that have integrated microelectrode sensors in the bottom of the wells. Adherent cells are cultured on the surface of the sensors. The presence or absence of cells affects the electronic and ionic exchange between cell culture media and the microelectrodes. The electrode impedance provides information about the biological status of the cells and can monitor phenomena such as proliferation, morphological changes, and cell death.

Impedance-based technology has been validated in several cell-based assays.[1,2] In this application note, a series of experiments is described to demonstrate how the system may be used to monitor functional activation of GPCRs in living cells.

Materials and methods

Cell culture

All cells used in this study were purchased from ATCC unless otherwise indicated. The cells were cultured in a standard humidified incubator at 37 °C with 5% CO_2. C6 cells and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine and 1% penicillin/streptomycin. Chinese hamster ovary (CHO) cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine and 1% penicillin/streptomycin. Cell lines stably expressing human recombinant histamine receptor 1 (H1), human vasopressin 1a (V1a), and human recombinant dopamine receptor 1 (D1) were obtained from Euroscreen and maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1%

penicillin/streptomycin and 400 μg/mL G418. A cell line stably expressing human recombinant 5-hydroxytryptamine receptor 1A (5-HT1A) was obtained from Euroscreen and maintained in Ultra-CHO medium supplemented with 1% penicillin/streptomycin and 400 μg/mL G418.

Reagents

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Loratidine, mirtazepine, mepyramine, triprolidine, and tiotidine were purchased from Tocris (Ellisville, MO).

Measurement of cell-electrode impedance

The detailed experimental procedures have been described in studies by Abassi et al and Solly et al.[1,2] Briefly, 50 μL of selective medium was added to wells of E-Plates to obtain background readings, then 150 μL of cell suspension containing the indicated number of cells was added. The E-Plates containing the cells were incubated at room temperature for 10 minutes, then placed on the device station (in a 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 6 to 8 hours, to reach a stable baseline before agonists were added. Typically, 5 μL of a 40X stock solution of agonist was then gently added to the well and recording was resumed. For pharmacological and mechanistic studies, antagonists or inhibitors were added to the cells 5 to 10 minutes before agonist application. Unless otherwise indicated, results were expressed as a normalized CI, derived from the ratio of CIs before and after addition of the compounds. For concentration-dependent studies, maximal response (usually 1 to 2 hours after the ligand application) to a given concentration of the compound was used to plot a concentration-dependent

curve. EC_{50} and IC_{50} were calculated by Prism (San Diego, CA).

Fluorescence microscopy

H1 cells and V1a cells were seeded in 16-well Lab-tec chamber slides and allowed to attach and spread for 24 hours. The cells were stimulated with 100 nM histamine for five minutes and washed three times with PBS before fixation. The cells were fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.2% Triton X-100. After they were washed, the fixed cells were blocked with PBS containing 0.5% BSA. The cells were stained with FITC-conjugated phalloidin and a monoclonal anti-paxillin antibody from Sigma (St. Louis, MO). The stained cells were washed three times with PBS, then photographed with an epifluorescence microscope.

Results and discussion

Dynamic monitoring of GPCR activation in living cells

To demonstrate that the impedance-based system can be used to dynamically monitor the functional activation of GPCRs, CHO-K1 cells expressing the human H1 histamine receptor (H1) and 1321-N1 cells expressing the human vasopressin receptor (V1a) were seeded on the E-Plates and stimulated with histamine and vasopressin (AVP), respectively (Figure 1). Both histamine and vasopressin induced an immediate and transient increase in CI (Figure 1A and C). The maximal response for H1 cells was at 40 minutes after histamine addition, while for V1a cells it was at 90 minutes (Figures 1A and 1C).

Both histamine and vasopressin act through Gq and have also been reported to modulate the actin cytoskeleton and its regulatory proteins, such as focal adhesion kinase (FAK) and paxillin.
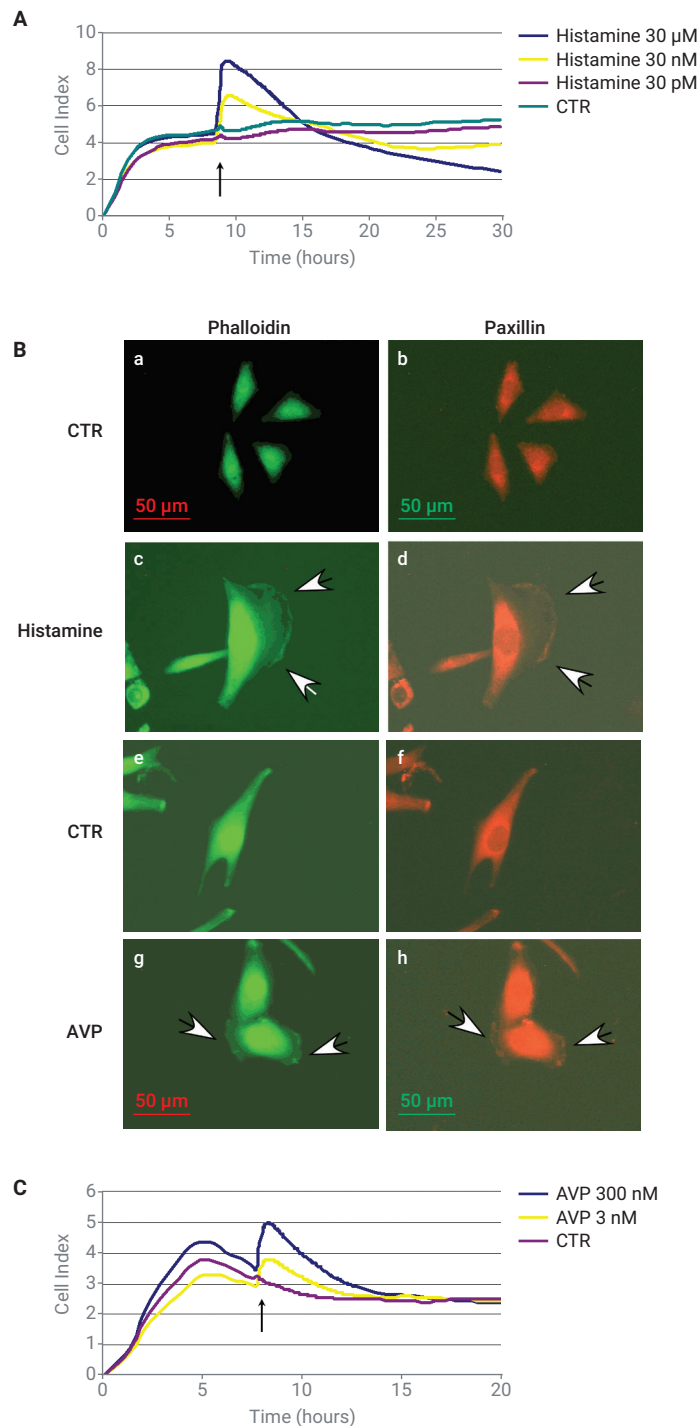

Figure 1. Characterization of activation of overexpressed GPCRs. Human recombinant histamine 1 receptor (H1) and human recombinant vasopressin 1a (V1a) receptor (panel A and panel C, respectively) were seeded at 50,000 cells per well on E-Plates, and the cells were continuously monitored. At the indicated time point, increasing concentrations of histamine or vasopressin (AVP) were added to the cells and the cell response was monitored every three minutes. A typical set of CI traces from the H1 and the V1a cell lines are shown. Arrows indicate the addition of the ligand. Plotting the peak Cell Index response versus the corresponding log concentration allows calculation of the EC_{50}. Panel B (the eight square images) shows cell ruffling and cell adhesion enhancement five minutes after application of the indicated ligand. Histamine increases cell ruffling (c, arrow head) and cell adhesion assembly (d, arrow head) in H1 cells. AVP increases cell ruffling (g, arrow head) and cell adhesion assembly (h, arrow head) in V1a cells.

To determine if histamine and vasopressin lead to modulation of the actin cytoskeleton and its signaling proteins, H1 cells and V1a cells were stimulated with histamine and vasopressin, respectively, then fixed and stained with FITC-phalloidin and anti-paxillin mAb (Figure 1B). Histamine treatment of H1 cells led to an immediate (five minutes) induction of membrane ruffles and translocation of paxillin to the site of membrane ruffles, which was indicative of active actin remodeling (Figure 1B). Similarly, vasopressin also induced formation of membrane ruffles and translocation of paxillin to these sites (Figure 1B). In summary, the system detected the functional activation of GPCRs based on its ability to modulate the actin cytoskeleton and cell adhesion.

Dynamic and quantitative monitoring of recombinant GPCRs coupled to different signaling pathways

To further extend the findings discussed previously, H1 cells were seeded in E-Plates, treated with increasing concentrations of histamine, and continuously monitored with the impedance-based system. As depicted in Figure 2A, histamine leads to a transient and dose-dependent increase in CI. To obtain a dose-response curve, the log concentration of histamine was plotted against the normalized CI (Figure 2B); the approximate EC_{50} value obtained from this plot was 1.7 nM. Similarly, vasopressin caused a transient and dose-dependent increase in CI (Figure 2C) with an EC_{50} of 155 pM (Figure 2D).
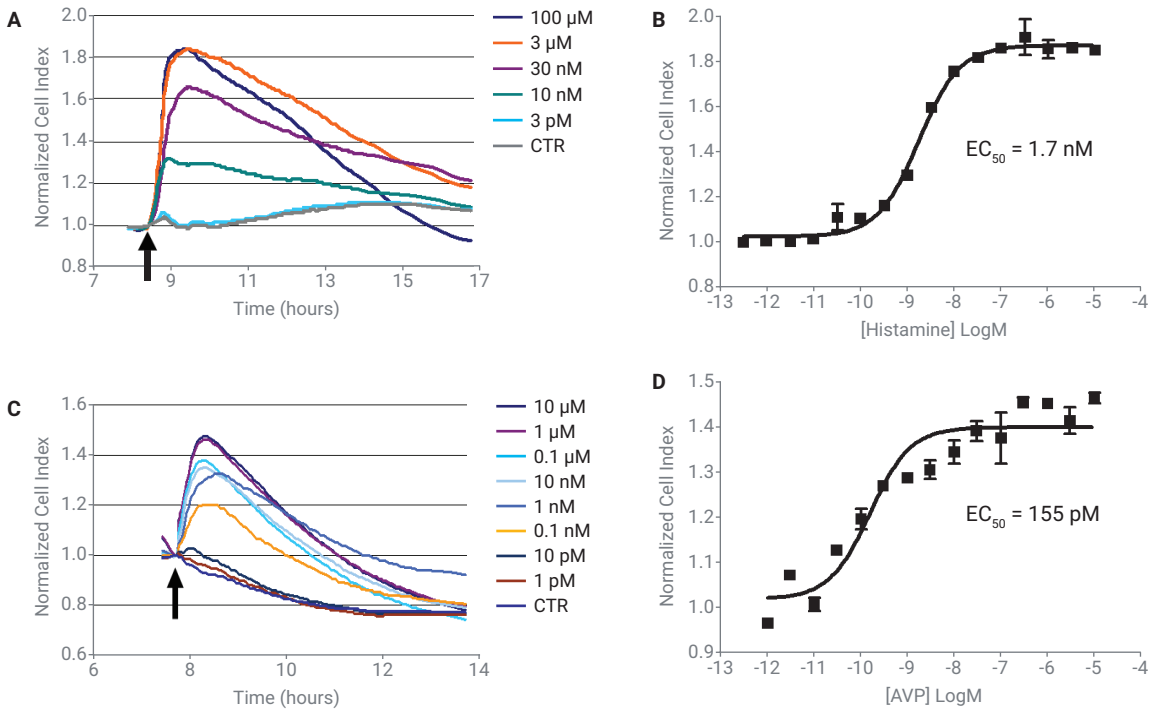

Figure 2. Pharmacological study of H1 and V1a cell lines coupled to Gq. Generation of EC_{50}: H1 and V1a cell lines were seeded at 50,000 cells per well on E-Plates, and the cells were continuously monitored. At the indicated time point (arrows), increasing concentrations of histamine (A) or AVP (C) were added to the cells and the cell response was monitored every three minutes. A typical set of normalized CI traces are shown. Plotting the peak normalized Cell Index responses versus the corresponding log concentrations allows calculation of the EC_{50} of histamine (B) and AVP (D).

CHO-K1 cells expressing the human dopamine 1 receptor (D1) coupled to Gs or the human 5HT1A receptor (5-HT1A) coupled to Gi were seeded on E-Plates. This was done to demonstrate that the new system could also be used to monitor functional activation of GPCRs coupled to other signaling pathways. They were stimulated with increasing concentrations of their respective ligands. As shown in Figures 3A and 3C, both cell types were activated in a dose-dependent manner. To generate a dose-response curve, the peak normalized CI value was plotted versus the log concentration of the agonist.

Stimulation of D1 receptor by dopamine (DA) gave an EC_{50} value of 0.79 pM (Figure 3B), while stimulation of 5-HT1A receptor by 5-HT gave an EC_{50} value of 12.4 nM (Figure 3D).

In summary, the impedance-based system detected the functional activation of GPCRs coupled to different signaling pathways. Monitoring each of these different signaling pathways traditionally required different detection technologies and instrumentation, such as calcium or IP3 measurement (Gq) and cAMP measurements (Gs and Gi).
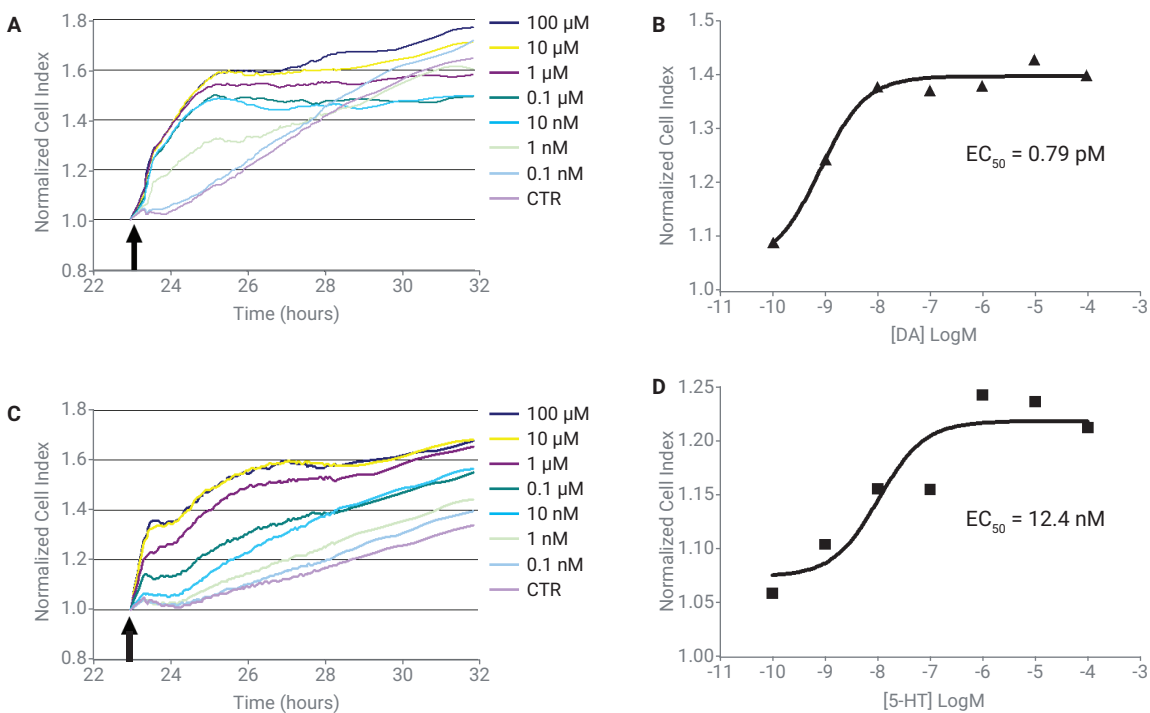
Figure 3. Pharmacological study of D1 cell line (Gs) and 5-HT1A cell line (Gi). Generation of EC_{50}: D1 and 5-HT1A cell lines were seeded at 25,000 or 12,000 cells per well, respectively, on E-Plates, and the cells were continuously monitored. At the indicated time point (arrows), increasing concentrations of dopamine (A) or 5-HT (C) were added to the cells and the cell response was monitored every three minutes. Normalized CI traces are shown. Plotting the peak normalized CI responses versus the corresponding log concentrations allows calculation of the EC_{50}s of DA (B) and 5-HT (D).

Dynamic monitoring of endogenously expressed GPCR in living cells

One of the challenges encountered with current label-based functional assays is that the cells must be engineered to express promiscuous G proteins that are coupled to, for example, the calcium pathway. Alternatively, receptors may need to be overexpressed to generate a measurable signal. While all these artificial manipulations may simplify the screening of large sets of compounds, the physiological relevance of any findings would have to be supported by other experiments.

The number of assays available to measure the functional activation of endogenous GPCRs in their natural and physiologically relevant settings is limited. It would therefore be useful to determine if impedance technology could monitor the functional activation of endogenous GPCRs coupled to different signaling pathways. HeLa cells were seeded in E-Plates and stimulated with increasing concentrations of histamine. As shown in Figure 4A and 4B, histamine stimulation of HeLa cells leads to a dose-dependent and transient increase in CI. The kinetics and amplitude of histamine-mediated response in HeLa cells were significantly different to those of H1 cells. Primarily, this can
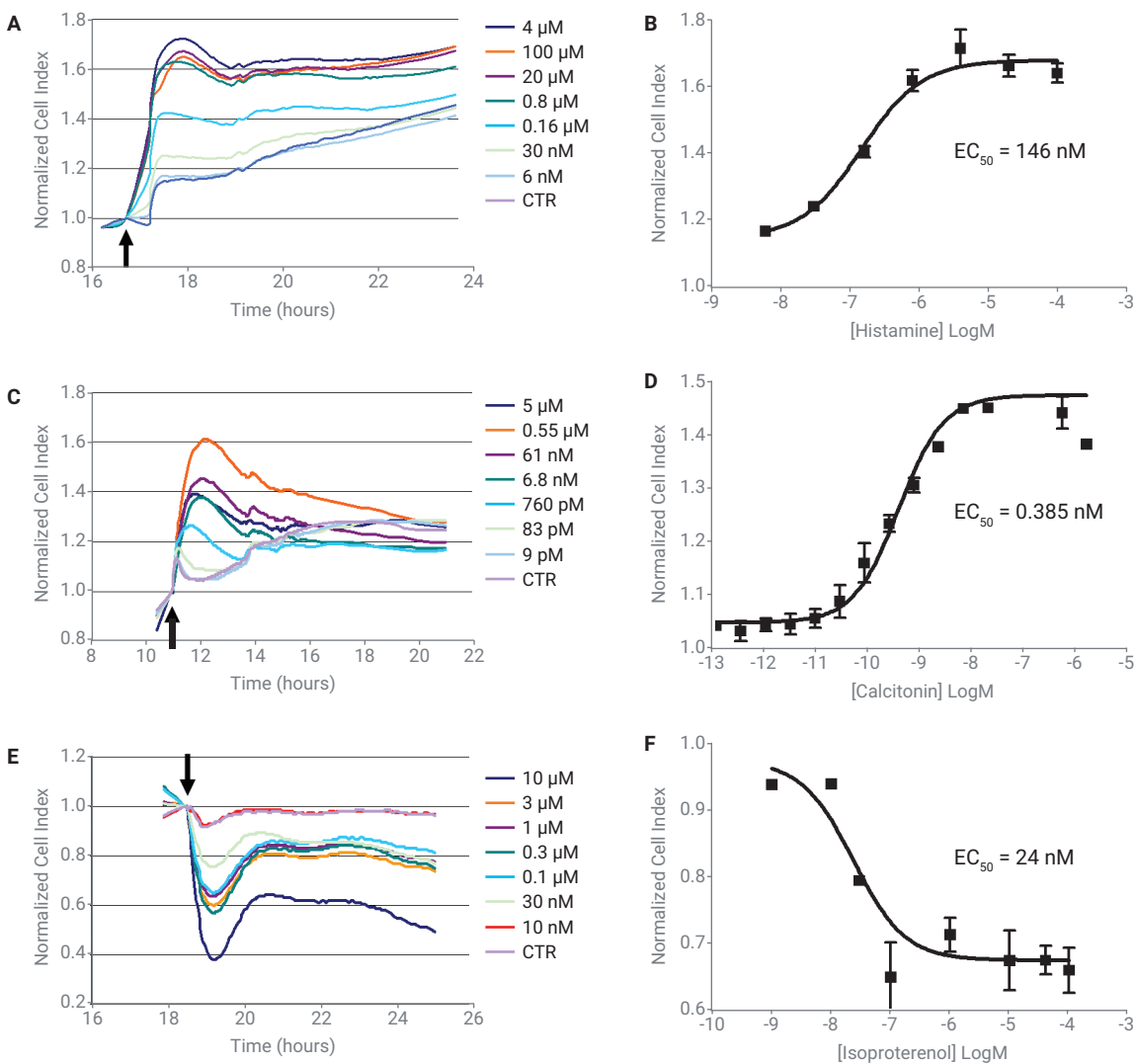

Figure 4. Pharmacological study of endogenous GPCRs with various ligands. Generation of EC_{50}: HeLa cells (A), CHO cells (C), and C6 cells (E) were seeded at 50,000 cells per well on E-Plates, and the cells were continuously monitored. At the indicated time point (arrows), increasing concentrations of histamine (A), calcitonin (C), or isoproterenol (E) were added to the cells and the cell response was monitored every three minutes. Plotting the peak normalized Cell Index responses versus the corresponding log concentrations allows calculation of the EC_{50}s of histamine (B), calcitonin (D), and isoproterenol (F), respectively.

be explained by cell type specificity in terms of histamine signaling. Also, other histamine receptors in addition to H1 may be present in HeLa cells, which could have contributed to the amplitude and duration of the response.

The calcitonin receptor is coupled to the Gs pathway. To demonstrate that endogenous activation of calcitonin receptors can be detected by the system, CHO-K1 cells were seeded on E-plates and stimulated with the indicated concentrations of calcitonin (Figure 4C). As shown in Figure 4C, calcitonin led to a robust and transient increase in CI. Plotting normalized CI versus log concentration of the ligand gave a dose-response curve with an EC_{50} of 0.385 nM (Figure 4D). In contrast, β1 adrenergic receptor stimulation in C6 cells by isoproterenol revealed an opposite effect on CI. As shown in Figure 4E, isoproterenol generated a dose-dependent decrease of CI with an EC50 of 24 nM (Figure 4F).

In summary, it has been demonstrated that the new impedance-based system can monitor the functional activation of GPCRs coupled to different signaling pathways for both endogenous receptors and recombinant receptors.

Quantitative analysis and ranking of histamine receptor antagonists and inverse agonists

A panel of well characterized and selective histamine antagonists was selected to validate the GPCR assay using impedance technology. The system was used to test and rank the potency of these antagonists in H1 cells stimulated with histamine. H1 cells were seeded in E-Plates, pre-incubated with the indicated concentrations of selective H1 antagonists (or H2 receptor antagonists as a control) and then stimulated with a fixed concentration of histamine (20 nM). The peak normalized CI values were plotted versus the log

concentrations of the antagonists. H1 receptor antagonists triprolidine, loratidine, mirtazepine, and mepyramine led to a dose-dependent inhibition of histamine-mediated cellular response (Figure 5A) with IC_{50}s of 9 nM, 24 μM, 343 nM, and 9 nM, respectively. In contrast, H2 selective antagonist tiotidine did not inhibit histamine-induced cell response up to 10 μM (the highest tested concentration was 100 μM, and showed a slight decrease).

These results strongly indicated that the impedance-based system could be used to rank the potency of antagonists in a receptor subfamily-specific manner.

Interestingly, the H1 receptor antagonists alone resulted in dose-dependent decreases of CI levels (Figure 5B). This observation could be explained by the fact that all these H1 receptor antagonists have a certain amount of activity as inverse agonists,[3] which will affect the basal activities of the H1 receptor. The selective H2 receptor antagonist tiotidine did not seem to affect histamine-mediated activation of the H1 cells, nor did it have activity as an inverse agonist. These findings indicate that the observed responses were specific.
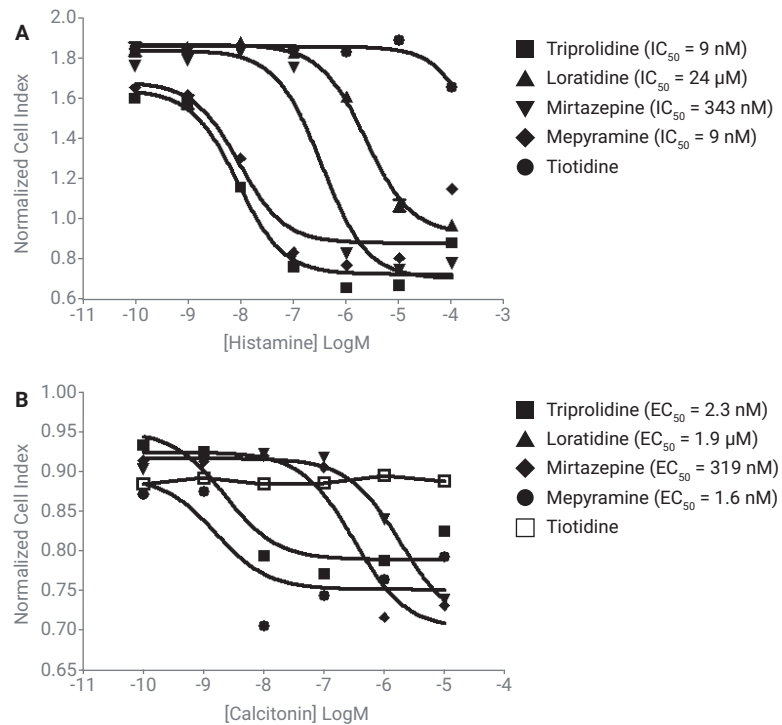

Figure 5. Ranking of potency of selective H1 antagonists on H1 cells and potential application for inverse agonists. A panel of selective H1 antagonists and one H2 antagonist were tested on H1 cells. H1 cells were seeded at 50,000 cells per well on E-Plates, and the cells were continuously monitored. Increasing concentrations of a given antagonist were added to the cells 10 minutes before the addition of a fixed concentration of histamine (20 nM), and the cell response was monitored every three minutes. Four chosen H1 antagonists (triprolidine, loratidine, mirtazepine, and mepyramine) blocked histamine-induced normalized CI increases in a concentration-dependent manner. These antagonists had different potencies, as reflected by their calculated IC_{50}s. H2 selective antagonist tiotidine did not block histamine-induced normalized CI responses up to 10 μM (A). Four chosen H1 antagonists (triprolidine, loratidine, mirtazepine, and mepyramine) caused concentration-dependent decreases of CI. Each had different potencies, as indicated by their calculated EC_{50}s. The H2 antagonist (tiotidine) did not induce any changes in CI at the highest concentration (10 μM) tested (B).

In summary, these results demonstrate that the impedance-based technology can be used to screen selective antagonists or inverse agonists of GPCRs. Furthermore, the basal CI displayed before agonist addition is an actual reflection of the basal and cumulative signaling taking place inside the cell. Therefore, any agent, such as an inverse agonist, that disrupts the basal signaling activity can potentially be detected with this new technology.

This application note has shown that an impedance-based system can be used for functional screenings of GPCR activity in cell-based assays. These assays can monitor engineered cell lines that express recombinant GPCRs coupled to different G-proteins and, more importantly, cell lines expressing endogenous levels of GPCRs.

The main features that distinguish the xCELLigence from other cell-based functional assays for GPCRs are:

1. No pre- or postlabeling of the cells is necessary, saving on time and costs.

2. The assay is noninvasive and therefore cellular destruction is not required. Multiple manipulations can be performed on the same cells in the same well. For example, multiple stimulations with the same agonist/antagonist or agonists/antagonists in different combinations can be carried out in the same well.

3. The assay monitors cellular processes in real time. The assay therefore provides a comprehensive representation of the entire assay period, allowing the user to make informed decisions about the timing of certain manipulations, such as ligand addition. The real-time kinetic readout also provides valuable information about the pathways being activated.

4. The detection method is not affected by endogenous compounds, which avoids a major problem encountered in most optical-based assays.

5. Since the system monitors cell attachment and cell morphology (integral components of cell viability) it can detect any compound that may be potentially cytotoxic or may have other adverse effects.

The impedance-based system provides a different method to monitor GPCR functions and adds an extra perspective to increase the understanding of GPCR functions.

References

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