

Cellular Dielectric Spectroscopy: A Powerful New Approach to Label-Free Cellular Analysis

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The past decade has seen a number of significant changes in identifying higher quality lead compounds earlier in the drug discovery process. Cell-based assay technologies yielding high-content information have emerged to achieve this goal. Although most of these systems are based on fluorescence detection, this article describes the development and application of an innovative cellular assay technology based on radio frequency spectrometry and bioimpedance measurements. Using this technique, the authors have discovered a link between cellular bioimpedance changes and receptor-mediated signal transduction events. By performing dielectric spectroscopy of cells across a spectrum of frequencies (1 KHz to 110 MHz), a series of receptor-specific, frequency-dependent impedance patterns is collected. These raw data patterns are used to determine the identity of the cellular receptor-signaling pathway being tested and to quantify stimulation endpoints and kinetics. The authors describe the application of this technology to the analysis of ligand-induced cellular responses mediated by the 3 major classes of G-protein-coupled receptors (GPCRs) and protein tyrosine kinase receptors. This single assay platform can be used with ease to monitor G_s , G_i , and G_q GPCRs without the need for chimeric or promiscuous G-proteins, fluorophors, or tagged proteins. In contrast to other methods of monitoring cellular signal transduction, this approach provides high information content in a simplified, noninvasive, and biologically relevant fashion. (*Journal of Biomolecular Screening* 2004:467-480)

Key words: cell-based assays, cellular dielectric spectroscopy, drug discovery, ligand-receptor interaction, bioimpedance

INTRODUCTION

THE PROCESS OF IDENTIFYING AND BRINGING a new drug to market is a complex and costly process taking many years. Traditionally, the early stages of drug discovery used in vitro-type assays that provided limited information on the ability of a potential drug compound to illicit an effect on the target of interest. Many of these potential leads fail when tested either in vivo in cell-based assays or further in the process at the preclinical/clinical trials, resulting in high attrition rates. Therefore, there is now strong demand to identify more druggable leads and to eliminate compounds with cytotoxic or other unwanted side effects earlier in the process.

In striving to achieve this goal, the past decade has seen a significant shift in the traditional drug discovery paradigm. This shift has been toward novel high-throughput screening (HTS) technologies. These technologies provide in vitro assays with dramatic increases in the number of compounds screened for any given target. Despite

these advances, substantial increases in the number of drugs coming to market have not been realized.

More recently, acceptance of innovative cell-based technologies that yield high information content has occurred. These platforms provide functional, kinetic cell-based information on the cellular consequences of receptor-ligand interaction. The data generated include information on signal transduction pathways, drug mechanisms of action, efficacy, selectivity, and cytotoxicity. The drug discovery community is now incorporating the routine use of these technologies in its drug development processes. This complex information is enabling end users to make better informed decisions on which leads to pursue early in the process.

Most of the existing technologies in this area are fluorescence-based imaging systems that require the use of fluorescent labels to monitor physiological pathways. Analysis focuses on evaluating discrete intracellular events with the overall integrated cellular response to drug activation incompletely captured. Although providing truly valuable high information content, the fluorescent labeling process and the need for artificial enhancements (e.g., transfection and overexpression) or the use of reporter gene systems could contribute in an adverse way to elucidating the real cellular physiology of the target of interest.

With this in mind, we focused our efforts on developing a label-free cell-based assay technology that would be noninvasive and

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yield information regarding the total integrated response of the cell to external stimuli. We chose to focus our initial work in the area of ligand-receptor interaction analysis, predominantly working in the areas of G-protein-coupled receptor (GPCR) and protein tyrosine kinase (PTK) receptor activation.

Cellular dielectric spectroscopy, or CDS, is based on an established technology with a long history of use in measuring material properties of cells.¹⁻¹⁰ The technology employs the application of alternating voltages at set frequencies to cellular components (membranes) and intact cells and monitors the resulting currents generated from the dipoles within the sample. At each frequency, the difference in magnitude of the reflected signal, as compared to the incident wave, is measured. In cell biology, this technology is better known as bioimpedance and has been successfully applied to monitoring cell membrane conductivity,¹¹⁻¹⁵ monolayer permeability,¹⁶⁻¹⁹ morphology,²⁰⁻²² migration,^{23,24} and cellular micro-motion.²⁵ Our goal was to extend the use of bioimpedance measurements in an embodiment of this technology to the label-free, noninvasive monitoring of the cellular consequences of ligand-receptor interaction. Most of the work in this field has centered on the use of frequencies in the radio-wave range from 10 Hz to 100 MHz.⁷⁻⁹ The principal thrust of this prior work has been to elucidate the basic dielectric properties of intact cells and cell membranes in an attempt to observe cellular behavior in a noninvasive fashion.⁷⁻⁹ In addition to these efforts, some researchers have attempted to monitor cellular ligand-receptor interactions using bioimpedance.²⁶⁻²⁸ This ligand interaction work has focused on the use of a single frequency and has shown that activation of a GPCR can be observed with this technique.²⁷ However, the results were of limited scope, with no consideration of how receptor specificity, if any, could be manifest in the measurements.

In our extended studies using bioimpedance measurements taken simultaneously across a large range of frequencies (from 1 KHz to 110 MHz) before, during, and after receptor activation, we observed that unique response patterns for different receptor classes were revealed. Thus, by working with multiple frequencies and multiple time points, these measurements provide high content information about the cellular consequences of receptor activation. With this approach, we have identified a methodology of monitoring the complex integrated response of cells to ligand-receptor interaction in real time and in a label-free fashion. In this article, we discuss this application of CDS to the investigation of the cellular consequences of receptor activation across members of the GPCR and PTK receptor classes. In doing so, we demonstrate the implementation of the technology across a variety of cell lines expressing both endogenous and transfected receptors. The technology may be applied to both adherent and gravity-settled nonadherent cell lines. Furthermore, we have developed this technique around a 96-well format, thereby giving us the advantage of enhanced throughput as compared to previous bioimpedance techniques.

The key value of the technology is the fact that the information contained within these bioimpedance measurements represents

complex cellular physiology. This article describes this complex information and how the data can be analyzed by different approaches depending on the information desired by the end user. A subset of the data may be used to apply the technology to applications such as hit identification and pharmacological profiling, including rank ordering of compound potency. Of greater significance is the ability to analyze the full complement of the complex data that enables classification of the receptor-ligand interaction response relative to the signaling pathway used by the activated receptor. This has the potential to provide the end user with information regarding the physiological consequences of receptor-ligand interaction. Furthermore, due to the label-free nature of this technology, assay development time is minimal, and the technique can be applied to any receptor system, both characterized and orphan. This article describes the broad applicability of the CDS technology as we have applied it to our research efforts and discusses potential future applications of this technique to the drug discovery and development industry.

MATERIALS AND METHODS

Cell lines

A549 (CCL-185), CHO_{kl} (CCL-61), CHO_{ml} (M1WT3; CRL-1985), COS-7 (CRL-1651), HEK293 (CRL-1573), HeLa (CCL-2), and U937 (CRL-1593.2) cells lines were obtained from ATCC (Manassas, VA). HeLa and HEK293 cells were grown in MEM, COS-7 cells in DMEM, and U937 cells in RPMI-1640. A549, CHO_{kl}, and CHO_{ml} cells were grown in Ham's F12K. All media were supplemented with 10% dialyzed fetal bovine serum (DFBS), 50 µg/mL streptomycin, and 50 U/mL penicillin. Stably transfected muscarinic receptor (m₁) expressing Chinese hamster ovary (CHO) cells (CHO_{m1}) were selected for growth in media containing 100 µg/mL geneticin. All other receptors stimulated in this study were endogenously expressed.

Reagents

Endothelin-1 (ET-1), isoproterenol, stromal cell-derived factor 1 alpha (SDF-1), epidermal growth factor (EGF), insulin-like growth factor (IGF), 5-hydroxytryptamine (5HT), calcitonin, forskolin, uridine triphosphate (UTP), prostaglandin E₂ (PGE₂), bradykinin, UK14304, carbachol, atropine, pertussis toxin (PT), PD142893, ipratropium Br, trihexyphenidyl, telenzepine, clozapine, p-fluoro-hexahydro-sila-difenidol (p-FHHSiD), probenecid, and geneticin were purchased from Sigma (St. Louis, MO). AG1478 was purchased from BIOMOL (Plymouth Meeting, PA). HANKS, HEPES, penicillin, streptomycin, and DFBS were from Invitrogen (Grand Island, NY). F12K, DMEM, MEM, and RPMI-1640 were purchased from Cellgro (Herndon, VA). Fluo-4 AM and Pluronic were from Molecular Probes (Eugene, OR). Whatman black-walled, clear-bottom 96-well plates were from VWR (West Chester, PA).

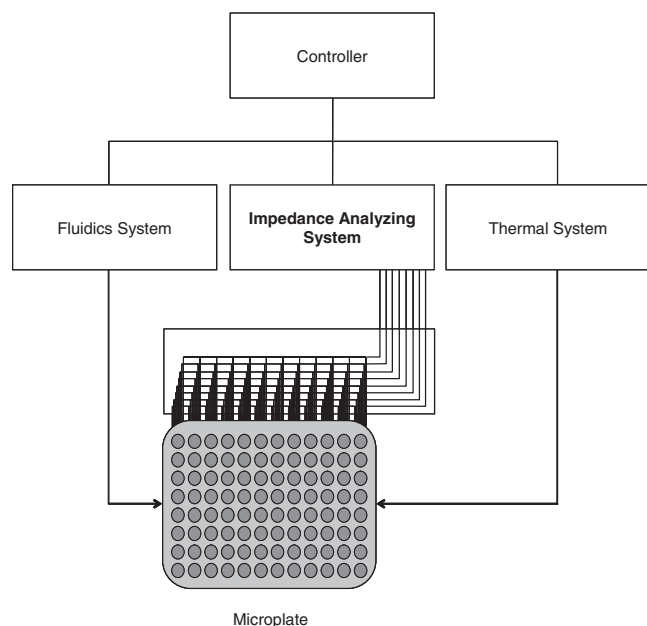


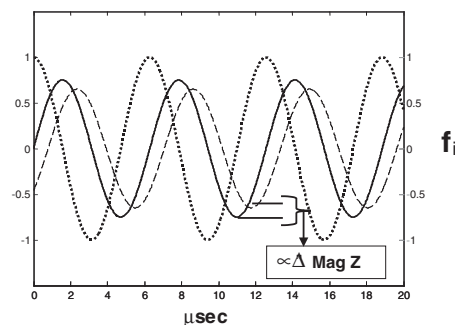
FIG. 1. Component diagram. Each well of a 96-well microplate contains interdigitated electrodes patterned onto the bottom of the well. These electrodes are connected to an impedance-analyzing device. The system contains a 96-well fluidics delivery device and a thermal system for temperature regulation. The entire system is controlled by a computer.

Instrument design

The principal components of our system are described in Figure 1. A 96-well microplate format was used. The microplates were custom fabricated using a bottomless well plate and a glass base plate containing 96 CDS electrodes. The electrodes make connections through the glass to a read head that is connected to Agilent (Palo Alto, CA) impedance analyzers. This read head can be thermally regulated so that experiments can be run between ambient and 37 °C. Onboard fluid additions and exchanges are handled by a 96-head delivery device with a range of 5 to 200 μ L. In addition to the cell plate location, the instrument has 2 additional stations for 96-well compound plates. All aspects of assay setup, execution, and data acquisition are controlled by computer software developed in-house.

General cell protocols

Adherent cells were plated into the wells in standard tissue culture media containing serum, and the plates were incubated overnight under standard incubator conditions (37 °C, 95% humidity, 5% CO₂). The cells adhered to and formed a monolayer on the electrodes during the course of the 18-h plating period and thus became part of an electric circuit. Typically, 4.0 to 4.5 $\times 10^4$ cells/well were used to form the monolayer, depending on the cell type. After the plating period, the plates were placed on the instrument, and the



Applied Voltage = ... f = Frequency (1 KHz to 110 MHz)
 Baseline Measured Current = ——— Z = Impedance ($|Z| = |V|/|I|$)
 Post Ligand Measured Current = - - - -

FIG. 2. Diagram of bioimpedance measurements. After an alternating voltage (V) (dotted line) of a set frequency (f_i) is applied to a monolayer of cells, the resultant current (I) is measured for a period of 5 min to generate a baseline (solid line). This is repeated for a series of 30 frequencies from 1 KHz to 110 MHz. At the end of the baseline period, ligand is added to the cells, and the change in the amplitude of the measured current (dashed line) is recorded. This change is proportional to the change in the magnitude of the impedance ($\propto \Delta \text{Mag } Z$), which is then calculated and displayed. This process is repeated over time to generate kinetic information about these changes. All 96 wells are scanned for all frequencies every 20 sec.

cell monolayers were washed 2 times with HANKS balanced salt solution containing 20 mM HEPES (HH). The cell monolayers were then allowed to equilibrate at room temperature (RT) for 15 min. At the end of this period, receptor activation experiments were initiated after the acquisition of 5 min of baseline measurements.

For experiments with nonadherent cells, the following protocol was used. Cells were harvested from stock tissue culture flasks and washed 3 times with HH. The cells were allowed to equilibrate at RT for 15 min in HH. Typically, 1.0 to 1.2 $\times 10^5$ cells were then added to each well in HH and allowed to settle for 30 min at RT. This resulted in a single layer of cells on top of the electrodes. Even though the cells were not adhered to the electrodes and were just resting on them, this interaction was sufficient for bioimpedance measurements in our system. After the cells settled, the plate was placed on the instrument, and receptor activation experiments were initiated after the acquisition of 5 min of baseline measurements.

Bioimpedance measurements

The principals underlying our bioimpedance measurements are diagramed in Figure 2. An alternating voltage of a set frequency was applied to a monolayer of cells, and the resultant current was measured. This was repeated for a series of 30 frequencies from 1 kHz to 110 MHz. The total time to scan all 96 wells for all frequencies was 20 sec. Readings were taken every 20 sec for a period of 5

min to establish a baseline. At the end of the baseline period, ligand was added to the cells, and the change in the magnitude of the measured impedance ($\Delta|Z|$) was recorded over time to collect kinetic information about these changes. Typically, 10 min of postligand data was acquired for any experiment.

Agonist and antagonist protocols

Typically, 135 μL of HH was left in the wells for equilibration. For receptor agonist studies, 15 μL of a 10 $\times$ concentration of agonist was added to this volume and mixed with an up-and-down motion 3 times. For nonadherent cells, the pipetting speed and pipette tip height were adjusted so as not to disturb the monolayer of settled cells. Readings were taken continuously during this procedure and for an additional 10 min after ligand addition. In the studies reported in this article, all experiments were conducted at 28 $^{\circ}\text{C}$.

For antagonist studies on adherent cells, after the wash step, the last 135 μL of HH was removed from the wells and replaced with HH containing a 1 $\times$ concentration of antagonist, and the wells were allowed to incubate at 28 $^{\circ}\text{C}$ for various times (see figure legends). At the end of the pretreatment period, 15 μL of a 10 $\times$ concentration of agonist was added to the wells, and the same procedure as described for agonist studies was carried out.

Calcium fluorescence assay

CHO_{mi} cells (7.5×10^5 cell/well) were plated into Whatman black-walled, clear-bottom 96-well microplates, and the cells were allowed to incubate overnight. The media were removed and replaced with 100 μL media containing 2.5 mM probenidol, 3 μM Fluo-4 AM, and 0.044% Pluronic. After 45 min at 37 $^{\circ}\text{C}$ to load the dye, the cells were washed 3 times with 135 μL media containing 2.5 mM probenidol alone. For antagonist studies, after the wash step, the last 135 μL of probenidol media was removed from the wells and replaced with probenidol media containing a 1 $\times$ concentration of antagonist, and the wells were allowed to incubate at 37 $^{\circ}\text{C}$ for 15 min. At the end of the pretreatment period, the plate was placed on an LJI Analyst (Molecular Devices, Sunnyvale, CA), and 15 μL of a 10 $\times$ concentration of agonist was added to the wells. Readings were taken every 10 sec in kinetic mode using 485-nm excitation and 530-nm emission filters. Measurements were normalized to background readings taken in control wells receiving no agonist. Sigmoidal-shaped inhibition curves were generated with increasing concentrations of antagonists. IC₅₀ values were calculated from the curves.

Data management and utilization

Using the predrug injection impedance measurement as a reference (at time t_{ref}), we calculated the differential change in the magnitude of the measured impedance due to the cellular reaction to the drug as a function of frequency and time:

$$\Delta|Z(f, t)| = |Z(f, t)| - |Z(f, t_{\text{ref}})|. \quad (1)$$

After transforming the frequencies to a domain of -1 to 1 using a logarithmic transformation,

$$x = a \log_{10}(f) + b, \quad (2)$$

such that

$$x(f_{\text{min}} = 1 \text{ kHz}) = -1; x(f_{\text{max}} = 110 \text{ MHz}) = +1,$$

we fitted the difference data with a polynomial approximation:

$$\Delta|Z(x, t)| = \sum_{n=0}^N c_n(t) P_n(x), \quad (3)$$

where the polynomials were the Legendre polynomials:

$$P_0(x) = 1,$$

$$P_1(x) = x,$$

$$P_2(x) = \frac{1}{2}(3x^2 - 1),$$

$$P_3(x) = \frac{1}{2}(5x^3 - 3x),$$

$$P_4(x) = \frac{1}{8}(35x^4 - 30x^2 + 3),$$

$$P_5(x) = \frac{1}{8}(63x^5 - 70x^3 + 15x),$$

$$P_6(x) = \frac{1}{16}(231x^6 - 315x^4 + 105x^2 - 5).$$

Therefore, the response at each time point could be characterized by 7 weighting coefficients, $c_n(t)$, or as a 7-element vector $\mathbf{c}(t)$. The length of the vector (the Euclidean norm) was used as a quantitative measure of response at any time, t :

$$\text{norm}(\mathbf{c})_t = \|\mathbf{c}(t)\|_2 = \sqrt{\sum_{n=0}^N c_n^2(t)}. \quad (4)$$

To quantify the overall cellular response to the drug addition, we used the maximum vector length (MVL) for all time t after drug injection ($\max_t \{\text{norm}(\mathbf{c})_t\}$), which can take place any time after the drug addition. For antagonist experiments, the relative MVL was determined by normalizing the MVLs to the MVL of the agonist positive control. The MVLs were normally distributed, and standard statistical analyses were performed on these values. For most receptors, the overall CVs of the MVLs are on the order of 10% to 15%.

RESULTS

Raw data

The initial graph displayed (Fig. 3) clearly illustrates the complexity of the information contained within the raw data obtained

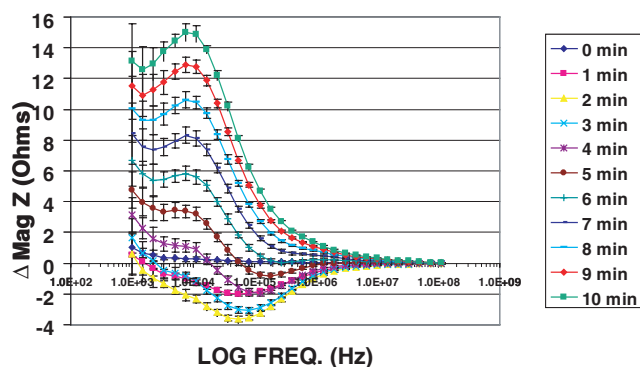


FIG. 3. Representative raw data generated with HeLa cells in the presence of endothelin-1 (ET-1). HeLa cells were stimulated with 400 nM ET-1, and the change in the magnitude of the impedance (as compared to baseline) for each frequency was recorded over time. The data are presented as the mean $\pm$ standard deviation from 3 wells.

using the CDS impedance technique. In this example, HeLa cells expressing the endothelin receptor were stimulated with ET-1. Following activation of the receptor, a change in the magnitude of the impedance is observed and graphed as a function of the log of the scanned frequencies. Represented within the graph is how the impedance changes across frequencies with time—in this instance, over a 10-min recording period. It is clear that there is both a time and frequency dependency to the data. During the first 2 min following ligand addition, $\Delta|Z|$ decreased below baseline, most notably in frequencies around 10^5 Hz. Beginning at 3 min, the response reversed direction and shifted to lower frequencies. By 4 min, the $\Delta|Z|$ began to increase above baseline at frequencies at and below 10^4 Hz. Finally, by 7 min, a new distinct peak arose at 10^4 Hz, and positive $\Delta|Z|$ values extended into the high frequencies, through 10^6 Hz. These data illustrate the complexity of the bioimpedance response following receptor stimulation, wherein the measured parameter decreases and increases around the baseline and shifts frequencies along the spectrum, all as a function of time after ligand-receptor interaction. Similar complex changes were observed after activation of multiple receptor members belonging to the GPCR and PTK receptor classes expressed in a range of cell lines (raw data not shown).

Data transformation and pattern generation

It is clear that to more easily interpret this complex information, it was necessary to mine the raw data. In an effort to gain the most information from the kinetic aspects of it, the frequency component was transformed as described in Materials and Methods. The data were then fitted by use of the Legendre polynomials. Seven coefficients were determined to be adequate to produce a good fit, the values of which change as the measured impedance changes over time. These values were then plotted as a function of time. Figures 4 through 7 represent raw data transformed in this manner for a range of GPCR and PTK receptors evaluated in this study.

Figure 4 depicts the results of these types of transformations for 2 different cell lines (HeLa and CHO) expressing a range of ligand-activated endogenous receptors. Figure 4A represents the transformation of the raw data previously illustrated in Figure 3. The coefficient values plotted over time produce a CDS pattern that represents the cellular response to stimulation of a particular receptor—in this case, the endothelin receptor (a G_q -coupled GPCR). Within the first minute after ligand addition, the C0 and the C1 coefficient values change rapidly and in opposite directions, whereas the C6 coefficient remains relatively unchanged. By 4 min postligand addition, the C0 and C1 coefficients have reversed direction and are headed back to baseline, again with little change in the C6 coefficient. When the patterns generated by stimulation of a receptor of a different class within the same cell line were analyzed, it was noted that a unique CDS pattern is obtained depending on the receptor class activated. Figure 4B represents the pattern obtained when the β_2 -adrenergic receptor (a G_s -coupled GPCR) is activated with its agonist, isoproterenol. When the latter pattern was compared to the data of Figure 4A, it was noted that the C0 and C1 coefficients have similar profiles for the initial 3 min of data collection following ligand addition. However, at 5 min, for the β_2 -adrenergic receptor, their values return to and remain near baseline. In contrast, in Figure 4A, the C0 and C1 coefficients continue to move above (C0) or below (C1) baseline for that same time period. A close evaluation of the pattern generated for the CXCR-4 receptor (a G_i -coupled GPCR) in Figure 4C also shows a unique pattern that is distinguishable from the previous data. In this example, the C0 and C1 coefficients slowly rise and fall respectively after ligand addition and never change direction. Although only the differences in the C0 and C1 coefficients are described here, similar types of comparisons can be made for all 7 coefficients with the same outcome. Thus, the coefficient values change in multiple ways over time and appear to correlate with the receptor signaling pathway used. It is important to note that the uniqueness of these patterns is not a result of the transformation process, as unique patterns are also observed in the raw data. The transformation process is merely a convenient way to represent the data such that the kinetic nature of the information is more easily discernable.

Although the patterns for the G_q -, G_s -, and G_i -coupled receptors discussed above were clearly unique and distinguishable from each other, we have also observed cases in which the generated patterns show similarities despite the fact that the receptors belong to different receptor classes and are known to signal through different intracellular signaling cascades. In Figure 4D, EGF was used to stimulate the EGF receptor expressed in HeLa cells, a protein tyrosine kinase receptor that does not involve G-proteins. In this case, the resulting pattern was similar to that produced by stimulation of the CXCR4 receptor, a G_i -coupled GPCR, with SDF-1, as shown in Figure 4C. This result suggests that the complex cellular end result that is measured by CDS is the same when these cells are stimulated with SDF-1 and with EGF.

We have evaluated these unique CDS patterns across multiple cell types. Figure 4E,F shows the patterns generated following ac-

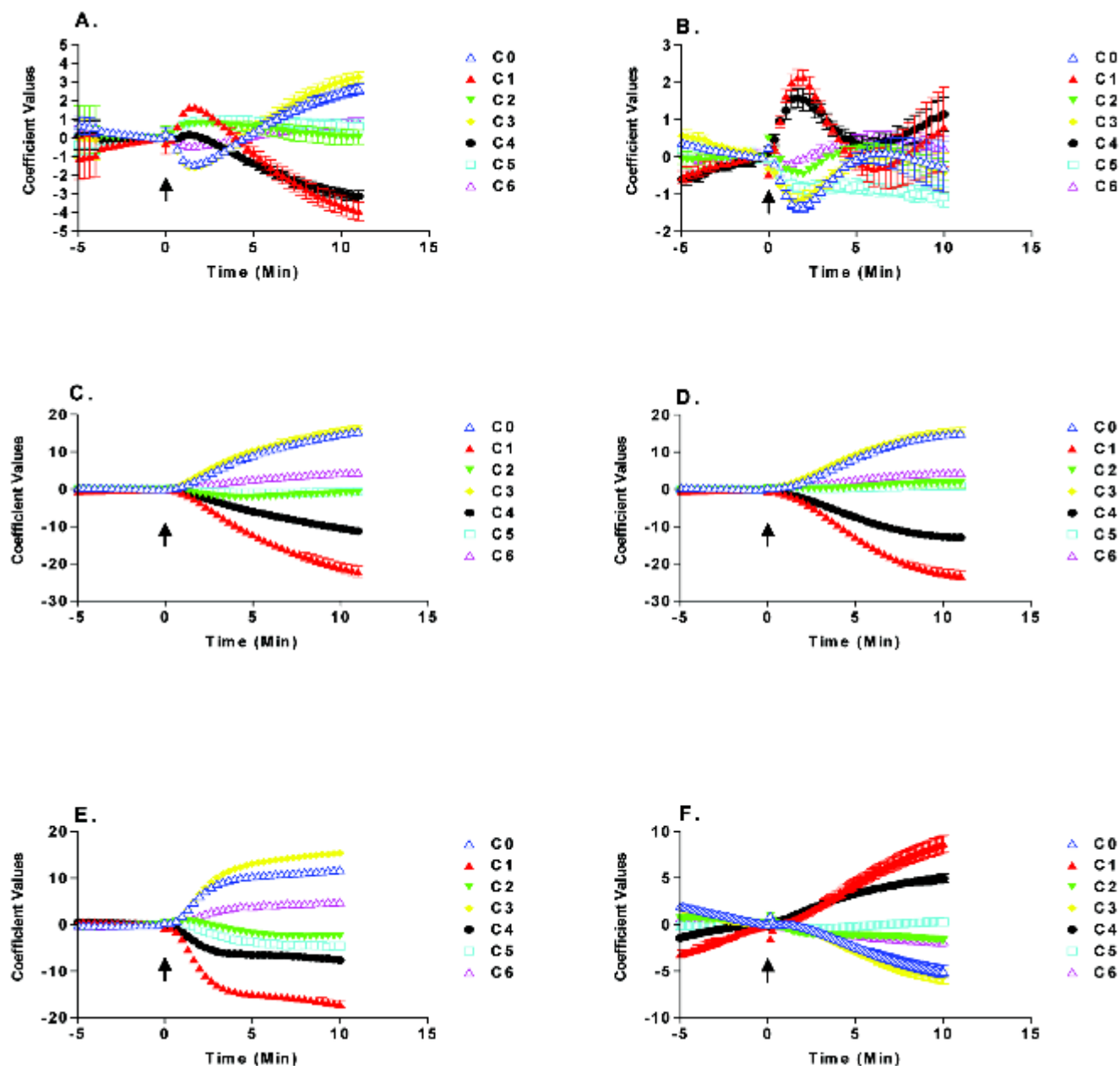


FIG. 4. Unique signaling response patterns generated by adherent HeLa and Chinese hamster ovary (CHO) cells after ligand activation of various G-protein-coupled receptors (GPCRs) and a protein tyrosine kinase (PTK) receptor. Legendre polynomial coefficients (C0-C6; see Materials and Methods) are plotted versus time for 5 min before, during, and for 10 min after addition of ligand (at zero time; arrow). HeLa cell patterns (A-D). CHO cell patterns (E, F). Response patterns generated for stimulation of (A) the endothelin receptor (G_i GPCR) with 400 nM endothelin-1 (ET-1), (B) the β_2 adrenergic receptor (G_s GPCR) with 10 μ M isoproterenol, (C) the CXCR-4 receptor (G_i GPCR) with 10 ng/mL stromal cell-derived factor 1 alpha (SDF-1), (D) the epidermal growth factor (EGF) receptor (PTK) with 10 ng/mL EGF, (E) the 5HT1b receptor (G_i GPCR) with 10 μ M 5-hydroxytryptamine (5HT), and (F) the calcitonin receptor (G_s GPCR) with 10 μ M calcitonin. Readings taken every 20 sec are shown, and the data represent the mean $\pm$ standard deviation for 5 wells for each ligand.

tivation of the 5HT1b and calcitonin receptors expressed in CHO cells. These receptors represent G_i - and G_s -coupled receptors, respectively. Although within CHO cells receptors belonging to a given receptor class were found to generate reproducible and con-

sistent patterns, the pattern for a given receptor class was not necessarily conserved across cell lines. This is not an unexpected result, given the unique complement of signaling cascades that exist within each cell type, and is discussed further below.

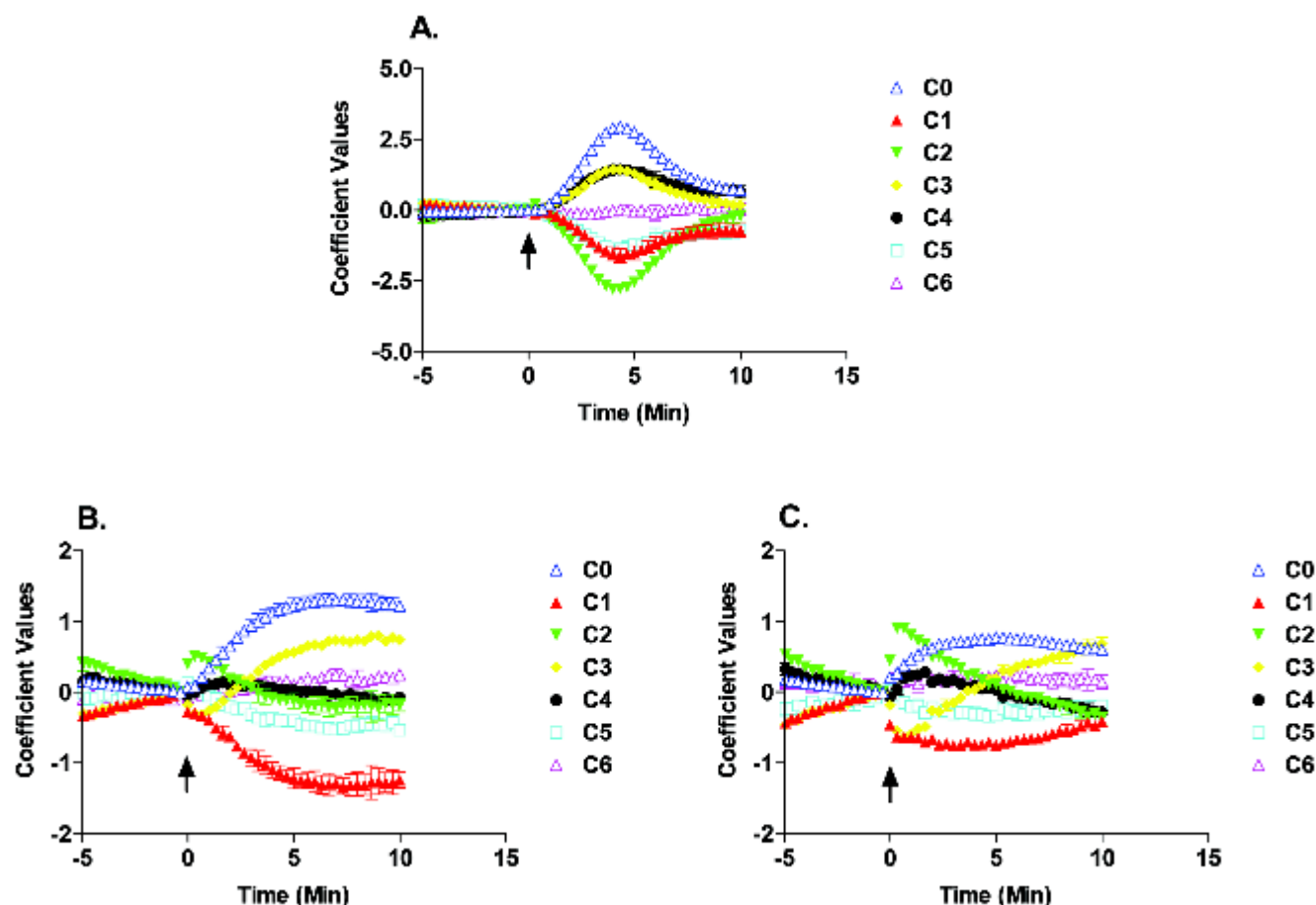


FIG. 5. Unique signaling response patterns generated by nonadherent U937 cells after ligand activation of G-protein-coupled receptors (GPCRs) and adenylyl cyclase activation with forskolin. Legendre polynomial coefficients (C0–C6) are plotted versus time for 5 min before, during, and for 10 min after addition of ligand (at zero time; arrow). Response patterns were generated for stimulation of (A) the P2Y receptor (G_q GPCR) with 10 μ M uridine triphosphate (UTP), (B) the EP4 receptor (G_s GPCR) with 10 μ M PGE2, and (C) adenylyl cyclase with 10 μ M forskolin. Readings taken every 20 sec are shown, and the data represent the mean $\pm$ standard deviation for 5 wells for each ligand.

The previous examples all involve receptors studied in adherent cell lines. However, these unique receptor-stimulated response patterns can also be generated using nonadherent cell lines such as U937, as shown in Figure 5. In addition, the use of mediators that mimic receptor activation events, such as the adenylyl cyclase activator forskolin, can also produce response patterns (Fig. 5C). PGE2 stimulation of the EP4 receptor (a G_s -coupled GPCR) (Fig. 5B) yields a pattern similar to that produced by forskolin, as expected (compare Fig. 5B,C).

Given these results, it is important to compare the patterns generated within and between different classes of receptors in a single cell line. In Fig. 6, additional representative examples of receptor-activated patterns from 2 classes of GPCRs in HeLa cells are shown. Fig. 6A,B shows the patterns generated from 2 different receptors of the G_q class of GPCRs. The patterns appear similar. Two different receptors of the G_i class of GPCRs are shown in Figure 6C,D. These patterns tend to be similar to one another and are distinctly different from those produced by receptors of another class

(compare Fig. 6A,B with Fig. 6C,D). If we now include in this comparison a G_s class pattern as shown in Figure 4B, an interesting trend is observed. For the major GPCR classes tested (G_q , G_i , and G_s), there appear to be distinct patterns. This result is expected within the realm of cellular physiology, as it is known that cells respond differently to activation of each of these signaling pathways. These data suggest that within a single cell line, different receptors of the same class tend to produce the same kind of pattern after activation with ligand, whereas receptors of different classes produce different patterns. However, as demonstrated in Figure 4, activation of a PTK receptor (the EGF receptor) produced a pattern that was similar to that produced by a G_i -coupled GPCR (Fig. 4C,D). These data suggest that CDS is monitoring appropriate cellular physiological changes, as it is known that PTK and GPCR signaling pathways overlap and that similar cellular physiological outputs will manifest from stimulation of members of each of these families.^{29,33} Therefore, the overall conclusion from all of the data is modified to include consideration of the receptor family. Thus,

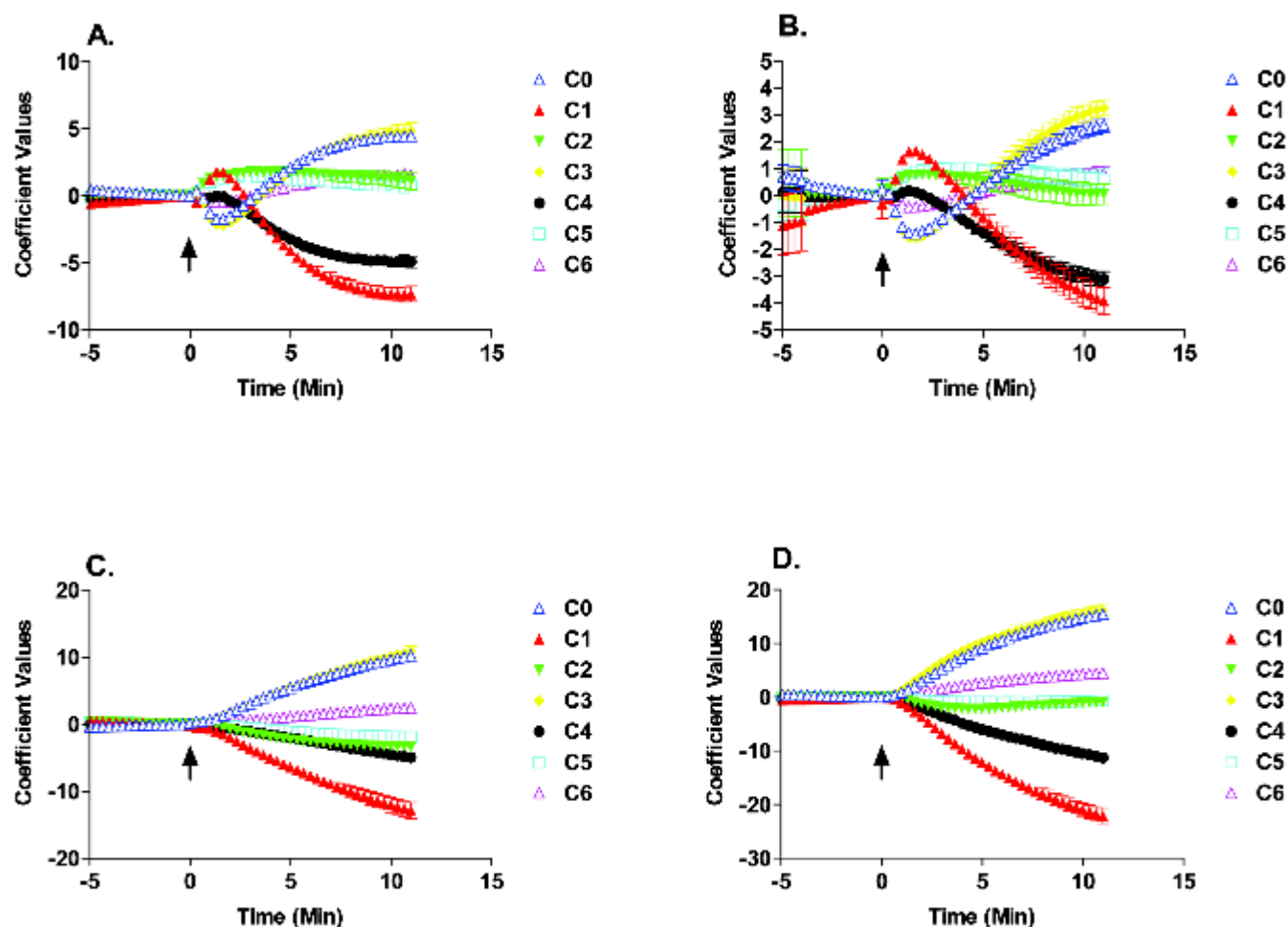


FIG. 6. Response pattern comparisons within and between different classes of G-protein-coupled receptors (GPCRs) in HeLa cells. Legendre polynomial coefficients (C0-C6) are plotted versus time for 5 min before, during, and for 10 min after addition of ligand (at zero time; arrow). Response patterns were generated for stimulation of 2 different G_q GPCRs: (A) the bradykinin receptor with 10 μ M bradykinin and (B) the endothelin receptor with 400 nM endothelin-1 (ET-1). In addition, response patterns were generated for stimulation of 2 different G_i GPCRs: (C) the α_2 -adrenergic receptor with 10 μ M UK14304 and (D) the CXCR-4 receptor with 10 ng/mL stromal cell-derived factor 1 alpha (SDF-1). Readings taken every 20 sec are shown, and the data represent the mean $\pm$ standard deviation for 5 wells for each ligand.

within a receptor family, different receptors of the same class tend to produce the same kinds of patterns, whereas receptors of different classes produce different patterns. However, patterns produced from receptors of 2 different families may be similar due to the convergent nature of the physiological responses elicited.

To date, we have analyzed GPCR and PTK receptor patterns from a significant number of different cell lines. Another important comparison to make is to determine if a particular receptor will produce the same response pattern across a number of different cell lines. A particular receptor expressed in different cell lines would be expected to produce a different response pattern depending on the cell line it is in. An example of this type of comparison is presented in Fig. 7. In this figure, we show the response of 5 differ-

ent cell lines to activation of the IGF receptor (a PTK receptor) with IGF. Three of the cell lines have human origin (A549, HeLa, and HEK293), one is from monkey (COS-7), and the other is from hamster (CHO_{kl}). The 3 human cell lines, although all epithelial in nature, come from different organs with widely varying functions (A549: lung; HeLa: cervix; HEK293: kidney) and would be expected to show different physiological outputs upon stimulation of the same receptor. The CDS patterns do appear to be different (compare Fig. 7A-C). In addition, other types of cells from other species would be expected to give different patterns upon stimulation of the same receptor. This can be seen in the comparison of the COS-7 and CHO_{kl} cells with the human cells (compare Fig. 7A-C with Fig. 7D,E). Similar results were obtained when we monitored

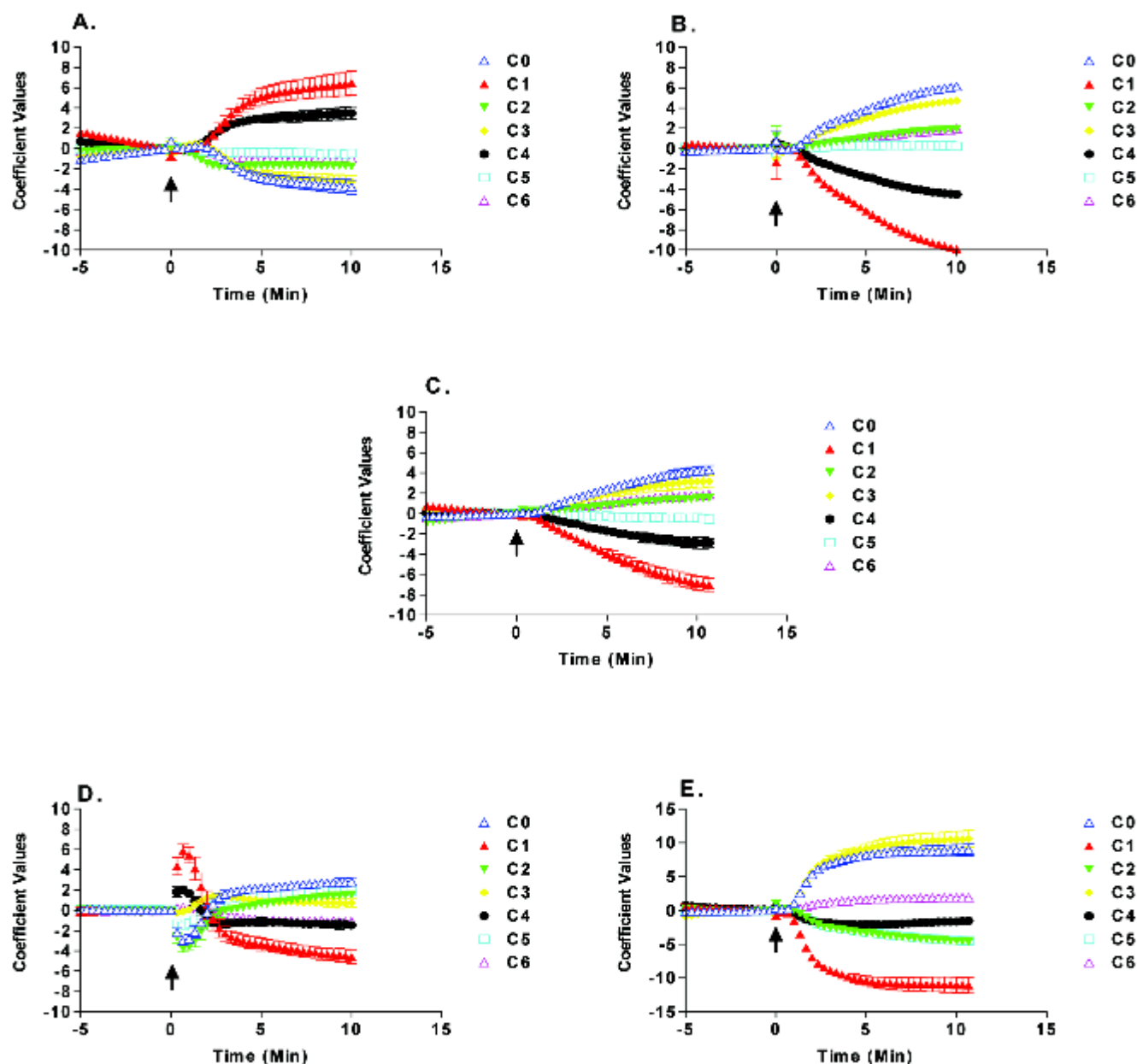


FIG. 7. Representative patterns for activation of the insulin-like growth factor (IGF) receptor in various cell lines. Legendre polynomial coefficients (C0-C6) are plotted versus time for 5 min before, during, and for 10 min after addition of ligand (at zero time; arrow). Response patterns were generated for stimulation of the IGF receptor using 100 ng/mL IGF. The cell lines used were (A) A549, (B) HeLa, (C) HEK293, (D) COS-7, and (E) CHO_{k1}. The data are the mean $\pm$ standard deviation for 3 wells for each cell line.

a GPCR receptor across different cell lines (data not shown). These results are consistent with known signal transduction information as different cells produce different physiological outputs from the same signaling molecules by using different scaffolding proteins to restrict the outcome to a specific result.³⁴ These results represent further evidence that CDS tracks expected cellular physiology.

Quantitative analyses: hit confirmation, physiological performance, pharmacological evaluation, and rank ordering

The overall cellular response to ligand activation of a receptor can be quantified from the Legendre coefficients as described in Materials and Methods. The generated MVL provides the system with the ability to quantify any cellular response to receptor activa-

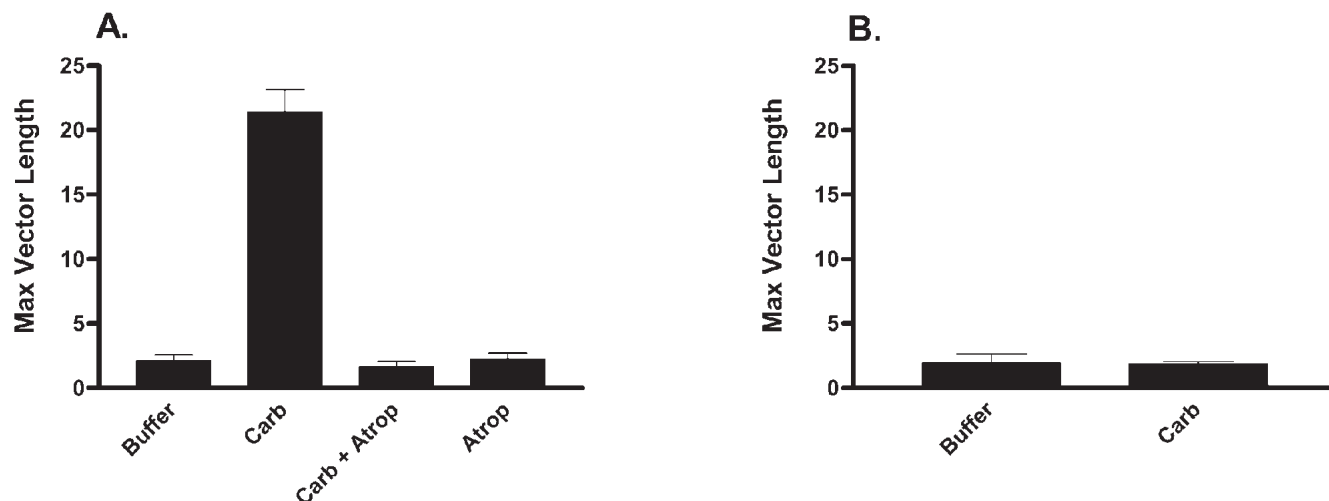


FIG. 8. Quantification of receptor-specific activation of the m_1 muscarinic receptor transfected into Chinese hamster ovary (CHO) cells. Maximum vector lengths (see Materials and Methods) were determined from Legendre coefficients at the time of maximal response after cells were stimulated with buffer alone (Buffer), 10 μ M carbachol (Carb), 10 μ M carbachol plus 1 μ M atropine (Carb + Atrop), or 1 μ M atropine alone (Atrop). Data are presented for the response of CHO cells transfected with the m_1 muscarinic receptor (A) and for untransfected parental CHO_{K1} cells (B). The data are the mean $\pm$ standard deviation for 3 wells for each reagent.

tion. This quantitation was applied to test receptor-specific activation using CHO cells transfected with the m_1 muscarinic receptor (CHO_{m1}) (Fig. 8). Upon addition of carbachol, an m_1 muscarinic receptor agonist, CHO_{m1} cells demonstrated a clear activation, as a large increase in MVL was observed compared to buffer control (Fig. 8A). This same concentration of carbachol had no effect on the CHO_{K1} parental cell line used for the transfection (Fig. 8B). In addition, the CHO_{m1} cell response to carbachol was completely blocked with the m_1 -specific antagonist atropine (Fig. 8A), whereas atropine alone had no discernible effect on the cells (Fig. 8A). These data clearly demonstrate that this system can quantitate receptor-specific activation and inhibition in a label-free fashion using a subset of the impedance data discussed above. These data also suggest that hit confirmation can be easily accomplished with this device.

Next, this quantitation was applied to test the reliance of the CDS responses on intact signaling pathways in cells. Disruption of 2 signaling pathways was tested in HeLa cells. AG1478 blocks the protein tyrosine kinase signaling mechanism of the EGF receptor. Pertussis toxin (PT) inhibits G_i -mediated signaling from G_i -coupled GPCRs. After the appropriate amount of incubation time with these inhibitors, cells were stimulated with EGF (to activate the EGF receptor) or SDF-1 (to activate the CXCR4 G_i -coupled receptor), and the relative MVL was determined (Fig. 9). AG1478 was effective at blocking the response to EGF but had no effect on the response to SDF-1. Conversely, PT completely inhibited the response of the cells to SDF-1 but had no effect on the response to EGF. These data further demonstrate that CDS tracks the physiological output of the appropriate intact signaling pathways that are

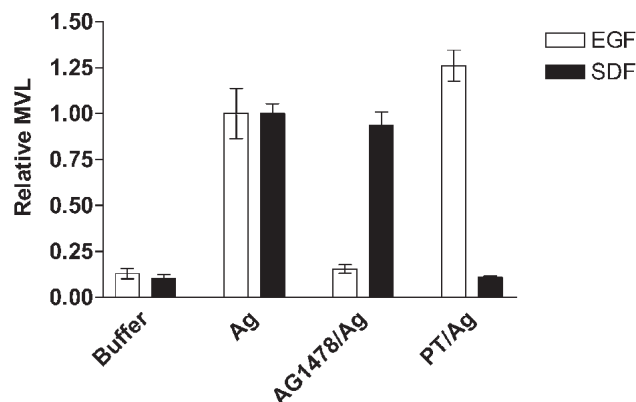


FIG. 9. Cellular dielectric spectroscopy (CDS) responses rely on intact signaling pathways. HeLa cells were stimulated with agonists (Ag), either epidermal growth factor (EGF) (10 ng/mL) or stromal cell-derived factor 1 alpha (SDF-1) (10 ng/mL), after pretreatment of the cells with 1 μ M AG1478 (AG1478) for 30 min or 100 ng/mL pertussis toxin (PT) overnight at 37 °C. Relative maximum vector lengths (see Materials and Methods) were determined from Legendre coefficients at the time of maximal response. The data are the mean $\pm$ standard deviation for 5 wells for each reagent.

associated with the receptors. These results are also important to the data presented in Fig. 4C,D. As previously discussed, the patterns generated by EGF and SDF-1 in HeLa cells looked very similar, and thus, potentially, the CDS device is measuring the end physiological result of activation of these receptors. The data from Fig. 9 suggest that this is true. The cells arrived at this end point

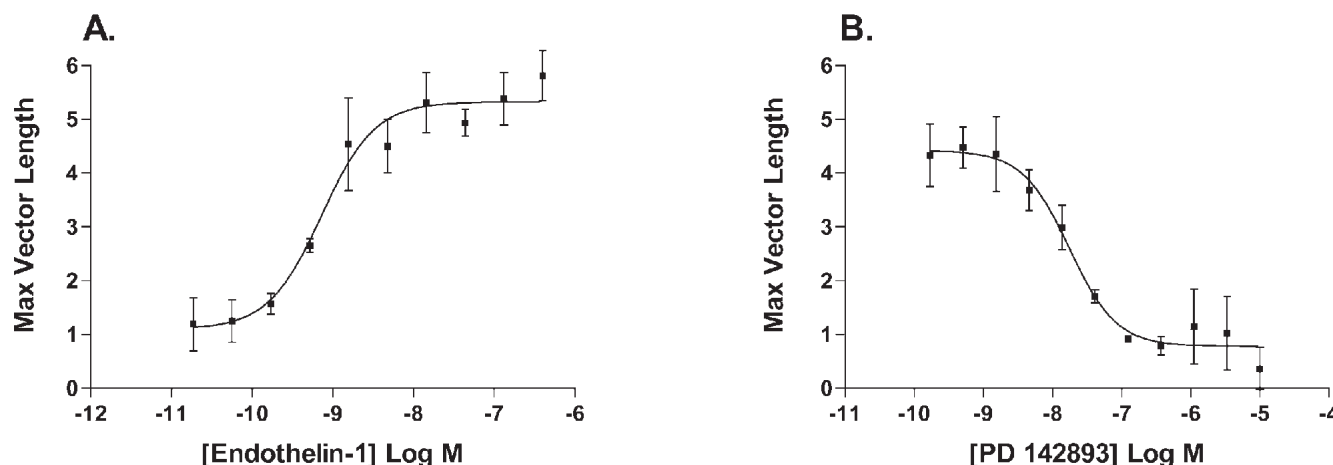


FIG. 10. Pharmacological evaluation of the endothelin receptor on HeLa cells using cellular dielectric spectroscopy (CDS). Maximum vector lengths (see Materials and Methods) were determined from Legendre coefficients at the time of maximal response after cells were stimulated with increasing concentrations of endothelin-1 (ET-1) (A). The EC_{50} value was determined to be 0.73 nM. The endothelin receptor antagonist PD142893, in a concentration-dependent manner, effectively inhibited the HeLa cell response to 0.8 nM ET-1 (B). The IC_{50} value was determined to be 17.5 nM. The data are the mean $\pm$ standard deviation for 5 wells for each concentration.

from 2 different pathways. The CDS response is manifested from an integrated cellular response as suggested by the complexity of the raw data (Fig. 3). Thus, CDS response measurement could become important in the field of integration biology.

Next, quantitation was applied to test the ability of the device to do pharmacological evaluations (Fig. 10). HeLa cells were stimulated with increasing concentrations of endothelin, and MVL was determined for each concentration. The data are shown in Figure 10A. The concentration response curve generated shows the expected sigmoidal shape, and the EC_{50} generated from the curve (0.73 nM) was within the range found in the literature for the endothelin receptor in this cell type.^{35,36} In addition, an inhibition curve was generated using the endothelin receptor-specific antagonist PD142893 (Fig. 10B). The generated curve showed an appropriate sigmoidal shape, and the IC_{50} generated from the curve (17.5 nM) showed good correlation to that reported in the literature for this inhibitor.^{37,38} These data indicate that CDS responses can be used to conduct pharmacological evaluations of receptor activity. Because this receptor was endogenous to this cell line, these data also indicate that overexpression of receptors by transfection is not necessary using this system. The data presented in Figure 10 are representative of the pharmacological evaluations (including agonist and antagonists) conducted on a variety of receptor targets using this device, and in all cases, the data obtained demonstrated good correlation to either data from secondary assays conducted in-house or that published in the literature.

Last, quantitation was applied to test the ability of the device to do rank ordering of antagonists of the m_1 muscarinic receptor on CHO_{m1} cells. A series of 6 different m_1 antagonists were tested against the m_1 agonist carbachol. MVLs were determined after

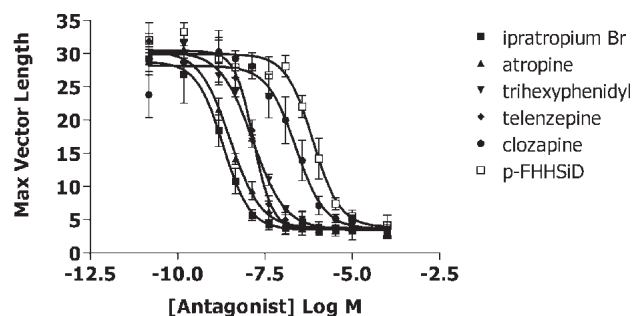


FIG. 11. Rank ordering of m_1 muscarinic receptor antagonists on transfected Chinese hamster ovary (CHO) cells. Maximum vector lengths (see Materials and Methods) were determined from Legendre coefficients at the time of maximal response after cells were stimulated with 10 μ M carbachol in the presence of increasing concentrations of various m_1 receptor antagonists. The data are the mean $\pm$ standard deviation for 5 wells for each concentration.

cells were stimulated with carbachol in the presence of increasing concentrations of the various antagonists. The data are presented in Figure 11. All antagonists evaluated in the study generated sigmoidal-shaped inhibition curves. The IC_{50} values were calculated from the curves and the compounds ranked accordingly. Ipratropium Br was the most potent, followed by atropine. Trihexyphenidyl and telenzepine were approximately equipotent and demonstrated the next highest potency. Clozapine was next potent, followed by p-FHHSiD, which was the least potent. To confirm and validate this ranking with our transfected cells, we performed a standard calcium fluorescence assay using an LJI Analyst (see Materials and Methods) and compared the results to

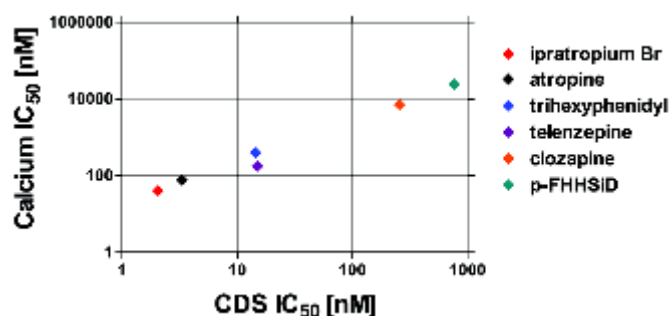


FIG. 12. Comparison of rank order potencies of m_1 muscarinic receptor antagonists using 2 different technologies. IC_{50} values for the above listed antagonists were generated using a standard calcium assay and compared to those values generated using maximum vector lengths (see Materials and Methods). The m_1 muscarinic receptor-transfected Chinese hamster ovary (CHO) cells were stimulated with 10 μ M carbachol in the presence of increasing concentrations of the various antagonists.

those from our technology. The results of this comparison are shown in Figure 12. This comparison shows that the same potency ranking order is achieved using both technologies. These data clearly indicate the applicability of the CDS technology to the rank ordering of potencies of compounds within the context of drug discovery and development.

DISCUSSION

One of the challenges of drug discovery is to identify better quality leads as early as possible in the discovery efforts. In an attempt to overcome this challenge, the community is recognizing and embracing the value of cell-based assays that yield high-quality information content on the consequences of target activation within the true biological context. By obtaining information of this nature, scientists can make better informed decisions on the lead candidate identified in the screening process.

Our goal at the beginning of this work was to develop a noninvasive, label-free cell-based assay technology capable of providing information about the total cellular integrated response of cells during GPCR and PTK receptor activation. We have shown that our device and technique can monitor receptor-specific activation on live cells in real time and have the sensitivity to detect responses from both endogenous and transfected receptors. This has been accomplished on adherent cells, and the potential exists that we perform similar analyses on nonadherent cell lines. We have demonstrated that the CDS technology is capable of doing pharmacological profiling, rank ordering of compounds, and hit confirmation with minimal assay development effort and without the requirement for molecular labels or special reagents. Although CDS is not the first label-free approach introduced for cell analysis in drug discovery, it benefits from a number of attributes that have hampered the universal adoption of these techniques (e.g.,

microphysiometry). These include improvements in throughput and ease of use, as well as compatibility with standard experimental buffers and reagents. Coupled with the volume of data and the uniqueness of the receptor-specific profiles, CDS offers appreciable advantages over other technologies for a number of discovery applications, as illustrated in this article.

The CDS responses obtained as a consequence of target activation have been shown to require intact signaling pathways and to track the expected cellular physiology. However, the most important result that we have shown is that this technology can monitor the complex integrated cellular response elicited by receptor activation. Using this technology, we can generate unique cellular activation response patterns that represent the unique underlying cellular physiology elicited by the activation.

This discovery is important for several reasons. The potential exists to be able to monitor all types of cellular events, including, but not restricted to, apoptosis and cytotoxicity, tumor cell progression, and stem cell differentiation. Ongoing work is progressing to see if unique patterns can be generated from these types of events. However, in the GPCR arena, the potential exists to correlate unique response patterns with specific receptor classes. With data of this nature, one can envision a canonical set of unique patterns that would represent activation of specific receptor classes in a particular cell line. This would enable classification of an unknown receptor using the canonical data set. Of added value to the drug discovery scientist is the fact that this information would be obtained in a completely innocuous fashion and would represent the real integrated cellular physiology elicited after receptor activation. The impact of this on the drug discovery industry can be illustrated in an example where we apply CDS technology to deorphanizing GPCRs. If there were direct correlations between response patterns and specific receptor classes, then in the process of fishing for ligands on cells transfected with the orphan GPCR, hits would produce a pattern that could be immediately assigned to a specific G-protein class. Thus, the ligand for the orphan GPCR and the G-protein system mediating its response would be revealed simultaneously. Because the information present in CDS measurements represents an integrated response, the end result of the ligand fishing process may be more relevant to intact cellular physiology than otherwise obtainable. Significant time and effort would be saved under these circumstances. In addition, once the ligand for the orphan GPCR has been discovered, the same CDS instrument could be used to pharmacologically characterize the ligand-receptor interaction and to search for antagonists. If all cellular events produce unique response patterns, then during the course of searching for antagonists, any compound that generated a cytotoxic pattern would be revealed. Compounds that cause cytotoxicity would be removed from consideration earlier in the drug discovery process. Again, this could have a significant effect on the time and effort expended in the search for druggable lead compounds.

From the data presented in Figures 4, 6, 7, and 9, we concluded that within a particular cell line and within a particular receptor

family, different classes of receptors give different patterns. Thus, the 3 major classes of GPCR could be distinguished from one another. We also concluded that due to the convergent nature of signaling pathways, different receptor families might give similar patterns depending on the resulting cellular physiology elicited. Thus, the response pattern elicited from PTK receptors is similar to that produced from G_i -coupled GPCRs (see Fig. 4C,D). However, these conclusions are based on visual inspection of the patterns. Work is progressing on developing computer algorithms to analyze these patterns and make use of subtle differences that will allow the separation of these responses into distinct categories in an automated fashion. In concurrence with this work, we are investigating the cellular physiological events that manifest themselves in the biophysical parameters that we measure. The literature has revealed that changes in the cell membrane,^{11,14,15} in cell-cell junctions,^{17,18} in the degree of cellular adhesion,^{16-19,21,23,24,25} and in the state of the actin cytoskeleton^{16,19} can all contribute to bioimpedance measurements. Work is progressing to define our biophysical measurements in terms of cellular physiological changes including, but not restricted to, those mentioned above.

In regards to converging signaling pathways and distinguishing subtle patterns, the more we learn about the underlying physiology of CDS measurements, the easier it will be to separate these types of responses. However, at present, we can still very easily distinguish like patterns by using biochemical approaches as demonstrated in Figure 9. In Figure 9, we were easily able to distinguish which ligand worked through the G_i pathway and which one worked through a PTK pathway. Because the patterns generated from G_q and G_s GPCRs are already distinguishable from the G_i /PTK pattern in this cell line, only the addition of a set of wells preincubated with pertussis toxin would be necessary to classify responses into G_q , G_s , G_i , and PTK. These data are representative of the use of biochemical modulators that alter the function of G_i and PTK receptors. Modulators of G_q and G_s receptors have also been used with equal effectiveness and ease (data not shown), and preliminary data suggest that like patterns from similarly coupled receptors can be dissected further using the appropriate modulators. Thus, this technology is very well suited for the reevaluation of potential hits in a discovery environment.

In addition to the aforementioned applications, this technology will be well suited to investigations in the area of integration biology. Since our output in an integrated response, the total cellular impact of perturbations of discreet signaling pathways will be more easily discernable. In addition, the results of multiple inputs on the total cellular response will be directly observable. This may lead to a better understanding of the actual effect of a drug within the context of a cell under the influence of multiple stimuli, as occurs in the intact organism.

In summary, we have created an extremely powerful technology with the capability to analyze multiple different receptor systems on a single platform. On this platform, one can perform hit confirmation, pharmacological profiling, and rank ordering on en-

dogenous as well as transfected receptors using adherent cells. This information is gathered in a label-free and completely innocuous way such that the cells could be reused for other assays after data acquisition. A clear advantage of the CDS technology is the ability to run these diverse applications for a range of cellular targets on a single platform with minimal assay development. More important, we have developed an analysis tool that can monitor the complex cellular physiology underlying the response of a cell to activation of a target in a noninvasive manner. It is envisioned that this complex cellular physiologic information will lead to a better understanding of the effect of a lead compound on the total integrated response of the intact cell.

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