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Evaluation of Dynamic Mass Redistribution Technology for Pharmacological Studies of Recombinant and Endogenously Expressed G Protein-Coupled Receptors

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Abstract: The Epic® cell assay technology (Corning® Inc., Corning, NY) uses a resonant waveguide grating optical biosensor to measure cellular response to ligands manifested through dynamic mass redistribution (DMR) of cellular contents. The DMR measurement is a noninvasive, label-free assay that can be used to assess the pharmacological properties of compounds. In this study, a panel of 12 compounds was evaluated against two G protein-coupled receptor (GPCR) targets in recombinant expressed cell lines using the Corning Epic system in 384-well microplates. The evaluation was performed in a double-blinded fashion such that the identity and properties of both the GPCR targets and compounds were unknown to the researchers at the time of the study. Analysis of the DMR response from cell stimulation was used to identify compounds that functioned as agonists or antagonists and to evaluate the associated efficacy and potency. DMR results were shown to have good agreement with data obtained from cyclic AMP and calcium flux assays for compounds evaluated. A further analysis was performed and successfully identified the signaling pathways that the two GPCRs activated. In addition, the DMR measurement was able to detect responses from an endogenous receptor in these cells. The Epic DMR technology provides a generic platform amenable to pharmacological evaluation of cellular responses to GPCR activation in a label-free live cell assay format.

Introduction

G PROTEIN-COUPLED RECEPTORS (GPCRs) are a superfamily of cell membrane proteins, which represent one of the largest classes of molecular targets for drug discovery and are targets of approximately 45% of the marketed human medicines.[1,2] These cell surface receptors are specifically activated by a diverse array of extracellular stimuli, including neurotransmitters, hormones, biogenic amines, neuropeptides, and lipids, among others. Their activations initiate various second messenger cascades that depend on the effector function. In addition to basic ligand–receptor binding assay, the GPCR signaling cascade opens opportunities to develop cellular HTS assays. Functional cell-based assays are more versatile than ligand–receptor binding assays; they provide additional information and enable the identification of allosteric modulators that act at sites other than the binding site of the endogenous ligand. Technology providers have responded with an increasing array of assays. However, assays that can report on multiple signaling pathways without the need to involve forced or promiscuous coupling through molecularly engineering would be particularly valuable to screening efforts.[3]

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ABBREVIATIONS: cAMP, cyclic AMP; CHO, Chinese hamster ovary; D3R, dopamine D_3 receptor; DMR, dynamic mass redistribution; EA, enzyme acceptor; EC_{50}, 50% effective concentration; ED, enzyme donor; EGF, epidermal growth factor; FBS, fetal bovine serum; G418, geneticin; GPCR, G protein-coupled receptor; IC_{50}, 50% inhibitory concentration; M1R, muscarinic acetylcholine M_1 receptor; N-DMR, negative-dynamic mass redistribution; P-DMR, positive-dynamic mass redistribution; POC, percentage of control; RWG, resonant waveguide grating.

Most current cell-based assays primarily rely on the measurement of a single event in a specific signaling pathway, including the second messengers (Ca^{2+} and cyclic AMP [cAMP]), translocation of a particular target tagged with a fluorescent label, or the expression of a reporter gene. These technologies require manipulations or molecular engineering of cells, such as overexpression of the target GPCRs and loading of fluorescent molecules into cells. These manipulations may alter the cellular physiology of the target receptors with possible interference from fluorescent and color compounds, leading to the identification of false-positive hits.

Ligand binding has been shown to initiate changes in GPCR conformation.[4] The functional change in receptor properties, in turn, leads to dynamic interactions with regulatory molecules at the cell plasma membrane and trafficking of many molecules and molecular assemblies, including the receptors to appropriate cellular compartments at various stages of the life cycle of a GPCR.[5,6] Such a movement results in dynamic redistribution of cellular contents, referred to as dynamic mass redistribution (DMR) in this report. The DMR response, measured by the optical biosensor, is a novel quantifiable and global representation signature for cell signaling.[7] The signal is an integrated cellular response that involves signaling pathways and cellular events, including cytoskeleton rearrangement, and/or movement of signaling cascade proteins. Unlike conventional methods, which are generally limited to measurements of a single signaling event such as direct visualization of the translocation of fluorescently tagged targets in cells, this technology utilizes the integrated signature of DMR to study cell signaling and the network interactions in cell signaling. This noninvasive, label-free DMR measurement of cellular contents resulting from ligand-induced receptor activation has been proposed to have great potential for the study of living cells.[8] This technology has further been proposed to be an integrated response that is a pathway-sensitive and a pathway-independent assay format for the study of endogenously expressed GPCRs.[9]

In the present study, a panel of 12 compounds was profiled against two GPCRs individually expressed in Chinese hamster ovary (CHO)-K1 cells. DMR measurements were performed in 384-well biosensor microplates using the Corning® (Corning, NY) Epic® system. The evaluation was performed in a double-blinded fashion such that the identity and properties of both the GPCR targets and compounds were unknown to the researchers at the time experiments were performed. DMR responses of these compounds were used to identify compounds that functioned as agonists or antagonists and to evaluate the associated potency and efficacy. The G protein-coupled signaling pathways for the two GPCRs were also evaluated and correctly identified using the DMR kinetic profiles induced by the reference agonists.

Materials and Methods

Materials

A panel of 12 compounds was provided under a numbering system (Compounds **1–12**; Table 1) for a double-blinded profiling study using the Corning Epic system and two recombinant cell lines, CHO-GPCRa (human dopamine D_3 receptor [D3R]) and CHO-GPCRb (human muscarinic acetylcholine M_1 receptor [M1R]). Compounds **1–12** and forskolin were purchased from Sigma Chemical Co. (St. Louis, MO). Compound **13** (ATP) was purchased from Calbiochem (La Jolla, CA). The two cell lines were obtained from Euroscreen (Gosselies, Belgium). Cell culture reagents including Ham's F-12 nutrient mixture, geneticin (G418), zeocin, L-glutamine, penicillin-streptomycin, fetal bovine serum (FBS), and EDTA (Versene™) were obtained from Invitrogen Corp. (Carlsbad, CA). Tissue culture flasks (150 and 225 cm^2) and Costar® microplates were purchased from Corning. L-15 medium was obtained from Lonza Inc. (Allendale, NJ). The cAMP assay kit, consisting of a cell lysis reagent, an enzyme acceptor (EA) reagent, an enzyme donor (ED)-cAMP reagent, an anti-cAMP antibody, and chemiluminescence β-galactosidase substrate reagents, was purchased from DiscoveRx® Corp. (Fremont, CA). This cAMP assay is an *in vitro* competitive immunoassay that relies on enzyme fragment complementation technology. Coelenterazine h luciferin was obtained from Assay Designs Inc. (Ann Arbor, MI).

Epic system

DMR measurements were performed using the label-free Corning Epic system, which consists of a microplate reader and 384-well lidded microplates that have resonant waveguide grating (RWG) optical biosensors integrated into each well.[9] The microplate reader illuminates the underside of each microplate at normal incidence with broadband light centered at 830 nm. It measures the reflected light from the biosensor, of which the wavelength is a sensitive function of the index of refraction of the composite biosensor and the target or cellular contents up to 150 nm above the biosensor surface. DMR events resulting from cell stimulation were manifest as a wavelength shift detectable with the Epic reader. The relocation of cellular contents towards the sensor surface contributes positively to the overall response, while the relocation of cellular contents away from the sensor surface contributes negatively to the overall response. The integration or aggregation of these redistribution events leads to the DMR signal measured.[7] Read time for an entire microplate was approximately 6 s in the present study, enabling kinetic measurements to be performed at this interval. The Epic system has a temperature control system to minimize interference associated with temper-

Table 1. Comparison of DMR Measurements to cAMP and Ca^{2+} Flux Results

		CHO-GPCRa				*CHO-GPCRb*			
		cAMP assay		*DMR*		*Ca^{2+} assay*		*DMR*	
Compound	*Name*	*EC_{50} (nM)*	*IC_{50} (nM)*	*EC_{50} (nM)*	*IC_{50} (nM)*	*EC_{50} (nM)*	*IC_{50} (nM)*	*EC_{50} (nM)*	*IC_{50} (nM)*
1	Dopamine	2.2 ± 0.7		20 ± 7					
2	Carbachol					32 ± 2		66 ± 32	
3	PD-128907	0.7 ± 0.2		3,500 ± 1,867				2,098 ± 141	
4	Spiperone		3 ± 2	N-DMR	5 ± 4		10,520 ± 8,641	4,782 ± 1,978	
5	Oxotremorine					9 ± 1		2 ± 2	
6	*R*(+)-7-Hydroxy-2-dipropylaminotetralin	0.3 ± 0.1		1 ± 3			534 ± 126		6,280 ± 2,917
7	Pilocarpine					391 ± 245		344 ± 122	
8	Amisulpride		122 ± 56	N-DMR	86 ± 25				
9	Haloperidol		9 ± 5	N-DMR	24 ± 8		2,290 ± 1,250	3,916 ± 1,470	
10	Telenzepine						10 ± 3		4 ± 2
11	Scopolamine						0.6 ± 0.1		2 ± 2
12	Atropine						0.7 ± 0.3		5 ± 2

The 12 compounds were tested in the cAMP and Ca flux assays in CHO-GPCRa (dopamine D3R) and CHO-GPCRb (muscarinic M1R), respectively, and data were compared to the Epic DMR results. Data are mean ± SD values of three separate determinations. Only compounds that show agonist or antagonist activity are shown here.

ature transients. The temperature inside the Epic system was maintained at 26 ± 0.2°C.

Measurement of Epic DMR response

CHO-GPCRa or CHO-GPCRb cells were seeded in a density of 12,000–15,000 cells per well in 384-well Epic microplates with 40 μl of growth medium (for CHO-GPCRa, Ham's F12 medium, 10% FBS, 0.4 mg/ml G418, and 1% L-glutamine/penicillin/streptomycin; for CHO-GPCRb, Ham's F12 medium, 10% FBS, 0.25 mg/ml G418, 1% penicillin/streptomycin, and 0.25 mg/ml zeocin) and cultured at 37°C in an atmosphere of 5% CO_2. The next day, cell culture media were manually removed, and the cells were gently rinsed three times, each with 30 μl of serum-free L-15 medium. The cells were incubated in the L-15 medium for 2 h in the Epic reader before an initial 5-min baseline activity was measured. After the baseline measurement, compounds in 10 μl of L-15 medium diluted to various concentrations were added to the wells, and DMR responses were monitored continuously for 45 min for agonistic activity measurement.

In the antagonist mode, the compounds were added and incubated with the cells for 3 h prior to a 5-min baseline measurement. At the end of the baseline measurement, 10 μl of the known agonist (compound **1** for CHO-GPCRa and compound **2** for CHO-GPCRb) at approximately the 70% effective concentration was added, and DMR responses were monitored for another 45 min continuously to determine the inhibition of the agonist-induced DMR responses.

In the analysis of G protein-coupled signaling pathways for GPCRa and GPCRb, the two cell lines were preincubated with 1 μ*M* forskolin or L-15 medium (control) for 1 h. After a 5-min baseline measurement was taken, 10 μl of compound **1** (50 n*M*) or compound **2** (500 n*M*) was added to CHO-GPCRa or CHO-GPCRb cells, respectively. DMR responses were monitored continuously for another 45 min.

Epic DMR data processing and analysis

In Epic DMR studies, compounds were tested at a concentration range of 0.02–10,000 n*M*. The DMR response was calculated by subtracting the baseline measurement at $t = 5$ min from the peak response intensity for each compound concentration tested. The 50% effective concentration (EC_{50}) and 50% inhibitory concentration (IC_{50}) values were determined by nonlinear regression analysis of the dose–response curves using Prism® (GraphPad Software, San Diego, CA).

Measurement of cAMP level in CHO-D3R cells

The cytosolic cAMP level in CHO-GPCRa (CHO-D3R) cells was determined using the DiscoveRx cAMP assay kit in 384-well white plates (Costar 3705, Corning). CHO-D3R cells were cultured in growth medium (Ham's F12 medium, 10% FBS, 1% L-glutamine/penicillin/steptomycin, and 0.4 mg/ml G418) at 37°C in an atmosphere of 5% CO_2. Before the experiment, cells were harvested by Versene, centrifuged, washed once with phosphate-buffered saline, and then suspended in a cell buffer (Ham's F12 medium, 30 m*M* HEPES, and 0.1% bovine serum albumin) at 500,000 cells/ml. Compounds to be tested were diluted with the cell buffer to the concentration required. The assay was performed according to the cAMP assay kit protocol provided by DiscoveRx. For agonistic response measurement, 20 μl of suspended cells (10,000 cells per well) was added to each well containing 5 μl of compound at various concentrations and incubated at room temperature for 30 min. This was followed by addition of 5 μl of forskolin in a final concentration of 30 μ*M* and incubation at room temperature for 30 min. A lysis buffer containing anti-cAMP antibody in 10 μl was added to the cells, followed by addition of 10 μl of an ED reagent 60 min later and incubation at room temperature for another 60 min. Finally 20 μl of EA and substrate reagents was added, and after a 2-h incubation at room temperature the luminescence signal was determined using a LEADseeker luminometer (GE Healthcare, Piscataway, NJ). In the antagonist studies, a D3R agonist dopamine in a final concentration of 15 n*M* was included in the forskolin solution at the second addition. All raw data were converted to percentage of control (POC) values prior to EC_{50} and IC_{50} analyses using XL-Fit® within ActivityBase (ID Business Solution Inc., Emeryville, CA). POC was defined as [100 × (test compound value − dimethyl sulfoxide control value)/(dopamine control value − dimethyl sulfoxide control value)].

Measurement of Ca^{2+} flux in CHO-M1R cells

Calcium flux was measured on an automated flash luminometer using a standard double-addition protocol in which compound was added prior to a known agonist. This double-addition protocol detects agonist or antagonist effect of test compounds in the same well. Light emission due to Ca^{2+} activation of aequorin in the cells was captured in 5-s intervals for 1 min in both agonist and antagonist modes. The emitted light peak intensity within the 1-min period was used in subsequent calculation of the compound response.

CHO-GPCRb (CHO-M1R) cells expressing M1R and apoaequorin were cultured in growth medium (Ham's F12 medium, 10% FBS, 0.25 mg/ml G418, 1% penicillin/streptomycin, and 0.25 mg/ml zeocin) at 37°C in an atmosphere of 5% CO_2. One day prior to the experiment, cells were seeded in 96-well clear-bottom black plates (Costar 3904, Corning) at a density of 25,000 cells per well in 80 μl of growth medium, and then the plates

were incubated at 37°C in an atmosphere of 5% CO_2. The next day before the experiment, the medium was manually discarded, and 60 μl of 10 μM coelenterazine solution diluted with the assay medium (Ham's F12 medium without phenol red and 15 m*M* HEPES, pH 7.5) was added. The plates were incubated at 34°C for 3 h. In the first compound addition, 20 μl of test compounds in various concentrations was added to the wells using a 96-well pipettor, and the emitted light due to cytosolic Ca^{2+} activation of aequorin was captured by the flash luminometer. After another 1.5 min, 120 μl of a M1R agonist carbachol in a final concentration of 67 n*M* was added, and the emitted light was captured for 1 min. All raw data were converted to POC values prior to EC_{50} and IC_{50} analyses using XLFit within ActivityBase. POC was defined as [100 × (test compound value − dimethyl sulfoxide control value)/(carbachol control value − dimethyl sulfoxide control value)].

Results

Identification of G protein-coupled signaling pathways for GPCRa and GPCRb

The addition of compound **1**, a known agonist for GPCRa, elicited a positive-DMR (P-DMR) response in a concentration-dependent manner. The maximum response intensity of 200 pm was observed at 600 n*M* (Fig. 1A). Similarly, compound **2**, a known agonist for GPCRb, induced a concentration-dependent P-DMR response that reached a maximum amplitude of 650 pm at 5 μM (Fig. 1B). The kinetic response profiles for CHO-GPCRa and CHO-GPCRb were notably different. At high concentrations of compound **1**, CHO-GPCRa cells responded with a rapid increase in P-DMR response, which peaked at 5 min and was followed by a gradual decline. On the other hand, CHO-GPCRb cells responded slower to compound **2** stimulation, with peak intensity reached

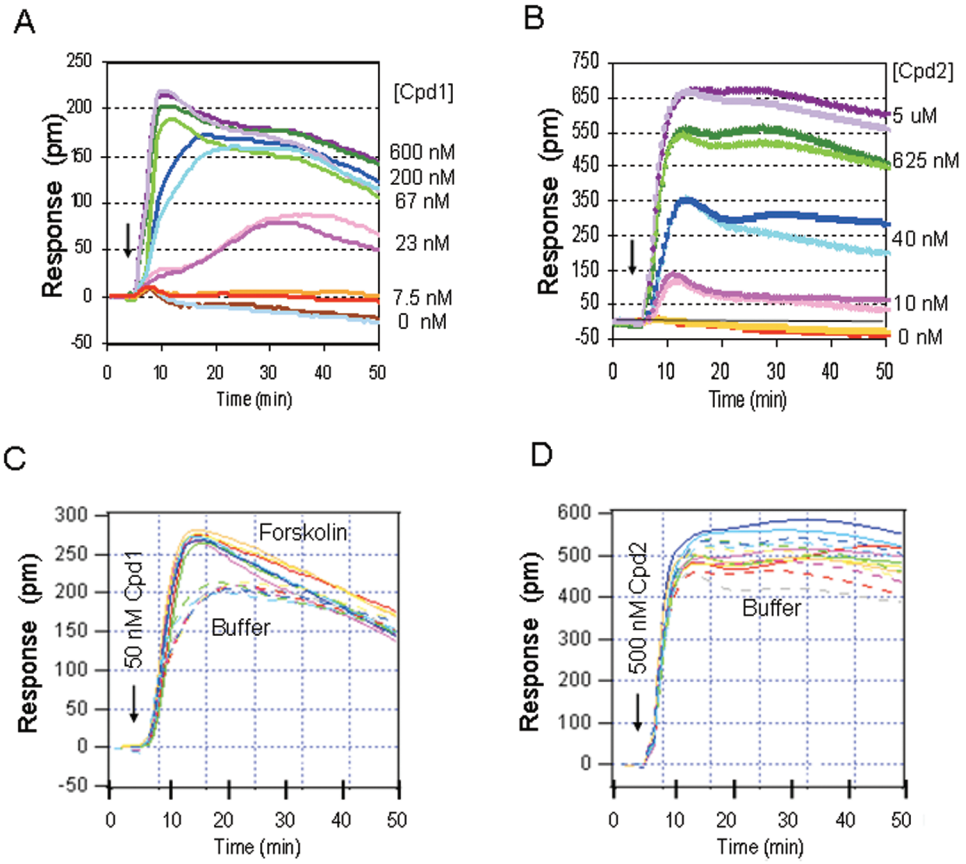


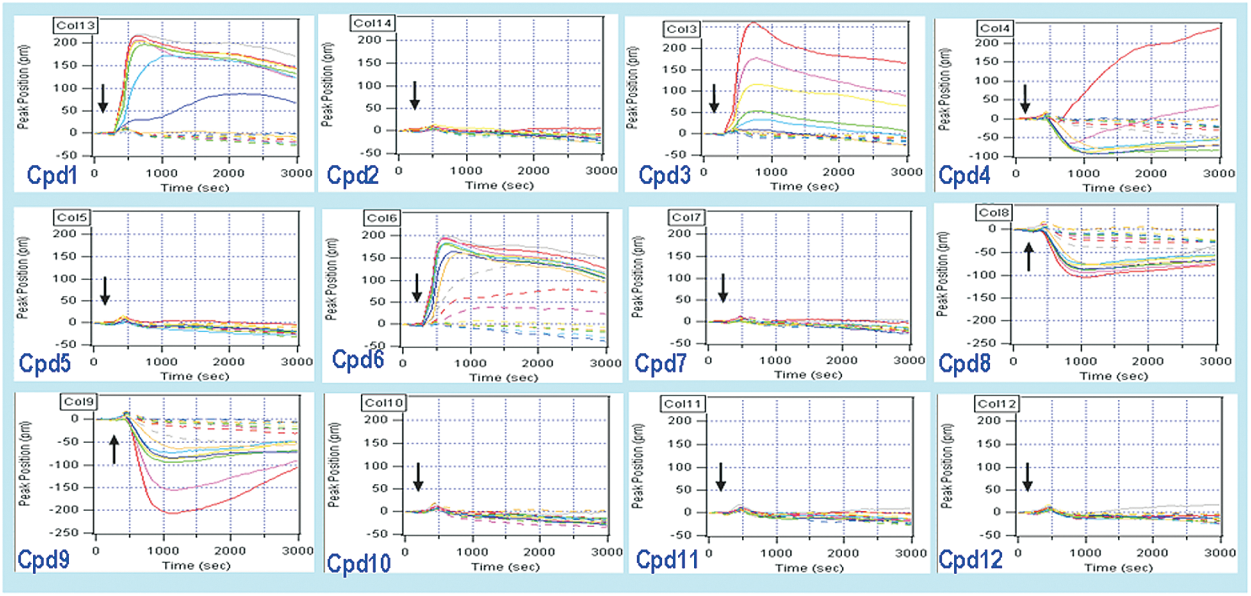
FIG. 1. Agonists induced DMR responses on CHO-GPCRa and CHO-GPCRb cells. Compound **1** or **2** was added to the wells after the plate had been equilibrated in the Epic biosensor for 2 h, and a 5-min baseline DMR was taken. The DMR response was monitored for another 45 min. (**A**) Compound **1**, an agonist for GPCRa, induced a concentration-dependent DMR response on CHO-GPCRa cells up to 600 n*M*; higher concentrations induced responses with similar magnitude as 600 n*M* and are not shown. (**B**) Compound **2**, an agonist for GPCRb, induced a concentration-dependent DMR response on CHO-GPCRb cells up to 5 μM; higher concentrations induced responses with similar magnitude as 5 μM and are not shown. Each curve was the DMR measurement of the cells in an individual well stimulated with various concentrations of the compound in duplicate. For the signaling pathway study, forskolin (1 μM; solid line) or assay buffer (dotted line) was incubated with the cells for 1 h prior to addition of (**C**) 50 n*M* compound **1** to CHO-GPCRa cells or (**D**) 500 n*M* compound **2** to CHO-GPCRb. Each curve was the DMR measurement of the cells in an individual well stimulated with the agonist ($n = 6$).

at about 10 min after the addition, after which the response leveled off for a period of 15 min before a gradual decline occurred. In order to evaluate the G protein signaling pathways for these two receptors, the cells were incubated with 1 μM forskolin for 1 h prior to addition of the corresponding ligand. On CHO-GPCRa cells, compound **1** induced a higher P-DMR response in the forskolin-pretreated cells (Fig. 1C), indicating that GPCRa is a Gi-coupled receptor. This is because forskolin, an adenylate cyclase activator, can increase the intracellular cAMP level, thus potentiating the Gi signaling due to heterologous sensitization. Conversely, for CHO-GPCRb cells, forskolin preincubation had no significant effects on the P-DMR response induced by compound **2** (Fig. 1D), suggesting GPCRb is a Gq-coupled receptor.

Identification of agonists and antagonists for GPCRa

The 12 compounds in various concentrations (0.02–10,000 n*M*) were added to CHO-GPCRa cells to identify compounds that functioned as an agonist at GPCRa. Compounds **3** and **6** induced a P-DMR response in a concentration-dependent manner similar to compound **1**, the known agonist for GPCRa (Fig. 2), suggesting they are agonists for GPCRa. Compound **2**, a known agonist for GPCRb, had no effect on CHO-GPCRa cells. Interestingly, CHO-GPCRa cells responded to compounds **4**, **8**,

A



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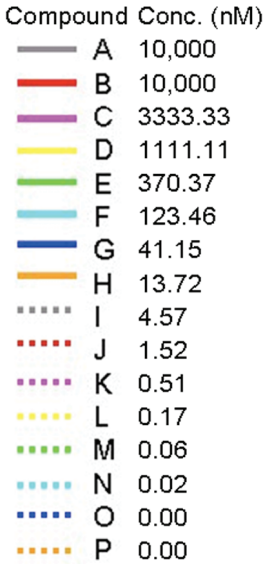

FIG. 2. The DMR profiles of 12 compounds on CHO-GPCRa cells in an agonist mode. (**A**) The individual compound in various concentrations (0.02–10,000 n*M*) was added to the wells after the plate had been equilibrated in the Epic biosensor for 2 h, and a 5-min DMR baseline was taken. The DMR response was monitored for another 45 min to identify compounds that stimulated a dose-dependent DMR response on CHO-GPCRa cells. Each curve was the DMR measurement of the cells in an individual well stimulated with the specified compound in various concentrations and color coding as indicated in (**B**) in wells A–P on each column of the 384-well plate. The same concentration range was used for compounds shown in Figs. 3–7.

and **9** with negative-DMR (N-DMR) responses. These effects were concentration-dependent, except that compound **4** also generated a P-DMR response at the highest concentration (10,000 n*M*).

In experiments to identify antagonists for GPCRa, CHO-GPCRa cells were incubated with individual compounds for 3 h at various concentrations (0.02–10,000 n*M*) prior to addition of the GPCRa agonist (compound **1** at 50 n*M*). Compounds **4**, **8**, and **9** attenuated the P-DMR response induced by compound **1** in a concentration-dependent manner (Fig. 3). Compounds **1**, **3**, and **6** also exhibited a concentration-dependent inhibition on compound **1**-induced response; this effect was likely due to receptor desensitization in the presence of GPCRa agonists.

Identification of agonists and antagonists for GPCRb

In a similar manner, the 12 compounds in various concentrations (0.02–10,000 n*M*) were added to CHO-GPCRb cells to identify compounds that behaved as an agonist at GPCRb. Compounds **5** and **7** induced a P-DMR response in a concentration-dependent manner and reached the maximum intensity of 600 pm similar to compound **2**, suggesting they are agonists for GPCRb (Fig. 4). Compound **1** was inactive on CHO-GPCRb cells. This indicates it is a selective agonist for GPCRa. At high concentrations of 1–10 μM, compounds **3**, **4**, and **9** induced a P-DMR response on CHO-GPCRb cells but with a lower amplitude that peaked between 300 to 400 pm.

In order to identify compounds that behaved as antagonists for GPCRb, CHO-GPCRb cells were incubated with individual compounds for 3 h at various concentrations (0.02–10,000 n*M*) prior to the addition of GPCRb agonist (compound **2** at 500 n*M*). Compounds **10**, **11**, and **12** showed a potent inhibition to the P-DMR response induced by compound **2**, suggesting they are antagonists of GPCRb (Fig. 5). Compounds **2**, **5**, and **7** also exhibited a concentration-dependent attenuation of compound **2** activation; this is likely due to receptor desensitization since these are GPCRb agonists. Compounds **3**, **4**, and **9** showed partial attenuation of the agonist-induced P-DMR response at 3.3 and 10 μM. This was in accordance to the weak agonistic responses observed on CHO-GPCRb cells. Compound **6** exhibited a similar inhibitory effect at micromolar concentrations, though it was inactive in the agonist mode, suggesting it is a weak antagonist to GPCRb.

*Effects of compounds **3**, **4**, and **9** on parental CHO-K1 cells*

The promiscuous activities of compounds **3**, **4**, and **9** on both CHO-GPCRa and CHO-GPCRb cells led to the testing of the three compounds in the parental CHO-K1 cells (Fig. 6). Compound **3** induced a P-DMR response in a concentration-dependent manner similar to those observed in CHO-GPCRa and CHO-GPCRb cells, suggesting this compound stimulated P-DMR via endogenous receptors or signaling events in CHO-K1 cells.

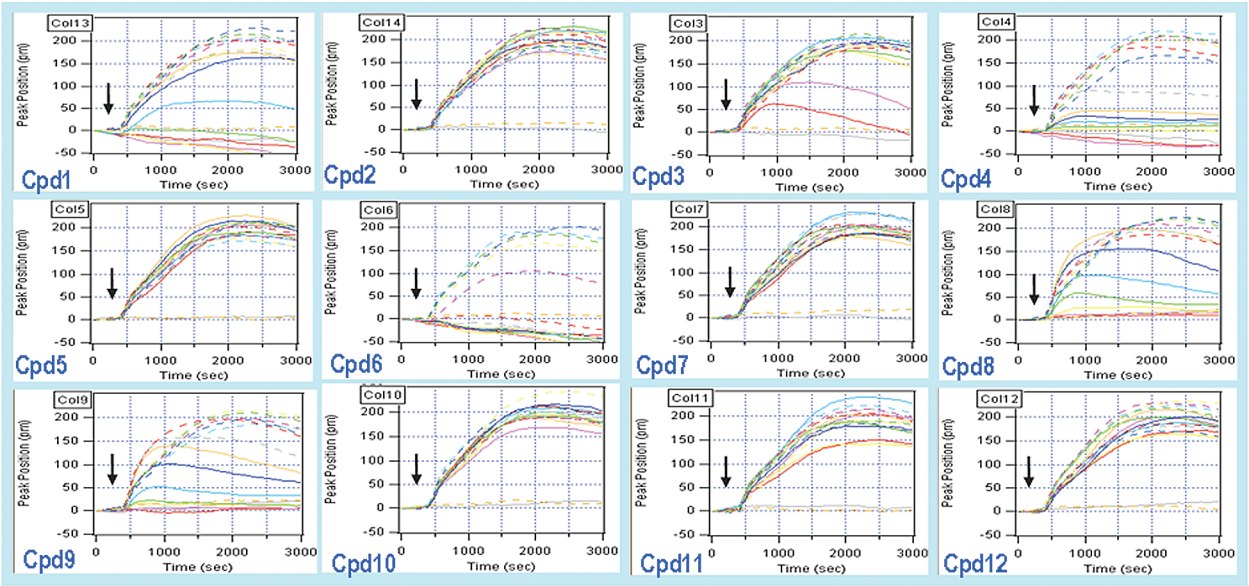

FIG. 3. The DMR profiles of 12 compounds on CHO-GPCRa cells in an antagonist mode. The cells in individual wells were preincubated with the specified compound in various concentrations (0.02–10,000 n*M*) for 3 h prior to taking a 5-min DMR baseline measurement. Compound **1** (50 n*M*) or assay buffer (to wells A and P) was added to the well, and the DMR response was monitored for another 45 min. Each curve was the DMR measurement of the cells in an individual well prior to and after compound **1** addition.

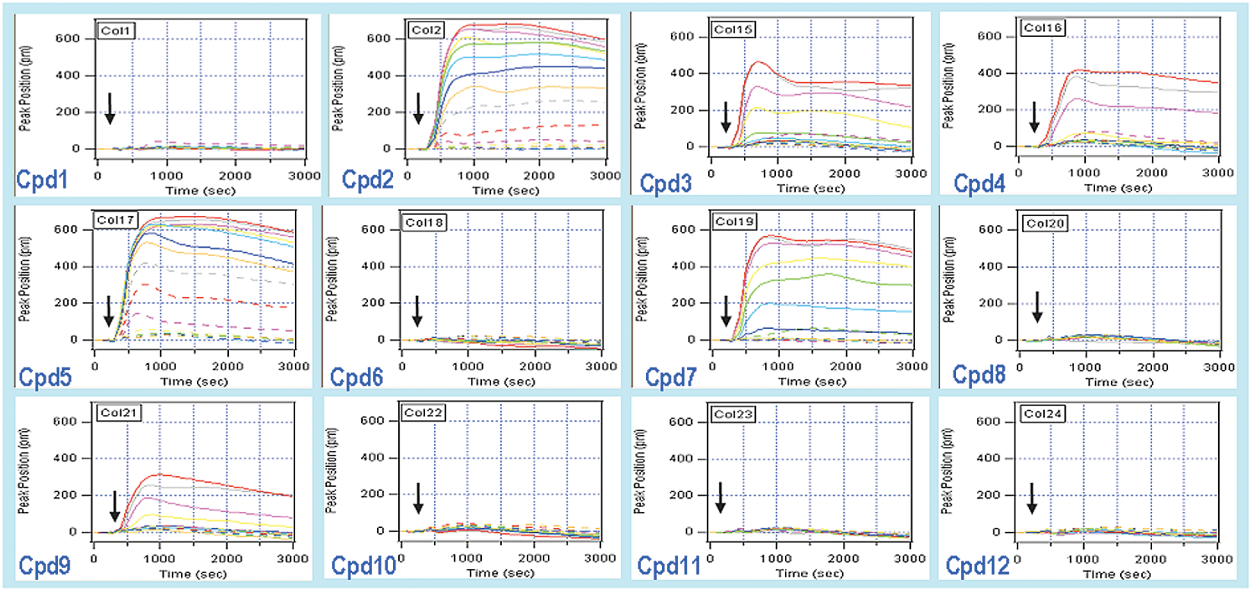

FIG. 4. The DMR profiles of 12 compounds on CHO-GPCRb cells in an agonist mode. The individual compound in various concentrations (0.02–10,000 n*M*) was added to the wells after the plate had been equilibrated in the Epic biosensor for 2 h, and a 5-min DMR baseline was taken. The DMR response was monitored for another 45 min to identify compounds that stimulated a dose-dependent DMR response on CHO-GPCRb cells. Each curve was the DMR measurement of the cells in an individual well stimulated with various concentrations of the specified compound.

Compound **4** stimulated a weak P-DMR response only at micromolar concentration. This response was much lower in magnitude as compared to its effect on CHO-GPCRb cells and different from its N-DMR response observed on CHO-GPCRa cells. This indicates compound **4** is active at GPCRa and GPCRb, as well as other endogenous receptors or signaling events in CHO-K1 cells. Compound **9** at the highest concentration elicited a N-

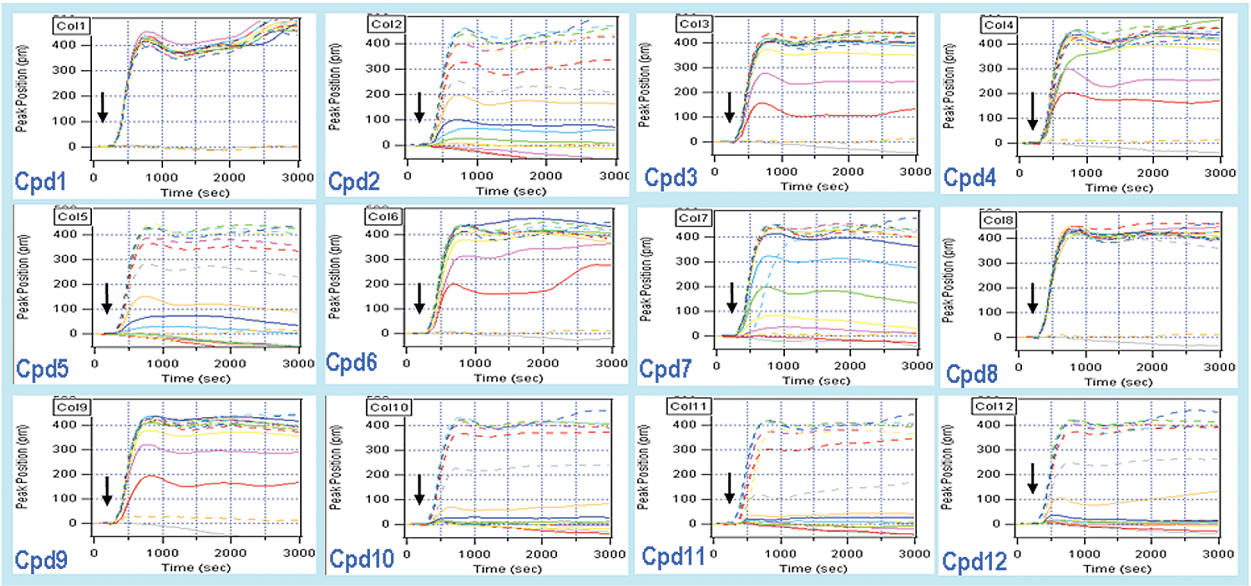

FIG. 5. The DMR profiles of 12 compounds on CHO-GPCRb cells in an antagonist mode. The cells in individual wells were preincubated with the specified compound in various concentrations (0.02–10,000 n*M*) for 3 h prior to taking a 5-min DMR baseline measurement. Compound **2** (500 n*M*) or assay buffer (to wells A and P) was added to the well, and the DMR response was monitored for another 45 min. Each curve was the DMR measurement of the cells in an individual well prior to and after compound **2** addition.

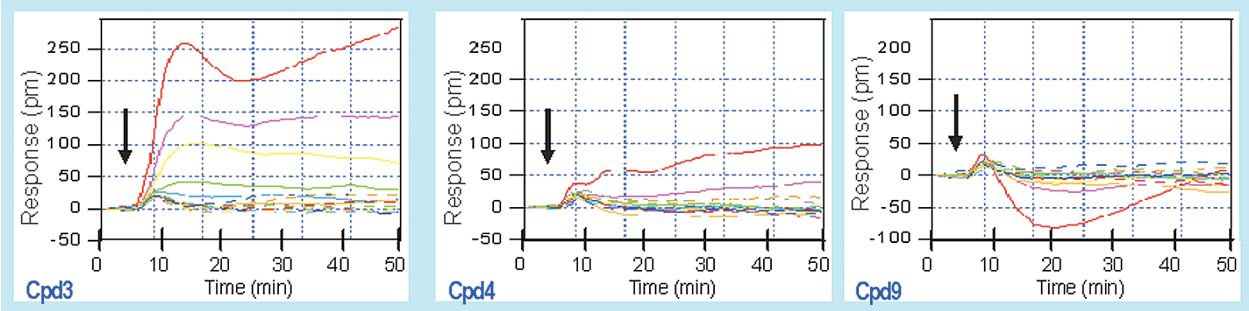

FIG. 6. The DMR profiles of compounds **3**, **4**, and **9** on CHO-K1 cells in an agonist mode. The individual compound in various concentrations (0.02–10,000 n*M*) was added to the wells after the plate had been equilibrated in the Epic biosensor for 2 h, and a 5-min DMR baseline was taken. The DMR response was monitored for another 45 min to determine if the compounds stimulated a dose-dependent DMR response on these cells. Each curve was the DMR measurement of the cells in an individual well stimulated with various concentrations of the specified compound.

DMR response on CHO-K1 cells. This response was similar to its effect on CHO-GPCRa cells but at much lower potency, which suggests compound **9** also stimulates a N-DMR response via endogenous receptors on parental CHO-K1 cells.

Comparison of DMR results to cAMP and Ca^{2+} flux data

The 12 compounds were tested on CHO-GPCRa (D3R) and CHO-GPCRb (M1R) cells using second messenger assays (cAMP and Ca^{2+} flux assay, respectively). Table 1 compares the EC_{50} and IC_{50} values obtained from the second messenger assays to DMR results. In the CHO-GPCRa cAMP assay, compounds **1**, **3**, and **6** functioned as agonists and inhibited forskolin-induced cAMP increase in these cells. Preincubation of CHO-GPCRa cells with the 12 compounds showed compounds **4**, **8**, and **9** antagonized the inhibitory effect of dopamine on forskolin-induced cAMP level. Compounds **1**, **3**, and **6** were more potent in the cAMP assay than in the DMR measurement. In the antagonist mode, compounds **4**, **8**, and **9** showed similar potency and rank order in both cAMP and DMR assays.

In the CHO-GPCRb Ca^{2+} flux assay, compounds **2**, **5**, and **7** acted as agonists; their potencies and rank order are in good agreement with results obtained from the DMR measurements. Both Ca^{2+} flux and DMR results showed compounds **10**, **11**, and **12** were potent antagonists against carbachol stimulation. Compound **6** also functioned as a weak antagonist on GPCRb in the Ca^{2+} flux assay, which is in accordance to the DMR result. In contrast to their agonistic responses observed in the DMR measurement, compounds **4** and **9** were micromolar antagonists in the CHO-GPCRb Ca^{2+} flux assay. Although compound **3** stimulated a P-DMR response on CHO-GPCRb cells with an EC_{50} value of 2.1 μM, it was inactive in the Ca^{2+} flux assay in both agonist and antagonist modes.

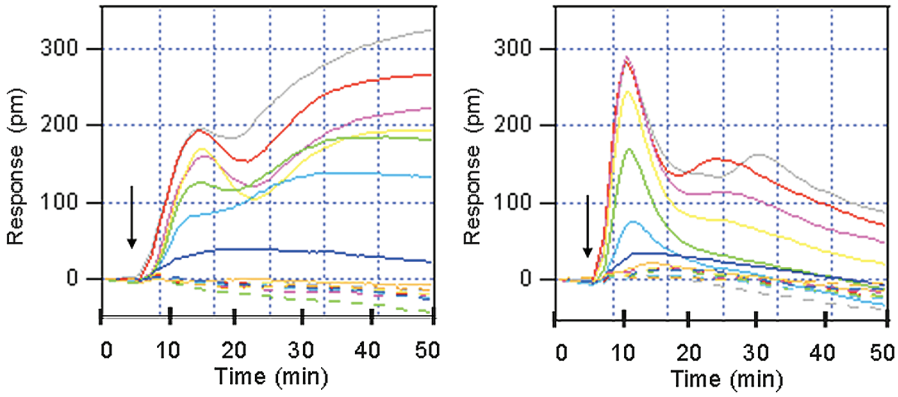

FIG. 7. Compound **13** stimulated dose-dependent DMR responses on (**left**) CHO-GPCRa and (**right**) CHO-GPCRb cells. After the plates had been equilibrated in the Epic biosensor for 2 h and a 5-min DMR baseline was taken, compound **13** in various concentrations (0.02–10,000 n*M*) was added to the wells, and DMR responses were monitored for another 45 min to determine if the compound stimulated a dose-dependent DMR response on these cells. Each curve was the DMR measurement of the cells in an individual well stimulated with various concentrations of compound **13**.

*DMR responses induced by compound **13***

Compound **13** induced a concentration-dependent P-DMR response on both CHO-GPCRa and CHO-GPCRb cells (Fig. 7). The DMR kinetic profiles elicited by this compound were different in the two cell lines. On CHO-GPCRa cells, a second and prolonged increase in the P-DMR response was observed, which had an intensity even higher than the first peak that reached 200 pm. On CHO-GPCRb cells, a rapid onset of P-DMR response that peaked at 300 pm at 5 min was observed after compound **13** addition. The response gradually declined to almost baseline level after 45 min. Dose–response curves were generated by plotting the intensity of the first peak against concentrations of compound **13**; the EC_{50} values of compound **13** on CHO-GPCRa and CHO-GPCRb cells are 127 ± 18 and 232 ± 52 n*M*, respectively.

Discussion

Conventional GPCR cell-based assays typically measure a specific intracellular response, including Ca^{2+} mobilization, cAMP production, and reporter gene expression, or the translocation of a tagged target protein. These assays measure a target-mediated event along a defined signaling pathway rather than an overall cellular response. The RWG biosensor and resultant DMR response enable the study of receptor biology and ligand pharmacology in living cells under physiological relevant conditions. The DMR measurement can be used as a novel class of physiological responses of living cells without the need for labels or cell manipulations. Similar to Ca^{2+} mobilization, the ligand-induced DMR responses are dependent on and saturable to ligand concentrations. It also exhibits classical desensitization patterns upon repeated agonist stimulations as shown by dopamine (compound **1**) on CHO-D3R cells (Fig. 3) and carbachol (compound **2**) on CHO-M1R cells (Fig. 5). The result shows that DMR technology can serve as a novel readout for monitoring GPCR activation.

GPCR signaling involves a series of highly regulated spatial and temporal events, leading to an ordered, regulated, and dynamic redistribution of cellular contents.[10] The DMR response observed is an integrated cellular response, and therefore distinct types of stimulation could lead to similar optical profiles. For example, two completely distinct stimuli such as epidermal growth factor (EGF), a natural agonist for the EGF receptor, and methyl-β-cyclodextrin (a cholesterol-depletion reagent) generated similar optical signatures.[11,12] The EGF-mediated DMR response in quiescent A431 cells is primarily linked to the Ras/mitogen-activated protein kinase pathway. Whereas the DMR response induced by methyl-β-cyclodextrin is a result of the transactivation of EGF receptors and involves multiple signaling pathways including mitogen-activated protein kinase, protein kinase C, and phosphatidylinositol 3-kinase. Many cell signaling events involve significant redistribution of intracellular contents, and the biosensor-based assays may have broad applications in different types of targets.

GPCR-mediated DMR response primarily consists of three components: trafficking of intracellular targets to the activated receptors and subsequently receptor internalization,[8] cytoskeletal rearrangement in between receptor activation and downstream signaling events,[10] and changes in cell adhesion property.[13] Analyses of activation of endogenous GPCRs in multiple cell lines have identified three types of DMR response profiles. Each response is correlated well with the activation of a class of GPCRs, depending on the G protein (Gq, Gs, Gi) with which the receptor is coupled.[9] In this doubled-blinded study a Gq-coupled M1R and a Gi-coupled D3R, both stably expressed in CHO cells, were examined. The activation of M1R or D3R led to a similar type of DMR response, which consists of an increase in P-DMR, but with distinct amplitudes (Fig. 1). Such similarity in DMR responses mediated through the activation of different classes of GPCRs added to the complexity in data interpretation, particularly for pathway identification, solely based on the observed features of their DMR responses. However, the coupled signaling pathways can be differentiated using the forskolin analysis, where forskolin pretreatment resulted in distinct differences on ligand-induced DMR responses between the Gq- and Gi-coupled receptors.

GPCRs coupled to the Gi and Gs signaling pathways lead to modulation of intracellular cAMP upon receptor activation, but manifested in opposite ways. Forskolin stimulated an elevation of cAMP level and potentiated the P-DMR response mediated by Gi-coupled receptor activation. This result was opposite to that observed in Gs-coupled GPCRs, where forskolin attenuated the agonist-induced N-DMR response.[9] Based on the impact of forskolin pretreatment on GPCR agonist-induced DMR responses, we correctly identified GPCRa as a Gi-coupled receptor (D3R) and GPCRb as a Gq-coupled receptor (M1R). Furthermore, the influence of the forskolin pretreatment has on dopamine-induced DMR response in CHO-GPCRa cells suggests that the response is not a measurement of receptor activation *per se*; it results from downstream signaling of cAMP mediated through the dopamine D3R.

A ligand-induced DMR response, measured using the label-free optical biosensor, is an integrated response that consists of contributions of many cellular events downstream from the activated receptor and leads to significant mass redistribution within the sensing volume of the biosensor.[7,8] Many ligands often exhibit "nonspecific" activity and activate more than one receptor in a cell type or cellular system.[9] Therefore, caution is warranted in an-

alyzing GPCR ligand pharmacology using the biosensor cellular assays. Conventional pharmacological and receptor biology techniques are required for systematical analysis of ligand pharmacology and evaluation of the signaling potentials of GPCR ligands. In the present study, the cellular activities of the same set of ligands were cross-examined in CHO-M1R, CHO-D3R, and their parental CHO-K1 cells using the biosensor cellular assays, in conjunction with conventional Ca^{2+} flux and cAMP assays. Our results showed that M1R agonists and antagonists were only active on CHO-M1R and not on CHO-D3R cells. This is consistent with results observed in the second messenger assays (Table 1), indicating these compounds are M1R-selective. In contrast, D3R agonists [PD-128907 and *R*(+)-7-hydroxy-2-dipropylaminotetralin] and antagonists (spiperone and haloperidol) were also active on CHO-M1R cells. Spiperone and haloperidol are nonselective dopamine D_2/D_3 receptor antagonists that also demonstrated low-affinity binding to muscarinic M_1 receptors.[14,15] Both compounds induced a weak P-DMR response on CHO-M1R cells and desensitized carbachol stimulation, suggesting they functioned as an agonist to the M1R at micromolar concentration. The two compounds were active in the Ca^{2+} flux assay with similar potency but only as an antagonist. GPCR signaling typically proceeds through a series of amplification steps. An agonist-mediated maximal response, a measure of agonist efficacy, is dependent on the cellular events measured. As a result, the closer to the receptor activation step the cellular events measured, the lower the sensitivity is to agonist-stimulated responses.[16,17] These results show the DMR technology is able to identify weak agonists that would have been missed in the Ca^{2+} flux assay platform.

PD 128907 (compound **3**) is a selective D3R agonist[18,19] and is potent in the cAMP assay. It is not clear why the compound generated a P-DMR response on CHO-D3R cells at a potency 1,000-fold lower than that observed in the cAMP assay. In DMR studies, PD-128907 also stimulated a P-DMR response at micromolar concentration in both CHO-M1R and parental CHO-K1 cells. The response from parental CHO-K1 cells was identical in amplitude and potency to those observed in CHO-D3R cells. This result suggests PD-128907 may activate an unknown signaling event common to Gi and Gq signaling pathways in order to cause desensitization of both dopamine- and carbachol-stimulated P-DMR responses (Figs. 3 and 5). Since DMR is a measurement of an integrated cellular response consisting of contributions from events involving dynamic redistribution of cellular contents, this could be complicated when the compound activates multiple GPCRs or regulates signaling events that have cross-activity with the target of interest.

Antipsychotics, such as spiperone, amisulpride, and haloperidol (compounds **4**, **8**, and **9**, respectively), induced a dose-dependent N-DMR response on CHO-D3R cells, which was opposite to that stimulated by the agonist dopamine. The effect of amisulpride was only observed on CHO-D3R and not CHO-M1R cells, suggesting the response is D3R-specific. Furthermore, both spiperone and haloperidol stimulated a P-DMR response at a lower potency on CHO-M1R cells, indicating the compounds functioned in a different manner at the M1R. The result suggests that N-DMR responses generated by these antipsychotics, which also desensitized the dopamine-induced DMR response in subsequent addition, are resulted from their activities on the D3R *per se*. Many antipsychotic drugs, including spiperone and haloperidol, have been shown to be inverse agonists at human dopamine D_2 receptors.[20] These compounds may behave as inverse agonists on CHO-D3R cells, and the observed N-DMR is a result of the net elevation of cAMP from suppressing the constitutively active D3R. This effect would not have been detected in the cAMP assay because of the assay sensitivity and the need to manipulate cAMP level with forskolin stimulation. The sensitivity of the DMR technology provides a label-free cell-based assay platform to evaluate the inverse agonist property of drugs. A recent study suggests as many as 85% of antagonists tested behave as inverse agonists.[21] The ability to determine the antagonist and inverse agonist properties of a drug is particularly important since this may help to elucidate some of the observed drug responses, as well as guide a decision on the therapeutic regimen.

The family of Gq-coupled receptors may lead to the same type of optical signature; however, distinct members have their own fine characteristics, including the amplitudes and the kinetics of DMR responses, due to its own signaling network interaction.[8] Our results showed all M1R agonists (carbachol, oxotremorine, and pilocarpine) elicited P-DMR responses with similar amplitudes and kinetics on CHO-M1R cells (Fig. 4). However, the P-DMR response induced by ATP through activation of the endogenous purinergic P2Y receptors,[22] which are also Gq-coupled, is distinct in both amplitude and kinetics from the M1R activation (Fig. 7). These characteristics of P-DMR responses from the two Gq-coupled receptors may reflect the difference in expression levels of the receptors and the pattern of physiological responses from the two receptor classes on the signaling network.

Addition of ATP to CHO-D3R and CHO-M1R cells elicited dose-dependent P-DMR responses with similar potencies, suggesting the DMR technology is capable of and sensitive to detecting activation of endogenous receptors and subsequent signaling events. Although ATP activated the endogenous P2Y receptors in the two cell lines, there is a distinct difference in the kinetic profiles on the DMR responses, especially after the initial DMR peak. Since DMR measures an integrated cellular response, it is sensitive to the cellular background and cel-

lular content. The transfected GPCR (D3R vs. M1R) and corresponding signaling mechanism (cAMP vs. Ca^{2+}) are different in these two cell lines. As a result, subtle differences in clonally selected cell lines may have significant effects on the DMR response profile. The detection of ATP-induced response also suggests that the biosensor cellular assays can be used to screen compounds against an endogenous receptor. Such a capability could be advantageous and implies its feasibility in multiplexed agonist screening.[7] In contrast, the biosensor cellular assays face a hit specificity issue if utilized in target GPCR screening efforts. A cocktail of solutions can be applied, including the use of the parental cell line or the use of selective antagonists of the target receptor in the counterscreen to evaluate the specificity of the agonists identified, as well as molecular engineering the cell line for the assay to enhance the amplitude of the target-specific DMR response over those generated by endogenous receptor activation.

In conclusion, qualitatively the DMR-generated pharmacological data are in most instances comparable to those measured by traditional cAMP and Ca^{2+} flux assays. The potency ranking of agonists and antagonists tested is very similar to that obtained by second messenger assays except for one compound. The DMR technology provides a noninvasive and continuous recording of cellular activity with high sensitivity. It is a valuable tool in the drug discovery process for the evaluation of compound efficacy, mechanism of action (antagonist vs. inverse agonist), and specificity. The technology can potentially enable the analysis of endogenous GPCRs in bio- and disease-relevant samples without the need of dyes and complex reporter systems.

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