

Accelerated Articles

Real-Time Monitoring of Morphological Changes in Living Cells by Electronic Cell Sensor Arrays: An Approach To Study G Protein-Coupled Receptors

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G protein-coupled receptors (GPCRs) constitute important targets for drug discovery against a wide range of ailments including cancer, inflammatory, and cardiovascular diseases. Efforts are underway to screen selective modulators of GPCRs and also to deorphanize GPCRs with unidentified natural ligands. Most GPCR-based cellular screens depend on labeling or recombinant expression of receptor or reporter proteins, which may not capture the true physiology or pharmacology of the GPCRs. In this paper, we describe a noninvasive and label-free assay for GPCRs that can be used with both engineered and nonengineered cell lines. The assay is based on using cell–electrode impedance to measure minute changes in cellular morphology as a result of ligand-dependent GPCR activation. We have used this technology to assay the functional activation of GPCRs coupled to different signaling pathways and have compared it to standard assays. We have used pharmacological modulators of GPCR signaling pathways to demonstrate the specificity of impedance-based measurements. Our data indicate that cell–electrode impedance measurements offer a convenient, sensitive, and quantitative method for assessing GPCR function. Moreover, the noninvasive nature of the readout offers the added advantage of performing multiple treatments in the same well to study events such as desensitization and receptor cross-talk.

G-protein-coupled receptors (GPCRs) constitute the largest family of plasma membrane proteins that are involved in signal transduction across the plasma membrane.^{1–3} These receptors play a prominent role in regulating a variety of physiological functions such as sight, taste, and smell, as well as fluid electrolyte balancing, cardiovascular function, and neurotransmission.² The vast 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 $G\beta/\gamma$ complex, both of which can activate downstream effectors depending on the receptors being activated.² More recently, it has also become apparent that GPCR signaling either through or in addition to heterotrimeric G proteins also activate the Rho family of small GTPases.^{3,4}

The Rho family of small GTPases, which include Rho, Rac, and CDC42, are well-characterized effectors of oncogenes, growth factor and adhesion-mediated signaling pathways^{5,6} and are not classically thought of as being key effectors for GPCRs. Rho family GTPases participate in a number of cellular processes, the main one being regulation and maintenance of specific structures within the actin cytoskeleton framework.⁶ GPCRs have been shown to modulate the actin cytoskeleton and hence cell morphology in a very specific manner depending on the Rho family GTPase being activated.^{6,7} The current view of the actin cytoskeleton is that of

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a dynamic and plastic system that is a reflection and manifestation of the intracellular signaling and not simply a static structure designed to maintain cellular architecture.⁸ Since GPCRs couple to the actin cytoskeletal network and induce very defined morphological changes, it is possible to harness this information as a functional and biologically relevant readout for GPCRs.

Therefore, we have designed electronic cell sensor arrays embedded in the bottom of the well of microtiter plates that are capable of measuring minute changes in cell morphology.^{9–11} The electronic sensors measure changes in cell–substrate impedance as a result of the disruption of the ionic environment due to the presence of cell and cell morphology dynamics. The main advantages offered by using cell–substrate impedance and cell morphology as a readout are that both exogenously expressed and endogenous receptors can be assayed without the need for engineering the cell with promiscuous G proteins and reporters or labeling the cells with dyes. In addition, since the readout is noninvasive, multiple stimulations with the same ligand or different ligands can be performed to assess events such as desensitization and receptor cross-talk. Finally, another major aspect of using cell–substrate impedance and cell morphology as a readout is that potentially all GPCRs, regardless of the signaling pathways, can be functionally monitored.

In this paper, we explore the utility of using cell–substrate impedance as a quantitative and functional readout for GPCRs. We tested both exogenously expressed and endogenous receptors coupled to different signaling pathways and performed side-by-side comparison with standard assays such as IP₃ and cAMP measurements. The cells were continuously monitored using a real-time cell electronic sensing (RT-CES) system that we have developed. Using molecular, cellular, and biochemical assays, we demonstrate that the impedance readout correlates with specific changes in cell morphology and activation of signaling pathways and interfering with these pathways using pharmacological inhibitors abolishes the impedance readout. Furthermore, we have used cell–substrate impedance-based measurement of cell morphology to assay and characterize inverse agonists for the histamine H1 receptor and to assess its desensitization.

MATERIALS AND METHODS

Cell Culture. All the cells used in this study were purchased from ATCC unless indicated otherwise. Cells are cultured in a standard humidified incubator at 37 °C with 5% CO₂/ 95% air. HeLa and N1E-115 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 IU/mL penicillin and 100 µg/mL streptomycin. CHO cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 IU/mL penicillin and 100 µg/mL streptomycin. Cell lines stably expressing human histamine receptor 1 (H1), human dopamine receptor 1 (D1), and human vasopressin 1a receptor (V1a) in CHO-K1 cells were obtained from Euroscreen and maintained in Ham's/F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100

IU/mL penicillin, 100 µg/mL streptomycin, and 400 µg/mL G418. Cell lines stably expressing human 5-hydroxytryptamine receptor 1A (5-HT1A) and 5-hydroxytryptamine receptor 2B (5-HT2B) in CHO-K1 cells were obtained from Euroscreen and maintained in Ultra-CHO medium supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 400 µg/mL G418.

Reagents. All the reagents were purchased from Sigma unless indicated otherwise. Loratidine, mirtazepine, mepyramine, triprolidine, tiotidine, clemastine, dimethidene, doxepin, ketotifen, SFK 38393, and 8-OH-DPAT were purchased from Tocris.

RT-CES Experimental Setup. The detailed experimental procedures have been described previously.¹¹ Briefly, 95 µL of media was added to wells in 16× E-plates to obtain background readings followed by the addition of 100 µL of cell suspension containing the indicated number of cells. The E-plates containing the cells were allowed to incubate at room temperature for 10 min before being placed on the device station in the incubator for continuous recording of impedance as reflected by cell index (CI, see detailed definition below). The cells were allowed to attach and spread typically for 6–8 h to reach a stable baseline before the addition of agonists. Typically, 5 µL of 40× stock solution of agonist was 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. The results were expressed by normalized CI, unless indicated otherwise, which are derived from the ratio of CIs before and after the addition of the compounds. For concentration-dependent study, maximal response of a given concentration of the compound was used to plot the concentration-dependent curve and EC₅₀ or IC₅₀ were calculated by Prism.

Impedance and Cell Index Measurements by RT-CES System. The electrodes in the E-plates are made out of thin gold film using lithographical methods. Gold is deposited on glass slides and is then patterned to form electrode arrays. The “circle on line” design used in this technology was chosen because it allows for maximal sensitivity for the detection of the cells with relatively uniform distribution of the electric field while maximizing the coverage area of each well; the electrodes cover ~75% of the total area of the well. Impedance measured between electrodes in an individual well depends on electrode geometry, ionic concentration in the well, and whether there are cells attached to the electrodes. In the absence of the cells, electrode impedance is mainly determined by the ion environment both at the electrode/solution interface and in the bulk solution. In the presence of the cells, cells attached to the electrode sensor surfaces will alter the local ionic environment at the electrode/solution interface, leading to an increase in the impedance. The more cells there are on the electrodes, the larger the increase in cell–electrode impedance. Furthermore, the impedance change also depends on cell morphology and the extent to which cells attach to the electrodes.

To quantify cell status based on the measured cell–electrode impedance, a parameter termed cell index is derived, according to

$$CI = \max_{i=1, \dots, N} \left(\frac{R_{\text{cell}}(f_i)}{R_b(f_i)} - 1 \right)$$

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where $R_b(f)$ and $R_{cell}(f)$ are the frequency-dependent electrode resistances (a component of impedance) without cells or with cell present, respectively. N is the number of the frequency points at which the impedance is measured. Thus, CI is a quantitative measure of the status of the cells in an electrode-containing well. Under the same physiological conditions, more cells attached onto the electrodes leads to a larger $R_{cell}(f)$ value, leading to a larger value for cell index. Furthermore, for the same number of cells present in the well, a change in the cell status such as morphology will lead to a change in the cell index. For example, an increase in cell adhesion or cell spreading leads to larger cell–electrode contact area, which will lead to an increase in $R_{cell}(f)$ and thus a larger value for cell index. A “normalized cell index” at a given time point is calculated by dividing the cell index at the time point by the cell index at a reference time point. Thus, the normalized cell index is 1 at the reference time point.

Fluorescence Microscopy. H1/CHO and V1a/1321-N1 cells were seeded in 16-well Lab-tec chamber slides and allowed to attach and spread for 24 h at 37 °C. The cells were stimulated with 100 nM histamine or μ M vasopressin for 5 min, followed by fixation in 4% paraformaldehyde for 10 min and permeabilization in PBS containing 0.2% Triton X 100 for 10 min. After washing, the fixed cells were blocked in PBS containing 0.5% BSA for 30 min. The cells were stained with phalloidin conjugated with FTIC (10 μ g/mL final concentration in blocking buffer) and a monoclonal anti-paxillin ascites fluid (1:500 final dilution in blocking buffer) for 1 h. The cells were washed 3 times with PBS, and the cells stained with anti-paxillin ascites fluid were further stained with goat-anti-mouse IgG conjugated to TRITC (Sigma, 20 μ g/mL final dilution) for 1 h. All incubations were carried out at room temperature. The slides were mounted, visualized, and imaged using a Nikon E400 epifluorescence microscope and Nikon ACT software.

Immunoprecipitation and Western Blotting Analysis for Phosphorylated Paxillin. H1/CHO cells were stimulated with vehicle or 20 nM histamine for 5 min. The cells were lysed with RIPA buffer containing phosphatase and protease inhibitors, and the lysate was centrifuged for 10 min at 14 000 rpm in a Beckman microcentrifuge. The soluble lysate was incubated with 2.5 μ g of anti-paxillin monoclonal antibody and immunoprecipitated with the addition of protein A–Sepharose. The immunoprecipitates were washed, eluted in 2 $\times$ SDS sample buffer, and fractionated on 4–20% SDS–PAGE gel (Invitrogen). The gel was transferred to a nitrocellulose membrane and immunoblotted with either PY-20 HRP (Santa Cruz Biotech) or anti-paxillin antibody. The blot was developed using SuperSignal West Dura Extended Duration Substrate (Pierce), and images were captured by a CCD camera (UVP BioImaging System).

IP₃ and cAMP Assays. To quantify the functional activities of H1/CHO cells, a modification of the described method¹² based on the measure of the depletion of [³H]-phosphatidylinositol was used. Briefly, 2.8×10^6 cells were loaded overnight with [2-³H-(N)]myo-inositol (10 nCi/plate), washed 3 times, and detached with PBS–EDTA. A total of 2.5×10^5 cells were resuspended in 400 μ L of LiCl buffer (121.5 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 24.93 mM Tris-HCl, 15 mM glucose, and

10 mM LiCl, pH 7.4) and incubated with 100 μ L of histamine at different concentrations for 30 min. The reaction was stopped by adding 500 μ L of methanol/HCl (88 mL of ethanol/12 mL 1 M HCl) and quick frozen at –20 °C. The cells were filtered on GF/B filters preincubated in 0.1% PEI, and [2-³H(N)]myo-inositol was counted in a TriCarb counter. Results shown are expressed as a decrease of membrane-bound [³H]PI content (in counts/min).

The effects of increasing concentration of SKF 38393 on D1/CHO-K1 cells were tested by seeding cells the day before the experiment (2.5×10^4 /well in a 96-well plate). The cells were incubated in KRH + IBMX buffer for 15 min at 37 °C. After this preincubation, increasing amounts of SKF 38393 were added to the wells and incubated for 20 min at 37 °C. The reaction was stopped by removal of the supernatant, and 100 μ L of lysis buffer was added on each well. The plate was incubated for 30 min at 37 °C and finally spun down for 3 min at 1200 rpm. The supernatant were recovered, and cAMP concentrations were determined using a Tropic kit (Applied Biosystems), according to manufacturer's specifications.

To assess the functional activation of 5-HT_{1A} receptor, 5-HT_{1A}/CHO cells were grown for 20 h in media without antibiotics and detached by PBS–EDTA. After centrifugation, cells were suspended at 6.25×10^5 cells/mL in KRH-IBMX (5 mM KCl, 1.25 mM MgCl₂, 124 mM NaCl, 25 mM HEPES pH 7.4, 13.3 mM glucose, 1.25 mM KH₂PO₄, 1.45 mM CaCl₂, and 0.5 g/L BSA supplemented with 1 mM IBMX). The 96-well plates (Costar) are then successively filled with KRH-IBMX, cells (7.5×10^3 cells/well), increasing concentrations of agonist (diluted in KRH-IBMX), and forskolin (10 μ M). The plate was then incubated for 30 min at room temperature. After addition of the lysis buffer, cAMP concentrations are estimated by an HTRF kit from Cis-Bio International.

RESULTS

Principle of Cell Electrode Impedance and Measurement of Cell Morphological Dynamics in Response to GPCR Activation. We have designed interdigitated microelectrodes that are embedded in the bottom of the wells of microtiter plates (E-plates) that are used in conjunction with an RT-CES system.^{10,11} As illustrated in Figure 1, in the absence of cells, the impedance value (Z) is derived from the electrode/solution interface (Z_0). The presence of cells on the electrode will affect the local ionic environment as well as the electrode/solution interface leading to an increase in electrode impedance. The impedance difference between the presence and absence of cells in the well reflects the pure impedance due to the presence of the cells (Z_{cell}). The addition of the agonist for different GPCRs will affect cell morphology in a very specific manner depending on the pathways being activated, and further change in impedance is a reflection of the morphological dynamics that are induced by the ligand ($Z_{activated\ cell}$). Because the readout can be sampled in as little as 50 ms/well, the rate of change, magnitude, and duration of the response can be precisely measured.

To demonstrate the utility of impedance measurement of cell morphology as a functional readout for GPCR activation, CHO-K1 cells expressing the human H1 histamine receptor (H1/CHO) and 1321-N1 cells expressing the human vasopressin receptor (V1a/1321-N1) were seeded on the E-plates and stimulated with histamine and vasopressin, respectively (Figure 2). Both histamine

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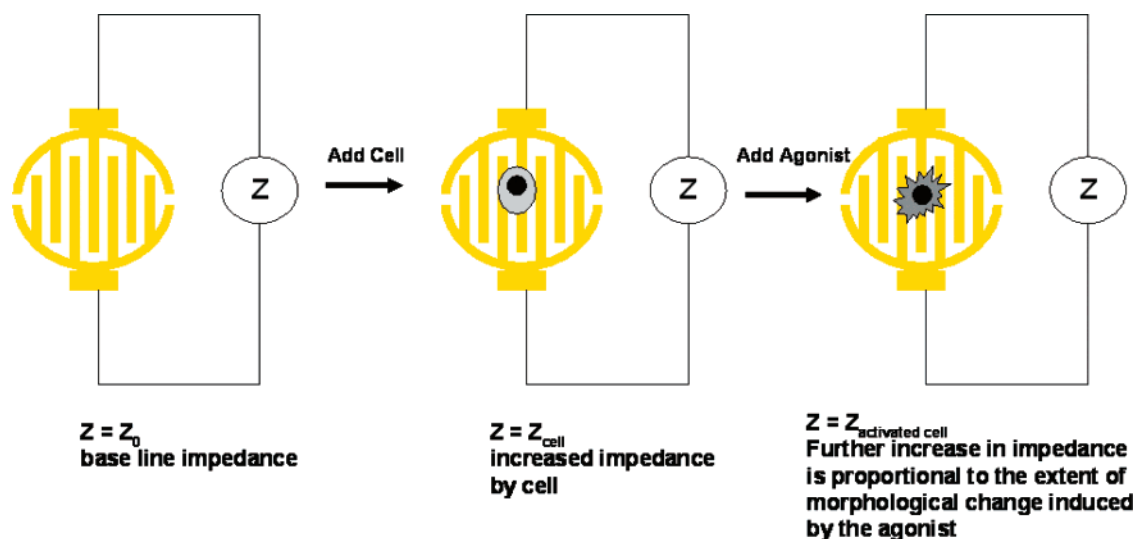


Figure 1. Schematic representation of microelectrodes and the principle of utilizing cell–electrode impedance to noninvasively measure GPCR-mediated modulation of cell morphology. In the absence of cells, the baseline impedance (Z_0) value is determined by the software. Addition of cells to the sensor microelectrodes leads to changes in impedance signal (Z_{cell}) that is directly proportional to the number of cells seeded on the sensors. Agonist-mediated stimulation of GPCRs expressed on the cell surface leads to transient changes in cell morphology that further increase or decrease the impedance value ($Z_{\text{activated cell}}$) depending on the activated signaling pathways.

and vasopressin induced an immediate (within 5 min) and transient increase in impedance, which is represented as normalized CI. The maximal response for H1/CHO cells was at 40 min after histamine addition (Figure 2A), while it was at 36 min after vasopressin addition for V1a/1321-N1 cells (Figure 2C). Both H1 receptor and V1a receptor act through Gq and can modulate the actin cytoskeleton and its regulatory proteins such as focal adhesion kinase and paxillin.^{13–15} To determine whether histamine- and vasopressin-induced increases in normalized CI correlate with modulation of the actin cytoskeleton and its associated proteins, H1/CHO cells and V1a/1321-N1 cells were stimulated with histamine and vasopressin, respectively, fixed, and stained with FITC–phalloidin and anti-paxillin mAb. Histamine treatment of H1/CHO cells led 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 2B, lower right panel). Similarly, vasopressin also induced formation of membrane ruffles and translocation of paxillin to these sites (Figure 2D, lower right panel). Furthermore, as shown previously, histamine-mediated stimulation of H1 receptor leads to tyrosine phosphorylation of paxillin, a major regulator of actin cytoskeleton (Figure 2E). In summary, impedance-based measurements of GPCR-mediated actin cytoskeleton dynamics and morphological changes can serve as a useful readout for ligand-mediated GPCR activation.

Specificity of Ligand-Mediated Changes of Impedance as a Functional Readout for GPCR Activation. To establish the specificity of impedance measurements represented as normalized CI as a functional readout for GPCR activation, we undertook a multifaceted approach including utilization of specific antagonist,

pathway blockers, and pathway modulators. Starting at the level of the H1 histamine receptor in H1/CHO cells, a number of selective H1 receptor antagonists were screened and ranked according to their relative potencies. As shown in Figure 3A, all H1 receptor antagonists blocked histamine-induced CI increases in H1/CHO cells in a concentration-dependent manner. Plotting the peak responses against concentrations of antagonists allows for the calculation of IC_{50} s (see caption of Figure 3. for IC_{50} s). No significant effect of tiotidine, a selective H2 receptor antagonist, was found at the highest concentration used (100 μ M).

It has been well established that activation of Gq leads to stimulation of phospholipase C and consequently protein kinase C (PKC) pathways. Stimulation of Gs activates adenylate cyclase and hence increases cAMP while Gi is negatively coupled to cAMP.² Therefore, we attempted to test the specificity at the pathway level. First, we demonstrated that blocking the PKC pathway by bisindolylmaleimide, a specific inhibitor of PKC, completely abolished histamine-induced CI increases (Figure 3B) in H1/CHO cells, indicating the involvement of the PKC pathway. Second, dopamine receptor 1, overexpressed in CHO-K1 cells (D1-CHO, coupled to Gs) was stimulated with its natural ligand dopamine in addition to forskolin. As shown in Figure 3C, dopamine and forskolin, an adenylate cyclase activator that bypasses the receptor and artificially activates adenylate cyclase, lead to CI increases with similar kinetics and comparable amplitudes, indicating that cAMP production may result in increases in CI. Finally, to demonstrate the specificity of serotonin (5-hydroxytryptamine, 5-HT) on serotonin receptor (5-HT1A receptor) overexpressed in CHO-K1 cell (5-HT1A/CHO, coupled to Gi), the cells were pretreated with pertussis toxin, a potent and selective inhibitor of Gi¹⁶ before 5-HT was added. As shown in Figure 3D (left panel), pertussis toxin pretreatment selectively blocks serotonin-induced 5-HT1A activation but does not affect serotonin-induced stimulation of 5-HT2B cells, which is coupled

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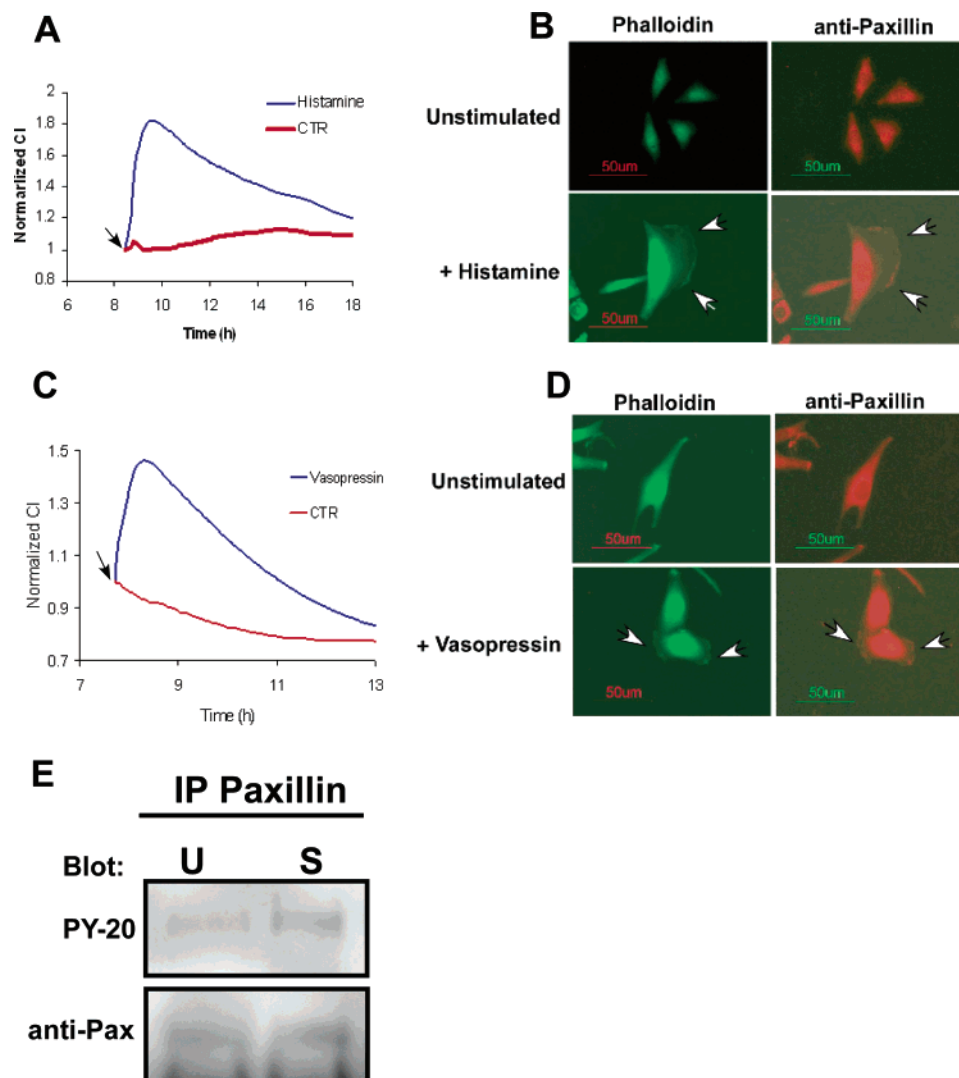


Figure 2. Correlation of agonist-induced increases of CI and cell morphology dynamics. (A) H1/CHO cells were seeded on cell sensor microelectrodes and stimulated with various concentrations of histamine (indicated by the arrow). Cell sensor impedance as displayed by normalized CI was continuously monitored every 3 min for the duration of the experiment. (B) H1/CHO cells were seeded on 16-well Lab-tec chamber slides and stimulated with vehicle or 100 nM histamine for 5 min. After stimulation, the cells were fixed and processed as described in Materials and Methods. The arrows indicate the site of membrane ruffles and paxillin translocation as a result of histamine treatment. The scale bar is in micrometers. (C) V1a/1321-N1 cells were seeded on cell sensor microelectrodes and stimulated with vehicle or 1 μ M vasopressin. Cellular response was continuously monitored every 3 min as described above. (D) V1a/1321-N1 cells were seeded on 16-well Lab-tec chamber slides and stimulated with vehicle or 1 μ M vasopressin for 5 min. After stimulation, the cells were fixed and processed as described in section of methods. The arrows indicate the site of membrane ruffles and paxillin translocation as a result of vasopressin treatment. The scale is in micrometers. (E) H1/CHO cells were stimulated with 100 nM histamine for 15 min. After the stimulation, the cells were processed as described in Materials and Methods. Upper panel shows the phosphorylated paxillin detected by PY-20 antibody in unstimulated (U) and stimulated (S) cells. Lower panel shows the total paxillin after immunoprecipitation.

to Gq and is pertussis toxin insensitive (Figure 3D, right panel). Taken together, these results strongly support the notion that cell–electrode impedance measurement as displayed by CI is dependent on the stimulation of specific signal transduction pathways mediated by a specific receptor/ligand pair of GPCRs.

Monitoring Activation Kinetics of Exogenous and Endogenous GPCRs in Live Cells: Comparison with Traditional Assays for EC₅₀ Values. To measure functional activation of GPCRs by cell–electrode impedance measurement, both recombinant GPCRs and endogenous GPCRs were tested with their respective agonists. For recombinant GPCRs, three representative categories of GPCRs were tested by the RT-CES system, namely, H1/CHO (Gq), D1/CHO (Gs) and 5-HT1A/CHO (Gi). The cells

were seeded on E-plates and treated with the indicated concentrations of either respective natural agonist (histamine, Figure 4A, upper panel) or receptor subfamily selective agonists (SKF 38393, D1 selective, Figure 4A, middle panel and 8-OH-DPAT, 5-HT1A selective, Figure 4A, lower panel). For each treatment, the cellular responses from four agonist concentrations are shown. Ligand application led to an immediate and sharp increase (H1/CHO and D1/CHO) in normalized CI, which reached maximal response between 30 min and 1 h depending on the receptors. Plotting the maximal response against each concentration allows for the calculation of EC₅₀ for each agonist as shown in Figure 4B. To further validate these findings, we performed functional GPCR assays using standard methods including a [³H]-phosphatidyl-

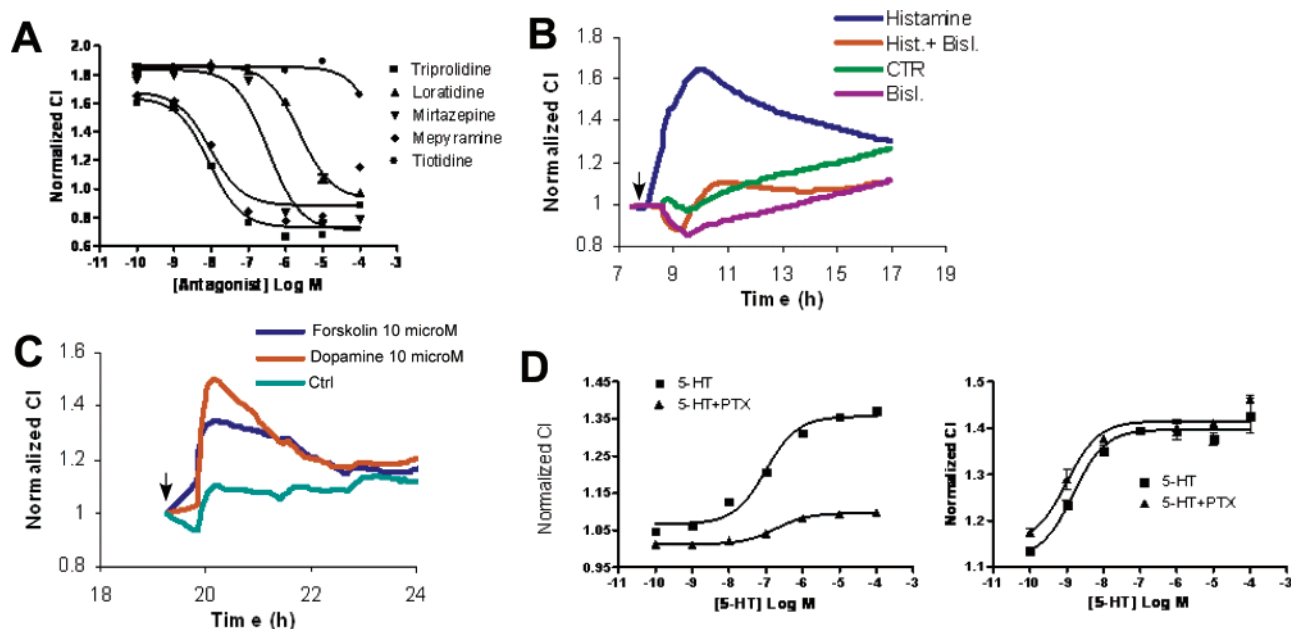


Figure 3. Specificity characterization of GPCR-mediated CI increases in three CHO cell lines stably expressing three representative GPCRs. (A) Selective H1 receptor antagonists but not H2 receptor antagonist (tiotidine) blocked histamine-induced CI increases in a concentration-dependent manner. IC_{50} s (in nM) for triprolidine, loratidine, mirtazapine, and mepyramine were 9, 24 000, 343, and 9, respectively. IC_{50} s (in nM) for brompheniramine, clemastine, dimethidene, doxepin, and ketotifen were 16, 5, 394, 8, and 2, respectively (curves not shown). IC_{50} for tiotidine was greater than 100 μ M (microM). (B) Complete block of histamine-induced CI increase in H1/CHO by PKC inhibitor bisindolylmaleimide. (C) Comparison of magnitude and duration of CI increases induced by dopamine and adenylylate cyclase activator forskolin in D1/CHO cells (coupled to Gs). (D) Pertussis toxin pretreatment abolished 5-HT-induced CI increases only in 5-HT1A/CHO cells (coupled to Gi; left panel) but not in 5-HT2B/CHO cells (coupled to Gq; right panel).

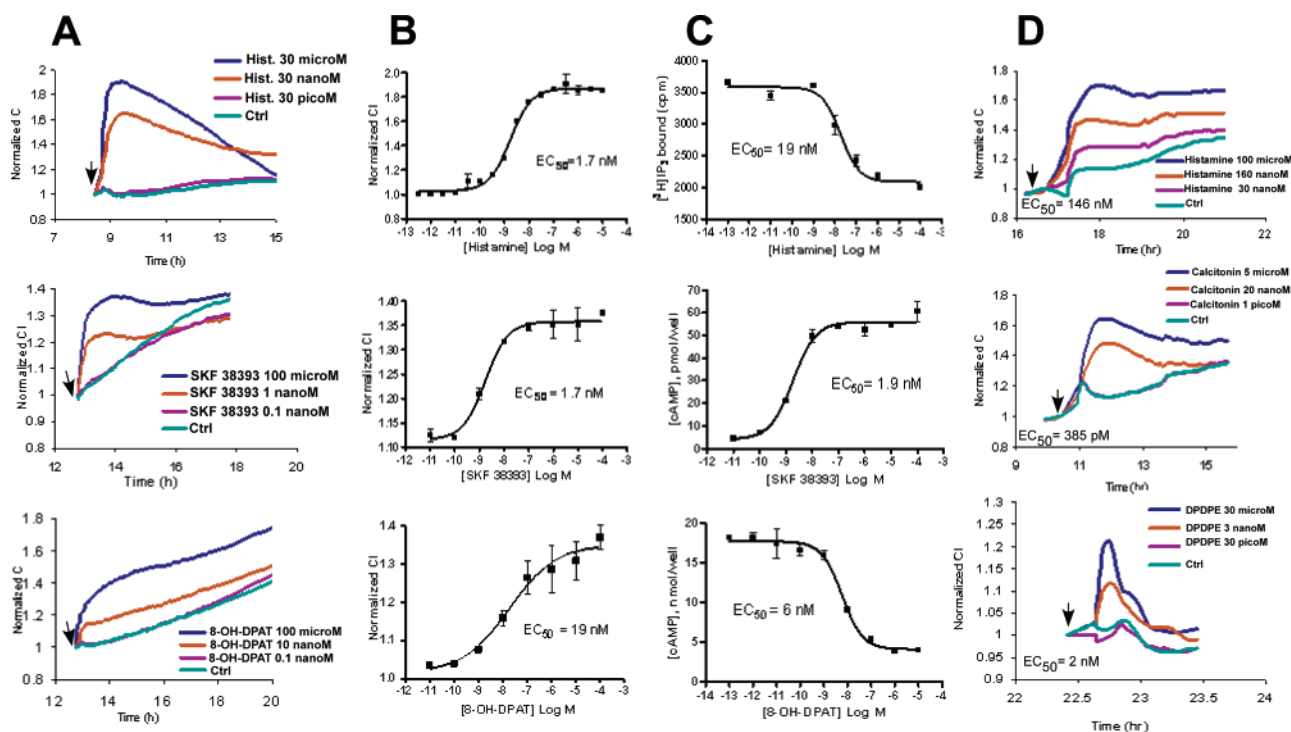


Figure 4. Comparison of RT-CES system with standard GPCR functional assays to obtain EC_{50} s of agonists in exogenous GPCRs and application for endogenous GPCRs. (A) Representative traces of CI of H1/CHO (upper panel), D1/CHO (middle panel), and 5-HT1A/CHO (lower panel) cells were stimulated by their ligands histamine, SKF 38393 and 8-OH-DPAT (arrows), respectively. The cells were continuously monitored. (B) Plotting the peaks of normalized CI responses versus corresponding agonist concentrations allows for the generation of concentration-dependent curves and calculation of EC_{50} s as shown in each panel. (C) Concentration-dependent curves and EC_{50} s generated from standard GPCR assays. Histamine on H1/CHO cells (upper panel), SKF 38393 on D1/CHO cells (middle panel), and 8-OH-DPAT on 5-HT1A/CHO cells (lower panel). (D) Measurement of activation of three representative endogenous GPCRs by RT-CES system. Response profiles and EC_{50} s for each agonist/cell pair are shown for histamine/HeLa cells (upper panel), calcitonin/CHO cells (middle panel), and DPDPE/N1E-115 cells (lower panel), which express endogenous H1 histamine receptor, calcitonin receptors, and opioid δ receptors, respectively.

nositol depletion assay for H1/CHO (Figure 4C, upper panel), cAMP measurement for D1/CHO (Figure 4C, middle panel) and

inhibition of forskolin-induced cAMP for 5-HT1A/CHO (Figure 4C, lower panel). EC_{50} values obtained by cell–electrode imped-

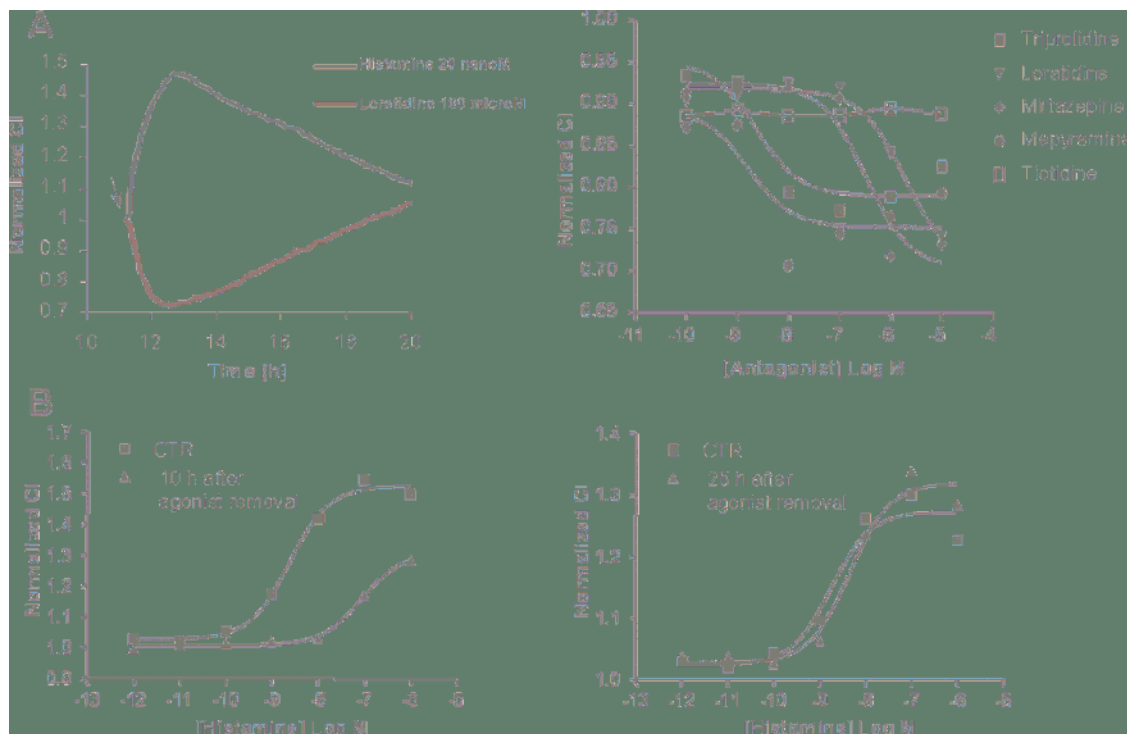


Figure 5. Utilization of RT-CES system to characterize inverse agonists and study desensitization of GPCRs. (A) Selective H1 antagonist loratidine (left panel) or other H1 receptor antagonists (right panel) alone were incubated with H1/CHO cells. Plotting the lowest values of normalized CI responses versus corresponding agonist concentrations allows for the generation of concentration-dependent curves. Tiotidine, a selective H2 antagonist did not affect CI. (B) Dynamics of H1 receptor desensitization. Ten hours after the initial activation by histamine, the medium of H1 cells was removed and the cells were washed twice and replaced with fresh medium. Ten (left panel) and 25 h (right panel) after removal of media, various concentrations of histamine were reintroduced to the cells and responses of impedance were monitored continuously. Concentration-dependent curves were generated, and EC_{50} s were calculated by plotting the maximal responses versus the concentrations of histamine.

ance measurements (Figure 4B) are comparable to the EC_{50} values obtained by the traditional assays (Figure 4C) indicating that the assays are equally sensitive.

One of the challenges encountered by current label-based assays such as calcium measurements is that the cells have to be engineered to express promiscuous G proteins coupled to the calcium pathway.¹⁷ Alternatively, receptors may need to be overexpressed in order to obtain sufficient measurable signal.¹⁸ All these artificial manipulations may ease the burden of screening large sets of compounds; however, physiological and pharmacological relevance of the findings will need to be supplemented with additional experiments. The number of assays available to measure the functional activation of endogenous GPCRs in their natural and physiologically relevant settings is limited. To determine the utility of impedance-based measurements in monitoring activation of endogenous receptors, three different cell lines expressing endogenous levels of histamine receptor, calcitonin receptor, and δ opioid receptor were selected. We tested histamine on HeLa cells (Gq), calcitonin on CHO cells (Gs), and DPDPE (selective δ opioid receptor agonist, Gi) on N1E-115 mouse neuroblastoma cells. Similarly, all the ligands generated transient increases of CI (Figure 4D). Peak CIs of each concentration of the agonist were chosen to plot the concentration-dependent curves and to extrapolate the EC_{50} s for histamine (Figure 4D,

upper panel) calcitonin (Figure 4D, middle panel), and DPDPE (Figure 4D, lower panel). In summary, these results indicate that cell–electrode impedance measurements can be used to functionally assess both endogenous and exogenous GPCRs dynamically without the need for labeling or engineering the cells to express reporter proteins. Furthermore, receptors coupled to the three common pathways, Gq, Gs, and Gi, can be assayed by the same technology platform.

Application of the RT-CES System To Characterize Inverse Agonists and To Study Desensitization of H1/CHO. Interestingly, the majority of the H1 receptor antagonists have been shown to stabilize the inactive state and serve as inverse agonists of the H1 histamine receptor^{19–21} by decreasing the basal levels of IP_3 and calcium.²² Incubation of H1/CHO with H1 receptor “antagonist”, loratidine alone (Figure 5A, left panel) or other H1 receptor antagonists (Figure 5A, right panel) lead to a dose-dependent decrease in CI. Incubation of the same compounds with CHO-K1 cells had no effect (data not shown). These results strongly support the notion that cell morphology is a very sensitive indicator, and manifestation of intracellular signaling and impedance measurements of cell morphology can be potentially used to discover and characterize inverse agonists of different GPCRs.

Last, we took advantage of the noninvasive nature of impedance readout to assess the desensitization of H1 histamine receptor. Ten hours after the initial histamine stimulation, the media were removed and the cells were washed. Various concentrations of histamine were reintroduced to the cells 10 and 25 h after the

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removal of the media containing histamine, respectively. As shown in Figure 5B, 10 h (left panel), after the removal of histamine, the magnitudes to histamine restimulation were 57% of the control with a 37-fold (89 vs 2.4 nM) increase of EC₅₀ and 25 h (right panel) afterward with comparable magnitudes and 2.5-fold (4.5 vs 1.8 nM) increases of EC₅₀. These results clearly demonstrated that RT-CES system is a simple and effective means to study receptor desensitization.

In summary, we have shown that impedance measurements can be used for pharmacological characterization of GPCR. According to our data, impedance readout is as sensitive as some of the standard functional assays for GPCR activation, such as IP₃ and cAMP measurements. The major advantages of impedance as a functional readout for GPCR activation are that the cells do not need to be labeled or engineered with promiscuous G proteins and reporters. Therefore, cells expressing both recombinant and endogenous receptors can be assayed. Furthermore, potentially all classes of GPCRs, regardless of the pathways they are coupled to, can be assayed on the same technology platform. In addition, since the readout is noninvasive, multiple stimulations with ligands, agonists, or antagonists can be conducted in the same well over time to assess receptor cross-talk or desensitization.

DISCUSSION

In addition to the canonical signaling pathways mediated by various G proteins, most GPCRs also have the capacity to modulate the actin cytoskeleton through the Rho family GTPases.^{3,8} The manner in which the actin cytoskeleton and hence cell morphology is modulated by GPCRs largely depends on the Rho family member(s) being activated. Each member of the Rho family GTPase affects the actin cytoskeleton in a very specific manner.⁷ For example, activation of Rho induces stress fiber formation and cellular contraction and rounding; Rac is mainly responsible for membrane ruffles and lamellapodia formation, which are extensions of the plasma membrane; CDC42, another member of the Rho family GTPases, induces the formation of filipodia, which are also extensions of the plasma membrane but distinct from lamellapodia.^{5,6} In this paper, we have described a noninvasive and label-free method based on measurement of cell-electrode impedance. The RT-CES system has the capacity to quantitatively measure minute changes in cell morphology. Due to the kinetic aspects of the measurements, agonist stimulation of GPCRs leads to receptor-specific profiles that depend on the signaling pathways being activated.

To establish the specificity of using cell morphology changes in conjunction with impedance measurements as functional readout for GPCRs, we screened several well-established antagonists of the H1 histamine receptor. According to our data, the histamine H1 receptor antagonists were able to dose-dependently and selectively block histamine-mediated stimulation of H1/CHO cells, whereas selective H2 (tiotidine) receptor antagonist did not have any effect, demonstrating the specificity of the response (Figure 3A). In addition, we were able to demonstrate that

selective PKC inhibitor, bisindolylmaleimide, was able to completely block the H1/CHO cell response to histamine stimulation, indicating that the PKC pathway plays an important role in H1 receptor signaling pathways leading to morphological changes (Figure 3B). This finding is corroborated by earlier findings that PKC plays an important role in histamine H1 receptor-mediated responses such as cytokine secretion and activation of transcription.^{23,24} The specificity of the D1/CHO receptor activation, which is coupled to Gs activation of adenylate cyclase and production of cAMP, was demonstrated by using forskolin to artificially activate adenylate cyclase and cAMP production (Figure 3C). The response profiles of D1/CHO to forskolin were very similar to those by dopamine in terms of amplitude and duration (Figure 3C), indicating that the cAMP pathway may be responsible for D1 receptor-induced cellular response on the electronic sensors. Finally, the specificity 5-HT1A/CHO (coupled to Gi) upon stimulation with 5-HT was demonstrated by using pertussis toxin (PTX), a specific inhibitor of Gi. PTX completely blocked 5-HT stimulation of 5-HT1A/CHO cell impedance response (Figure 5C, left panel) while 5-HT-induced responses in 5-HT2B/CHO (coupled to Gq) were not affected by PTX treatment (Figure 5C, right panel).

We have taken several approaches to demonstrate the utility and versatility of impedance measurements as a functional readout for GPCRs. First, we have demonstrated that different recombinantly expressed GPCRs coupled to the Gq, Gs, and Gi pathways can be monitored in an agonist-dependent manner in live cells using the RT-CES system (Figure 4). For histamine-mediated stimulation of H1/CHO cells, we have shown that the impedance signal generated corresponds to concurrent changes in cell morphology in the form of membrane ruffles, being indicative of Rac activation. Furthermore, histamine stimulation of H1/CHO cells is also accompanied by concomitant phosphorylation of paxillin, an actin-binding protein that is known to be involved in modulation of the actin cytoskeleton.²⁵ The versatility of the impedance measurements as a readout for GPCRs is demonstrated by the fact that receptors linked to three different pathways can be functionally monitored on the same system. Typically, several different technologies including those capable of measuring calcium or IP₃ and cAMP would be required to functionally assess all these receptors. Furthermore, our data indicate that measurement of cell sensor impedance upon agonist addition is as sensitive as traditional assays such as IP₃ and cAMP measurements (Figure 4), which are reflected by similar EC₅₀ values.

The other major benefit of impedance measurements with respect to GPCRs is their capability to monitor activation of endogenous GPCRs in live cells without the need for labels or reporters (Figure 4D). This is a much needed development in the GPCR assay field because it allows for the monitoring of GPCRs in their natural and relevant cell lines or even primary cells. Physiological relevance of current assays that employ labels, reporters, and overexpression of GPCRs needs to be interpreted with much caution because it may not necessarily reflect the actual signaling scheme of the receptors in their natural states.¹⁸ Overexpression of receptors can disrupt the stoichiometry of the

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receptor with respect to downstream signaling and can lead to aberrant measured responses. Furthermore, some receptors may be difficult to overexpress in cells in order to assess their function. Moreover, another pitfall with overexpression is the realization that certain receptors in their natural and physiological setting form heterooligomers with other receptors with very distinct pharmacological and regulatory properties.^{26,27} Therefore, while overexpression in a heterologous cell line may be convenient for the generic identification of agonists and antagonists, the signaling pathways employed in a heterologous system such as CHO cells may vary significantly when compared to a primary cell such as a neuron.

Interestingly, while screening H1 receptor antagonist, we observed that the antagonists alone dose-dependently decreased the baseline impedance values in H1/CHO cells (Figure 5A) but not in parental CHO cells (data not shown). This observation indicated to us the possibility that H1 antagonists may act as inverse agonists of the H1 receptor rather than a pure antagonist. Indeed, it has been shown in the literature that most H1 antagonists do function as inverse agonists through the H1 receptor serving to downmodulate basal signaling such as IP₃ production.^{19,21} This is an important observation and further

supports the premise that cellular morphology is a very sensitive and precise indicator of intracellular signaling events, and any perturbation in intracellular signaling, however subtle, is reflected by changes in cellular morphology.

We have demonstrated that stimulation of GPCRs leads to certain morphological changes, which can be sensitively and precisely measured by cell-electrode impedance measurement. The major advantages offered by impedance measurement over traditional GPCR functional assays is that it can be used in cells expressing both exogenous or more importantly endogenous receptors. The preclusion of labels, reporters, and promiscuous G proteins allows for a more physiologic assessment of GPCR functions. Furthermore, the change in cell cytoskeleton or cell morphology that is measured by cell sensor impedance is an inherent cellular response that reflects the status of intracellular signaling. The kinetic element of the assay system allows for receptor-specific response profiles that can potentially be used to characterize orphan GPCRs. In summary, impedance readout allows another vantage point to assess GPCR functions and in combination with standard technologies and other emerging technologies such as BRET and FRET will undoubtedly add to our understanding of GPCR biology and drug discovery.

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