Application Note

Cell Analysis

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High-Throughput GPCR Assay Development

xCELLigence RTCA HT instrument

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Introduction

G-coupled protein receptor (GPCR) modulators are estimated to make up approximately 40% of currently marketed drugs. They are among the largest class of drug candidates in development.[1] The rich complexity of GPCR signal transduction mechanisms is leading to new paradigms for GPCR drug discovery. Novel assay technologies have enabled the discovery of new GPCR family members as therapeutic targets.[2] The Agilent xCELLigence real-time cell analysis (RTCA) high throughput (HT) instrument offers a label-free solution for high-throughput screening of GPCRs, providing robust, real-time assays of recombinant and endogenously expressed receptors.

Many cell-based, high-throughput GPCR assay systems use recombinant cell lines to couple receptor activation to an artificial readout. An effective approach uses forced calcium coupling by promiscuous G protein overexpression or binding of a beta-arrestin fusion protein to the receptor. Biologically relevant cell-based assays measure an intermediate in one of the second messenger pathways involved in signaling. Gq-coupled receptors may be assayed using calcium release or metabolites downstream of phospholipase C activation. Gs- and Gi-coupled receptors may be assayed using cyclic AMP detection reagents. Agonist-mediated GPCR activation coupled to different signaling pathways will sometimes induce a rapid morphological change that can be assayed using the xCELLigence system.[3] The xCELLigence system can run GPCR assays without expressing additional reporter proteins. Real-time monitoring is possible using assays with endogenously expressed receptors in appropriate cell models.

This combination of attributes in the xCELLigence system can capture all the effects of GPCR modulation in the most biologically or disease-relevant cell types, resulting in higher-quality hits in a screening campaign.

The xCELLigence RTCA HT instrument consists of the RTCA HT control unit with RTCA HT software, the RTCA HT analyzer, and up to four RTCA HT stations. The RTCA HT station uses an Agilent E-Plate 384 to measure cell responsiveness to GPCR stimulation using electrical impedance. Cells are seeded one day before stimulation for attachment to the biosensors located in the bottom of the E-Plate well. When the E-Plate 384 is placed on the station, cellular interactions with these biosensors are detected by the RTCA HT analyzer as a change in electrical impedance. This is due to a low-voltage current running through the sensors in the bottom of the E-Plate. The impedance value is converted to a Cell Index (CI) by the RTCA HT software. The CI shows the number of cells and morphological parameters, such as cell size, shape, and degree of cell attachment to the substrate.

The sampling time can be as short as 15 seconds, enabling a real-time readout of rapid responses such as those mediated by GPCR activation. A heating unit in the RTCA HT station ensures that all assays are performed at physiological temperature, making the results as biologically relevant as possible. The compact footprint of the xCELLigence HT station is ready for integration into existing liquid handling and automation workflows.

Methods and materials

Background impedance measurements were taken after adding 20 µL of growth media to an E-Plate 384 and centrifuged briefly to eliminate any bubbles and to cover the entire bottom of the E-Plate. CHO-K1 cells expressing the human α-adrenergic 2A, histamine H1, or dopamine D1 receptors (available from Perkin Elmer) were then seeded at 12,000 cells per well, or HeLa cells at 6,000 cells per well, in a final volume of 40 µL, for a total of 60 µL per well before adding the agonist. The E-Plate 384 with cells was incubated at room temperature for 30 minutes for cell attachment, then incubated in a tissue culture incubator overnight.

For experiments performed in assay buffer, the growth media was replaced with assay buffer (HBSS containing 0.1% BSA and 20 mM HEPES), and incubated for 15 to 60 minutes at 37 °C in a tissue culture incubator before agonist treatment. E-Plates 384 with cells were then placed on the RTCA HT station, set at 37 °C, and monitored using impedance recordings at 30-second intervals. This was done for 2 to 5 minutes before agonist addition to determine the baseline values. Agonist was added in a volume of 5.5 to 6.7 µL (as a 10x stock diluted in assay buffer) using a liquid handling instrument (Beckman FX) with continuous monitoring. Plates were measured every 15 seconds for an additional 10 to 20 minutes.

Results and discussion

Agonist response assay for recombinant receptors

Representative receptors for each of the Gq-, Gi-, and Gs-coupled classes were chosen to assess the ability of the RTCA HT instrument when assaying GPCR function in recombinant cell lines. CHO-K1 cells expressing the human α-adrenergic 2A (Gi), histamine H1 (Gq), or dopamine D1 (Gs) receptors were plated on E-Plate 384 accessories, incubated overnight, then assayed using the xCELLigence system under two conditions. One condition used an assay buffer similar to those used in traditional GPCR assays. This has the advantage of eliminating trace amounts of GPCR modulators present in serum-containing media. It may also, in some cases, improve assay signal-to-background and simplify buffer matching when adding agonist. The other condition avoids the media change entirely to maximize throughput. All three tested receptors showed CI value changes immediately after agonist addition under both conditions (see Figures 1A, 1B, 1D, 1E, 1G, and 1H). The α-adrenergic 2A receptor-expressing cells showed an immediate increase in CI; a maximal response was observed within approximately 5 minutes. The histamine H1-expressing cells showed an immediate and transient decrease in CI followed by a gradual increase. The dopamine D1-expressing cells showed an immediate decrease in CI in assay buffer.

The signature responses observed were also influenced by cell background, cell density, and assay conditions. For example, in the absence of media change, dopamine D1-expressing cells exhibited an increase in CI after agonist addition (see Figure 1H). This suggests that the morphological changes detected using the impedance readout can be altered by assay conditions, indicating that the direction of the CI change is not always predictive of pathway coupling.

For each recombinant cell line, assay sensitivity was assessed using the dose-response curve function of the RTCA HT software to calculate the EC_{50} value for the agonist (see Figures 1C, 1F, and 1I). All three agonists showed EC_{50} values in the low picomolar (dopamine) to mid nanomolar (histamine) range, as expected based on cell providers'

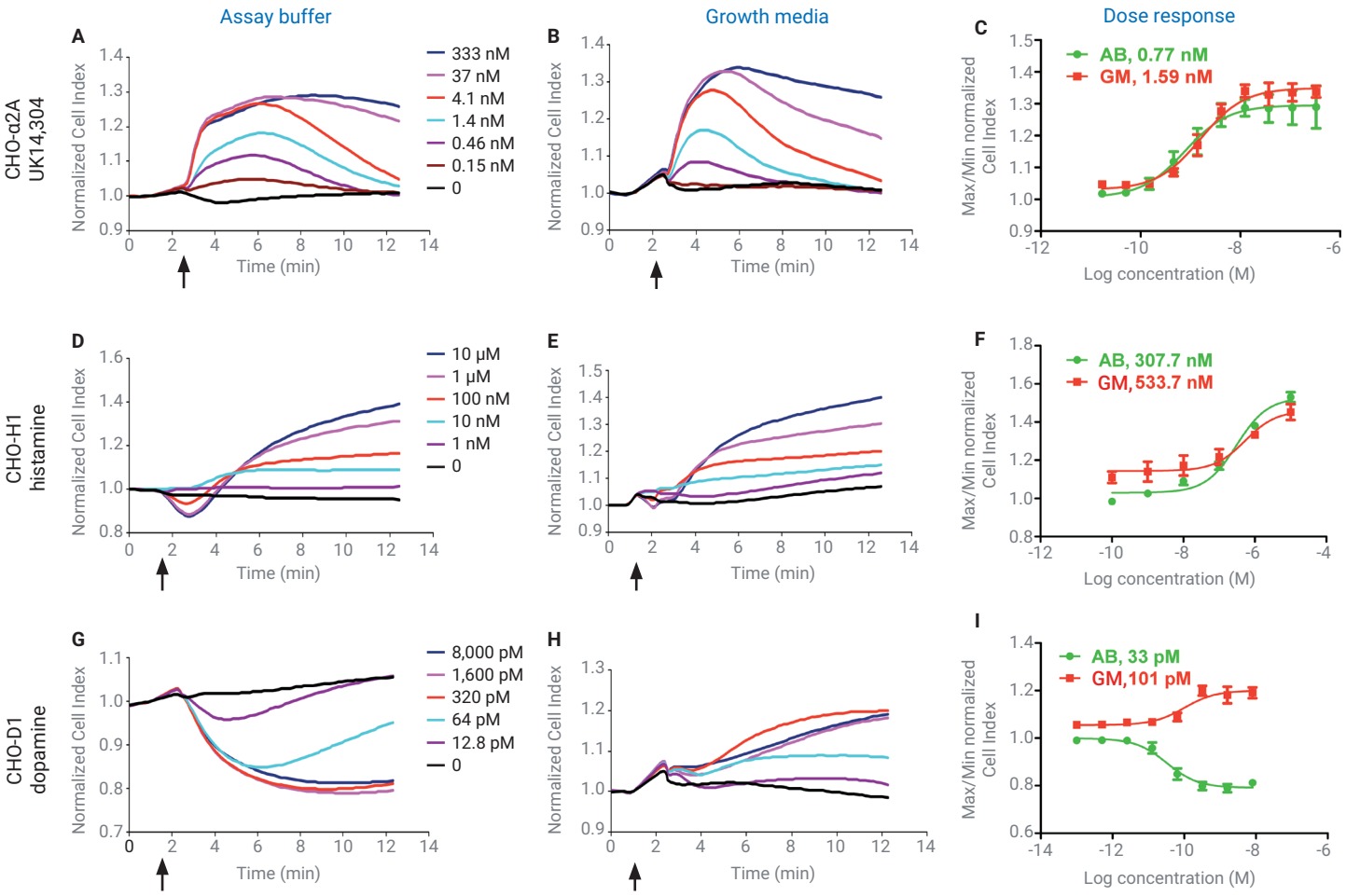

Figure 1. GPCR assays using recombinant cells. CHO-K1 cells expressing the α-adrenergic 2A (CHO-α2A), histamine H1 (CHO-H1), or dopamine D1 (CHO-D1) receptor were seeded at 12,000 cells per well on Agilent E-Plates 384, and grown overnight; growth media was changed for assay buffer (A, D, G) or left in place (B, E, H). After 15 to 60 minutes, cells were treated with the indicated concentrations of agonist and monitored for morphological responses using the Agilent xCELLigence RTCA HT system. Cell Index values were normalized for each well to the time point immediately before agonist addition, indicated by each arrow and plotted as the mean value from a minimum of four replicates (A, B, D, E, G, H). Maximal, or minimal, in the case of dopamine tested in assay buffer, normalized CI values were used to plot dose responsiveness as mean values with error bars representing one standard deviation (C, F, I).

data using standard assays. In addition, assay robustness was assessed by calculating the Z factor, which takes into account both the signal-to-noise ratio and well-to-well variation.[4] The Z factor was calculated using the time point and agonist concentration that produced maximal CI change, and a minimum of four replicate wells (see Table 1). All three cell lines produced Z factors greater than 0.5 in at least one assay condition, indicative of a very robust assay.

Table 1. Z factor calculation was performed at saturating agonist concentration and at the time point of maximal response.

Cells	Assay Buffer			Growth Media		
	Concentration	Time (min)	Z factor	Concentration	Time (min)	Z factor
CHO-α2A	12.4 nM	6.2	0.606	12.4 nM	6.2	0.600
CHO-H1	10 µM	11.9	0.862	10 µM	11.8	0.529
CHO-D1	1.6 pM	7.5	0.692	0.32 pM	12.3	0.341
HeLa-α2A	333 nM	10.1	0.823	333 nM	10.1	0.816
HeLa-H1	20 µM	17.1	0.802	20 µM	17.1	0.570
HeLa-D1	20 µM	9.6	0.619	20 µM	9.6	0.325

For the α-adrenergic 2A receptor, the maximal response was observed within the first six minutes, and for the other assays, a robust Z factor was obtained in at least one condition within 10 minutes. The one exception, the dopamine D1 receptor assayed without a media change (Z factor 0.36), showed an increasing response at 10 minutes, suggesting that a longer assay time may improve the Z factor. Taken together, these results indicate that the RTCA HT instrument can be used for rapid, sensitive, and robust assays of GPCR responses in recombinant cell lines.

Many receptors can be assayed without additional handling steps, such as a media change before screening.

Agonist response assay for endogenous receptors

The impedance-based readout used by the xCELLigence system offers the opportunity to assay endogenously expressed receptors in their native context, which has distinct advantages over recombinant systems.[2] The same agonists described earlier in this section were used to assay HeLa cells. HeLa cells treated with the α-adrenergic 2A receptor agonist UK 14,304 exhibited a rapid increase in CI, reaching a maximum CI approximately 5 minutes after compound addition, with or without a media change (see Figure 2). HeLa cells treated with histamine exhibited a common Gq response pattern,[3] consisting of a small drop in CI followed by a large increase. Interestingly, dopamine treatment of HeLa cells generated an increase in CI under both assay conditions (see Figure 2). This differs from the decreased CI observed in the recombinant dopamine D1 receptor-expressing CHO-K1 cells that were assayed after a media change, and

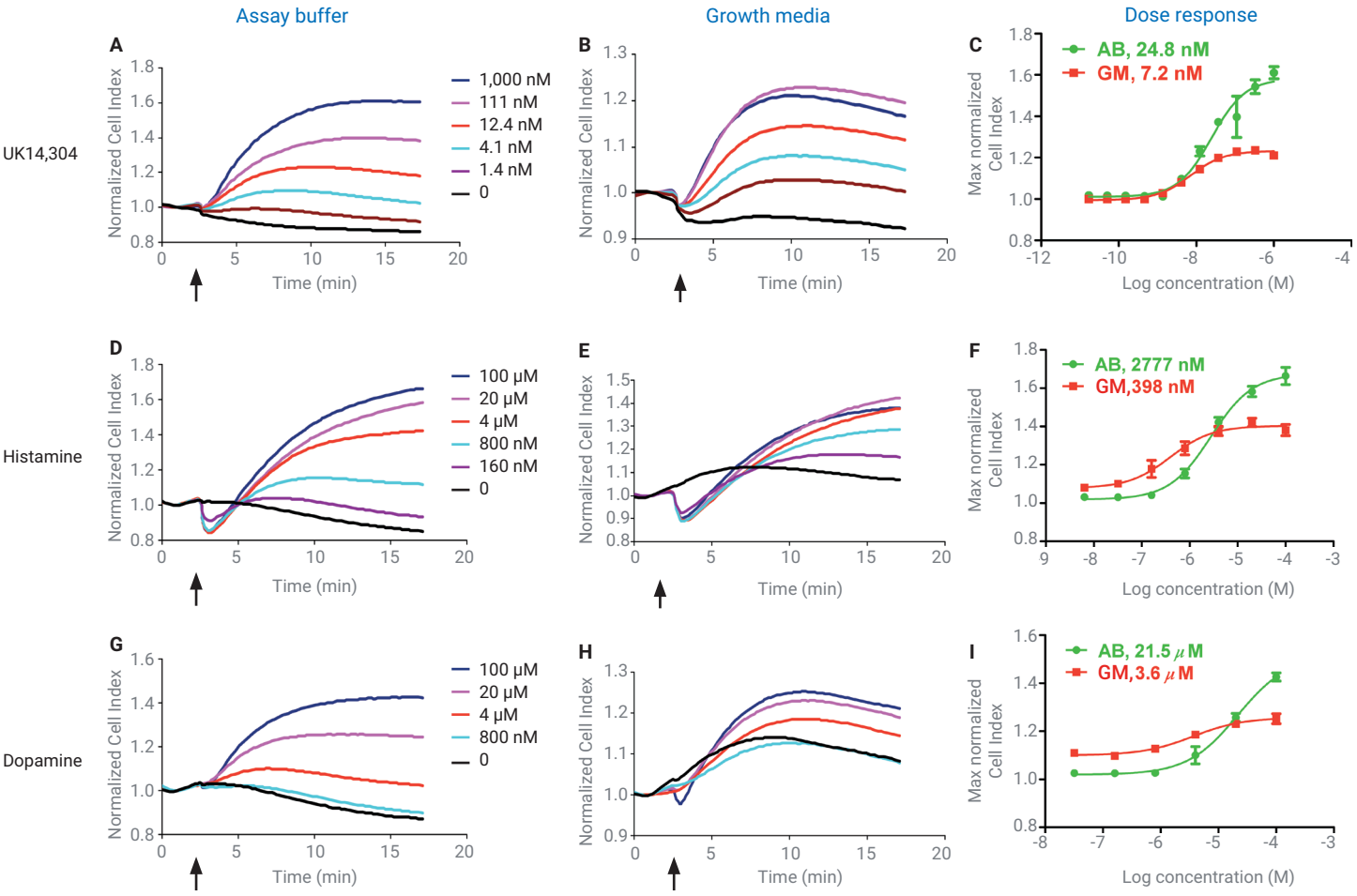

Figure 2. GPCR assays on endogenous receptors. HeLa cells were seeded at 6,000 cells per well on Agilent E-Plate 384 plates, and grown overnight. Cell cultures were then treated with the indicated concentrations of agonist and monitored for morphological responses using the Agilent xCELLigence RTCA HT system. Cell Index values were normalized for each well to the time point immediately before agonist addition (indicated by arrows), and plotted as the mean value from a minimum of four replicates (A, D, G). Maximal normalized CI values were used to plot dose responsiveness as mean values, with error bars representing one standard deviation (C, F, I).

may reflect the effect of other dopamine receptors expressed in HeLa cells. For example, the dopamine D2, D2S, and D3 receptors are all Gi-coupled, and all may be expressed in HeLa cells.[5] Alternatively, there may be differences in the way the activated signal transduction pathways couple to the morphology change in the two different cell lines, either at the level of the receptor/G protein-coupled step, or possibly at some point further downstream.

The endogenous receptor responses assayed in HeLa cells were robust, with at least one condition exhibiting a Z factor greater than 0.5, and some as high as 0.8 (see Table 1). Again, the exception was the dopamine response assayed without a media change, suggesting an effect of trace amounts of dopamine or other modulators in the serum-containing media. Dose-response curves revealed that the xCELLigence system assay for the α-adrenergic and histamine receptors endogenously expressed in HeLa cells exhibited a similar sensitivity to that of the recombinant cell lines, with EC_{50} dose response values in the low and high nanomolar ranges, respectively (see Figures 2C, 2F, and 2I).

In contrast, a much higher concentration of dopamine was required to activate endogenous receptors in HeLa cells than in the recombinant dopamine D1-expressing cell line under either assay condition. This could reflect lower expression levels or a different type of receptor in the HeLa cells. Further experiments with receptor subtype-selective agonists or antagonists should be able to distinguish between these possibilities. These findings underscore the fact that, as with any assay, each receptor must be optimized individually to obtain optimal signal-to-background and Z factor values.

Functional profiling of endogenous receptors

Directly assaying endogenous GPCRs allows for more biologically relevant assays and opens up the spectrum of cell types amenable to HT campaigns. To evaluate the utility of the RTCA HT instrument for assaying various GPCRs in the endogenous context, an assessment of the function of a set of 24 GPCR receptor families in HeLa cells using a panel of 43 agonists (see Figure 3). Using the baseline subtraction feature of the RTCA HT software, test well responses were assessed in relation to the buffer controls for each solvent type (DMSO for small molecules, 0.1% BSA for peptides). An arbitrary cutoff of three standard deviations from the control well mean value was used. Twelve of the tested agonists produced robust responses in HeLa cells, representing eight different GPCR receptor families (histamine, dopamine, prostanoid, CXC chemokine, endothelin, lysophospholipid, purinergic, and GPR119). This covered all the major coupling classes (Gi, Gs, and Gq). These results show that the RTCA HT instrument can be used to assay various endogenous GPCRs using appropriate cell backgrounds. The ability to assay GPCR function in the endogenous context is critical, because GPCR functionality can be modulated by both the repertoire and stoichiometric ratios of interacting proteins. This includes receptor heterodimers, coupled G-proteins, GPCR modulating kinases (GRKs) and beta-arrestins, all of which may exhibit altered functionality in receptor-overexpressing recombinant cell lines.

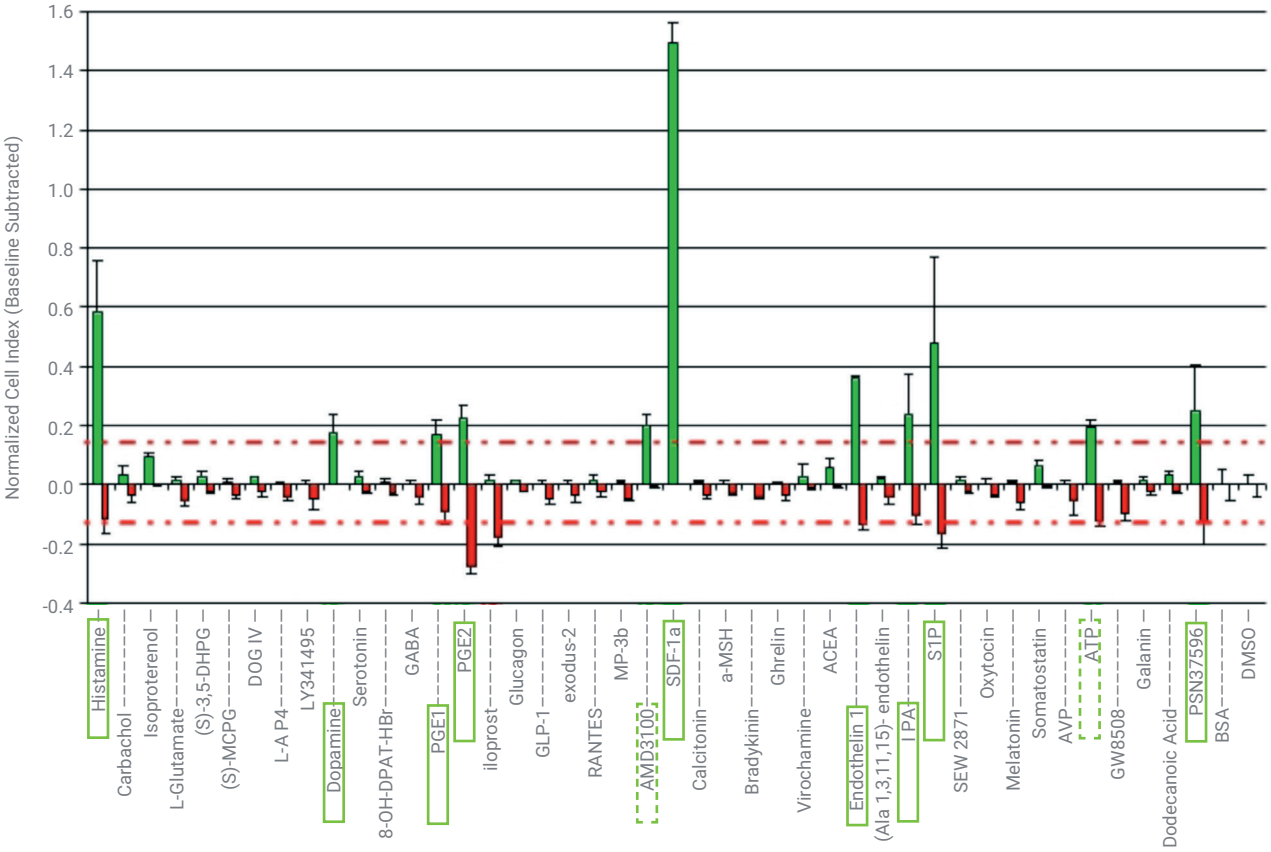

Figure 3. Functional profiling of endogenous receptors. HeLa cells were seeded at 6,000 cells per well on Agilent E-Plate 384, grown overnight, and the media was replaced with assay buffer. After 15 minutes, cells were treated with a panel of GPCR modulators at 7 µM (small molecules) or 0.7 µM (peptides), and monitored for morphological responses using the Agilent xCELLigence RTCA HT system. Cell Index values were baseline subtracted using the values of four replicate wells containing buffer controls, then normalized to the time point immediately before agonist addition using the RTCA HT software. Data were exported to Microsoft Excel, and maximum and minimum CI values were determined and plotted as the mean value from four replicate wells, with error bars representing one standard deviation. The red dotted line indicates a value three times the standard deviation of the buffer control wells used to determine active receptors. Solid green boxes indicate robustly active, while dashed green boxes indicate marginally active receptors based on maximum (in green) and minimum (in red) CI values.

GPCR antagonist assay

The potential therapeutic benefit of modulating GPCR activity comes from the antagonizing response to endogenous ligands. For example, antagonists of the α-adrenergic 2A receptor are useful for treating several conditions, including hypertension. To test the utility of the RTCA HT instrument in an antagonist screening mode, the α-2A receptor-expressing CHO-K1 cell line was pretreated with different adrenergic receptor antagonists and challenged with the α2A agonist UK 14,304. As expected, ARC 239, a selective adrenergic α2B receptor antagonist, had no effect on the morphological response to UK 14,304 stimulation, known to act through the α2A receptor (see Figure 4A).

In contrast, both BRL 44408 (a highly selective adrenergic α2A receptor antagonist), and rauwolscine (a potent but general adrenergic α2 antagonist), exhibited a dose-dependent inhibition of response, with IC_{50} values of 498 and 14 nM, respectively (see Figure 4A). The relative potencies of these inhibitors were nearly identical when tested in comparison to the endogenous adrenergic 2A receptor in HeLa cells (see Figure 4B). These results indicate that the RTCA HT instrument can be used to screen for selective antagonists for a given receptor, and that the degree of antagonism can distinguish among compounds with different potencies.

The RTCA HT instrument is a powerful tool for screening GPCR modulators. Sensitive and robust assays can be developed for various receptor families, including the three major coupling classes (Gi, Gs, and Gq) in both recombinant cell lines and in cell lines expressing endogenous receptors. Depending on the receptor assayed, robust responses (with Z factors greater than 0.5) can be achieved in as little as 5 to 10 minutes. Very high throughput capacity is possible, especially when combined with parallel processing using all four RTCA HT stations on the system at the same time. The findings show that in cases where the change of media for assay buffer produced a difference in assay quality, this effect, in general, improved assay robustness by increasing the signal-to-background ratio and decreasing assay variability (see Table 1 and Figures 1 and 2). Some GPCR assays showed no difference in the two different conditions performed (for example, CHOK1 cells expressing the α-adrenergic 2A receptor). For best results, each new assay should be tested under both conditions, weighing the importance of assay robustness against the decrease in throughput by performing media changes.

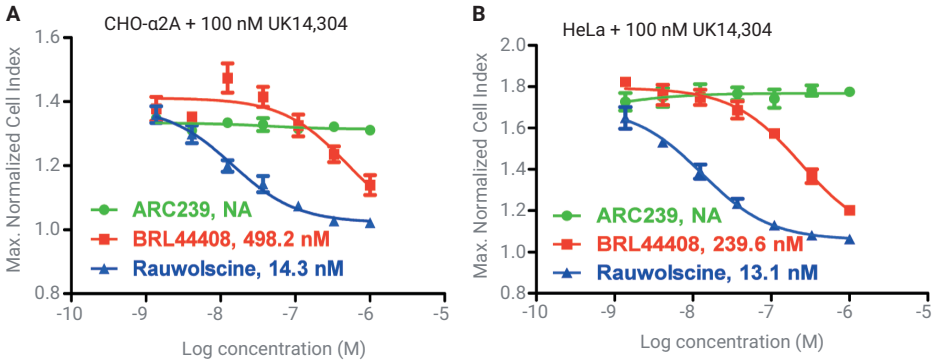

Figure 4. GPCR antagonist assay. A) CHO-K1 cells expressing the α-adrenergic 2A receptor were seeded at 12,000 cells per well on Agilent E-Plate 384 plates, grown overnight, and growth media was replaced with assay buffer. The indicated antagonist was added and after 1 hour incubation, the α-adrenergic 2A receptor-selective agonist UK 14,304 was added at 100 nM. Resulting dose-response relationships are plotted using mean values from four replicate wells; error bars indicate one standard deviation. ARC 239 is a selective adrenergic α2B receptor antagonist, BRL 44408 is a selective adrenergic α2A receptor antagonist, and rauwolscine is a potent, general adrenergic α2 receptor antagonist. B) The relative potencies of the inhibitors BRL 44408 and rauwolscine were nearly identical when tested in comparison to the endogenous adrenergic 2A receptor in HeLa cells.

Conclusion

The present study has shown that:

- The RTCA HT instrument can be used to screen modulators of GPCR activity in recombinant cell lines in up to four E-Plate 384 plates at one time. It can also be used for endogenously expressed receptors.
- These assays can be performed on a wide variety of GPCR families, including all coupling classes.
- These assays are highly sensitive and robust.
- These assays can be performed in agonist or antagonist mode.

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