



**xCELLigence System**

Application Note No. 3 / March 2008

## Dynamic Monitoring of G-protein Coupled Receptor Activation in Living Cells



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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 a variety of 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. The majority of GPCRs couple to and activate heterotrimeric G proteins and subsequently stimulate second-messenger systems. The heterotrimeric G proteins consist of a  $G\alpha$  subunit and a  $G\beta/\gamma$  complex, both of which can activate downstream effectors. Depending on its components and corresponding second-message systems, the  $G\alpha$  subunit can be further divided into 4 subtypes:  $G\alpha_s$  (Gs),  $G\alpha_i/o$  ( $G_i/o$ ),  $G\alpha_q/11$  ( $G_q/11$ ) and  $G\alpha_{12/13}$  ( $G_{12/13}$ ).

- Gs leads to stimulation of adenylate cyclase
- $G_i/o$  inhibits adenylate cyclase
- $G_q/11$  activates phospholipase C (PLC) and hence mobilization of intracellular calcium and activation of protein kinase C (PKC)
- $G_{12/13}$  triggers Rho kinase activation

More than 50% of the current therapeutic agents on the market are targeted against GPCRs. At its completion, the human genome sequencing project had identified approximately 720 genes that belong to the GPCR super-family. One hundred fifty of these are orphan receptors whose ligands and functions are unknown. Elucidation of the functions of existing and emerging GPCRs will not only provide new insights into cell biology and disease processes, but also offer hope for therapeutic intervention.

Cell-based assays monitoring functional activation of GPCRs are a crucial part of 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, and
- Measuring single molecular entities such as inositol phosphate, calcium or cAMP, depending on the pathways of interest.

In addition to labeling, the above mentioned assays also involve engineering the 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 closely examined and supported by other data. Furthermore, it is often difficult to correlate the discrete intracellular events with the overall integrated cellular response after the receptors are activated.

ACEA Biosciences has developed a unique, impedance-based system (RT-CES®) that allows label-free, real-time monitoring of cellular processes such as cell growth, proliferation, adhesion and morphology changes. The new system is composed of a device station that fits inside the cell culture incubator, an electronic analyzer and a computer which 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 sensitively and precisely affects the electronic and ionic exchange between cell culture media and the microelectrodes. Thus, the electrode impedance provides succinct information about the biological status of the cells and can monitor such phenomena as proliferation, morphological changes and cell death. This system is the predecessor of the new xCELLigence System jointly developed by Roche Applied Science and ACEA Biosciences.

The impedance-based technology has been thoroughly validated in several cell-based assays<sup>1,2</sup>. In this application note, a series of experiments is described to determine whether the system is suitable for monitoring 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 are cultured in a standard humidified incubator at 37°C with 5% CO<sub>2</sub>. 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, mirazepine, mepyramine, triprolidine and tiotidine were purchased from Tocris (Ellisville, MO).

### *Measurement of Cell-Electrode Impedance*

The detailed experimental procedures have been described previously<sup>1,2</sup>. 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 the 37°C CO<sub>2</sub> incubator). Impedance was continuously recorded and converted to a Cell Index (CI). The cells were allowed to attach and spread, typically for 6-8 h, 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 5-10 min prior to agonist application. Unless otherwise indicated, results were expressed as a normalized CI, which is derived from the ratio of

CI before and after addition of the compounds.

For concentration-dependent studies, maximal response (usually 1-2 h after the ligand application) to a given concentration of the compound was used to plot a concentration-dependent curve. EC<sub>50</sub> and IC<sub>50</sub> were calculated by Prism (San Diego, CA).

### *Fluorescence Microscopy*

H1 cells and V1a cells were seeded in 16-well Labtec chamber slides and allowed to attach and spread for 24 h. The cells were stimulated with 100 nM histamine for 5 min and washed 3 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 3 times with PBS, then photographed with an epifluorescence microscope.

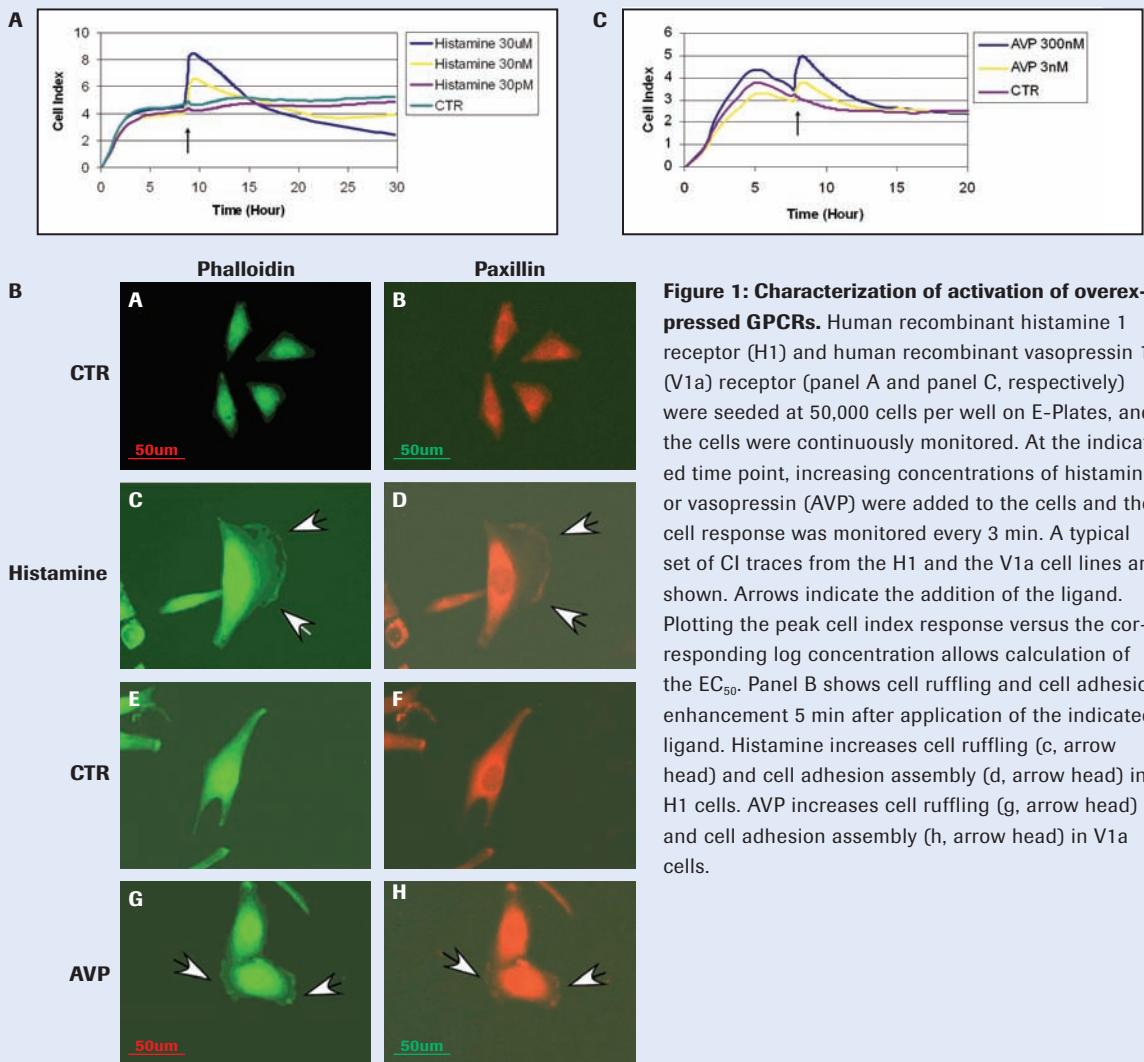
## Results and Discussion

### Dynamic Monitoring of GPCR Activation in Living Cells

To determine if 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 min after histamine addition, while for V1a cells it was at 90 min (Figure 1A and C).

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. 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 leads to an immediate (5 min) induction of membrane ruffles and translocation of paxillin to the site of membrane ruffles, which is 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 can detect the functional activation of GPCRs based on its ability to modulate the actin cytoskeleton and cell adhesion.

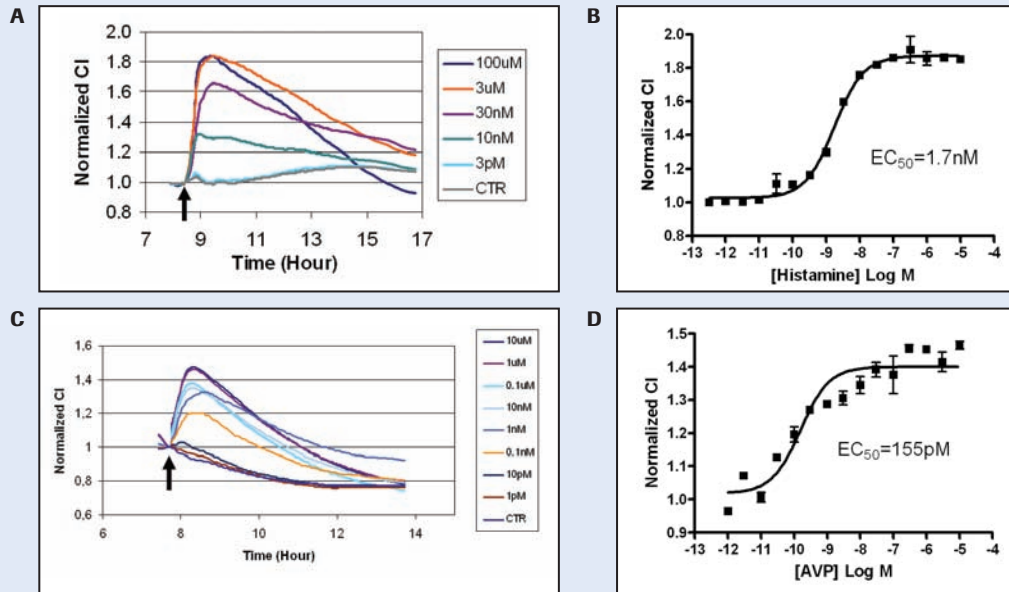


**Figure 1: Characterization of activation of overexpressed GPCRs.** Human recombinant histamine 1 receptor (H1) and human recombinant vasopressin 1a (V1a) receptor (panel A and panel C, respectively) were seeded at 50,000 cells per well on E-Plates, and the cells were continuously monitored. At the indicated time point, increasing concentrations of histamine or vasopressin (AVP) were added to the cells and the cell response was monitored every 3 min. 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 shows cell ruffling and cell adhesion enhancement 5 min 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.

*Dynamic and Quantitative Monitoring of Recombinant GPCRs Coupled to Different Signaling Pathways*

To further extend the findings discussed above, 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 causes a transient and dose-dependent increase in CI (Figure 2C) with an  $EC_{50}$  of 155 pM (Figure 2D).

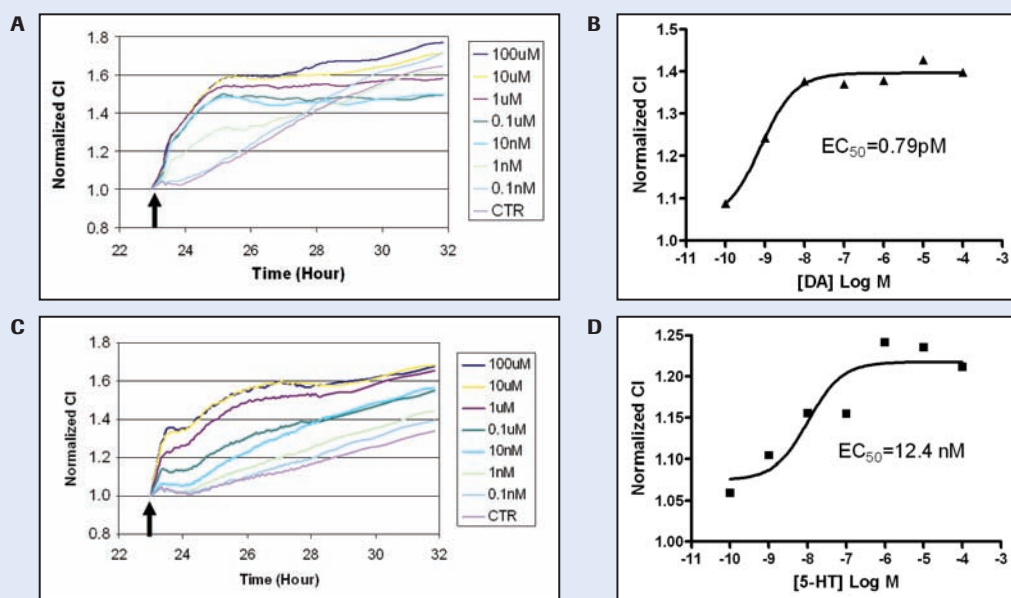


**Figure 2: Pharmacological study of H1 and V1a cell lines coupled to Gq.** Generation of  $EC_{50}$ : H1 and V1a cell lines were seeded at 50,000 cells per well on E-Plates, and the cells were continuously monitored. At the indicated time point (arrows), increasing concentrations of histamine (A) or AVP (C) were added to the cells and the cell response was monitored every 3 min. A typical set of CI traces are shown. Plotting the peak cell index responses versus the corresponding log concentrations allows calculation of the  $EC_{50}$  of histamine (B) and AVP (D).

To determine if this new system could also be used to monitor functional activation of GPCRs that are coupled to other signaling pathways such as Gs or Gi, 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 and stimulated with increasing concentrations of their respective ligands. As shown in Figure 3A and 3C, both cell types are 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 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 can detect the functional activation of GPCRs coupled to different signaling pathways. Monitoring each of these different signaling pathways traditionally requires different detection technologies and instrumentation, *e.g.* 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 3 min. CI traces are shown. Plotting the peak cell index 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, *e.g.* calcium measurements, is that the cells have to be engineered to express promiscuous G proteins that are coupled to *e.g.* the calcium pathway. Alternatively, receptors may need to be overexpressed in order 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. Therefore, it should be determined 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.

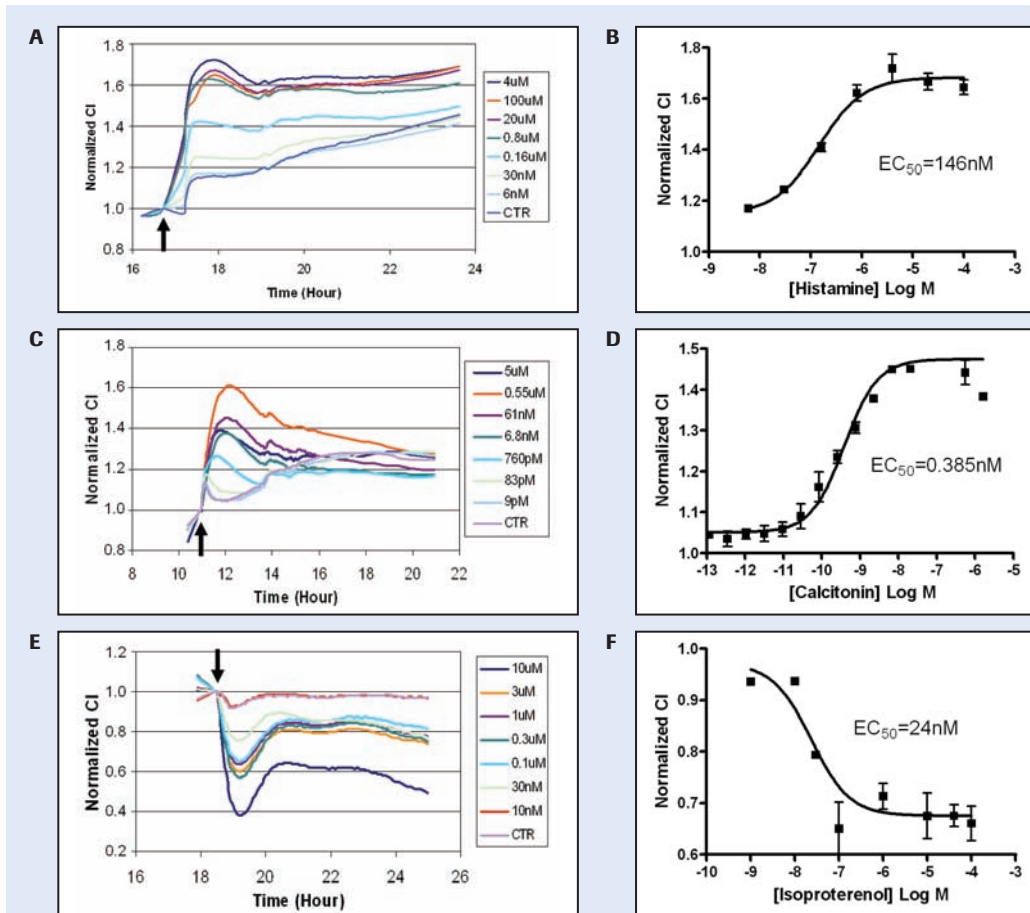
The kinetics and amplitude of histamine-mediated response in HeLa cells are significantly different

from those of H1 cells. Primarily, this can 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 can contribute to the amplitude and duration of the response.

The calcitonin receptor is coupled to the Gs pathway. To determine if endogenous activation of calcitonin receptor 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 leads to a robust and transient increase in CI;

plotting normalized CI versus log concentration of the ligand gives a dose-response curve with an  $EC_{50}$  of 0.385 nM (Figure 4D). In contrast,  $\beta_1$  adrenergic receptor stimulation in C6 cells by isoproterenol reveals an opposite effect on CI. As shown in Figure 4E, isoproterenol generated a dose-dependent decrease of CI with an  $EC_{50}$  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.



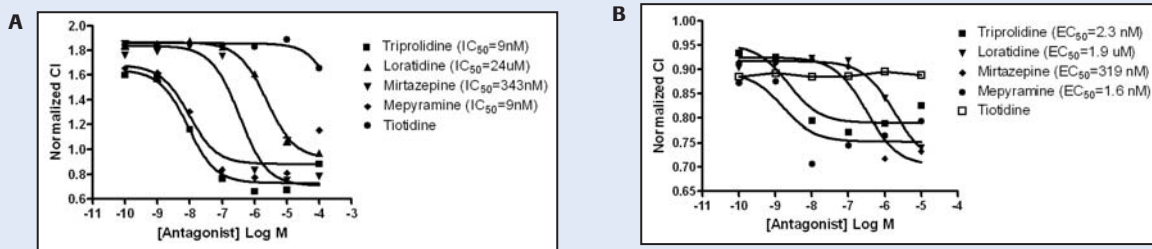
**Figure 4: Pharmacological study of endogenous GPCRs with various ligands.** Generation of  $EC_{50}$ : HeLa cells (A) CHO cells (C) and C6 cells (E) were seeded at 50,000 cells per well on E-Plates, and the cells were continuously monitored. At the indicated time point (arrows), increasing concentrations of histamine (A) calcitonin (C) or isoproterenol (E) were added to the cells and the cell response was monitored every 3 min. Plotting the peak cell index responses versus the corresponding log concentrations allows calculation of the  $EC_{50}$ s of histamine (B) calcitonin (D) and isoproterenol (F), respectively.

### Quantitative Analysis and Ranking of Histamine Receptor Antagonists and Inverse Agonists

In order to validate the GPCR assay using impedance technology, a panel of well-characterized and selective histamine antagonists was chosen. 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  $\mu$ M, 343 nM and 9 nM respectively. In contrast, H2 selective antagonist tiotidine did not inhibit histamine-induced cell response at the concentrations tested. These results strongly indicate that the impedance-based system can be used to rank the potency of antagonists in a receptor-sub-family-specific manner.

Interestingly, the H1 receptor antagonists alone gave rise to dose-dependent decreases of CI levels (Figure 5B). This observation can be explained by the fact that all these H1 receptor antagonists have a certain amount of activity as inverse agonists<sup>3</sup>, which will affect the basal activities of the H1 receptor. Furthermore, 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 the observed responses are specific.

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 prior to 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 perturbs the basal signaling activity can potentially be detected with this new technology.



**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 min prior to the addition of a fixed concentration of histamine (20 nM), and the cell response was monitored every 3 min. Four chosen H1 antagonists (triproli-dine, loratidine, mirtazepine and mepyramine) blocked histamine-induced CI increases in a concentration-dependant manner; these antagonists had different potencies, as reflected by their calculated  $IC_{50}$ s. H2 selective antagonist tiotidine did not block histamine-induced CI responses at the highest concentration (10  $\mu$ M) tested (A). Four chosen H1 antagonists (triproli-dine, loratidine, mirtazepine and mepyramine) caused concentration-dependent decreases of CI; these 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  $\mu$ M) tested (B).



This Application Note has shown that an impedance-based system can be used for functional screening 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 which distinguish the new system described here from other cell-based functional assays for GPCRs are:

1. No pre- or post-labeling of the cells is necessary, saving both expense and time.
2. The assay is non-invasive and therefore cellular destruction is not required. Thus, 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. Therefore, the assay 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. Furthermore, the real-time kinetic readout provides succinct and 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, which are integral components of cell viability, it can detect any compound that may be potentially cytotoxic or may have other adverse effects.

Since the impedance-based system provides a different way to monitor GPCR functions in living cells, it could provide another vantage point for increasing our understanding of GPCR functions.

## References

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**The original impedance-based assay system (RT-CES®), which was used to perform the experiments described in this application note, is the predecessor of the new xCELLigence System jointly developed by Roche Applied Science and ACEA Biosciences. While retaining the advantages of impedance-based technology described in this publication, the xCELLigence System will have improved functionality over the original system.**

**Notes:**

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The xCELLigence System is intended for life science research only. It is not intended for use in diagnostic procedures.

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