Duplexed Label-Free G Protein–Coupled Receptor Assays for High-Throughput Screening

ELIZABETH TRAN and YE FANG

This article describes duplexed label-free optical biosensor cellular assays for simultaneously assaying 2 endogenous receptors, the G_q-coupled histamine receptor (H_1) and the G_s-coupled β_2-adrenergic receptor (β_2AR), in A431 cells. The biosensor cellular assays consist of 2 sequential steps—an initial agonist screening using Sigma LOPAC (Library of Pharmaceutically Active Compounds) and a subsequent antagonist screening using a solution mixture containing the H_1 agonist histamine and the β_2AR agonist epinephrine. Results showed that costimulating A431 cells with histamine and epinephrine led to an optical response additive to individual responses. The agonist screening not only identified all full agonists for both the H_1 and β_2 receptors, but also detected pathway-biased ligands for the β_2AR. Furthermore, the succeeding antagonist screening documented all known antagonists in the library for either the H_1 or β_2 receptors. This is the 1st demonstration of a single cellular assay that is capable of screening ligands against 2 GPCRs coupled to distinct G proteins, and highlights the power of pathway-unbiased and label-free biosensor cellular assays for GPCR screens. (*Journal of Biomolecular Screening* 2008:975-985)

Key words: optical biosensor, G protein–coupled receptor, histamine H_1 receptor, β_2 adrenoceptor, high-throughput screening, multiplexing, ligand-directed functional selectivity, compartmentalization

INTRODUCTION

G protein–coupled receptors (GPCRs) are the richest class of drug targets in the human genome and remain a popular class of targets for the pharmaceutical industry. About 30 known GPCRs are the targets for about 40% of all currently marketed drugs, and many other functionally uncharacterized GPCRs are potentially druggable targets and represent an untapped resource in drug discovery.[1-3] Efforts to bring new GPCR drugs to the market have seen a revolution of assay methods, particularly functional cellular assays.[4-8] However, current assays are mostly pathway biased and only measure "points of contact" in complex signaling pathways.[9] Given the recent realization of the complexity of GPCR signaling[10,11] and of the ligand-directed functional selectivity,[12,13] these pathway-biased assays tend to result in false negatives.[4,9] Furthermore, many conventional approaches typically assay a single target at a time because of their pathway-biased nature and limited capacity for multiplexing. Multitarget screens that examine the activity of compounds against multiple targets simultaneously are logically suited to address compound selectivity.[14,15]

Label-free optical biosensors including surface plasmon resonance, resonant waveguide grating (RWG), and plasmon-waveguide resonance are routinely used for biomolecular interaction analysis.[16,17] Recently, we had applied RWG biosensors for whole cell sensing and found that these biosensors are capable of monitoring endogenous receptor activation, leading to high information and physiologically relevant measures of a receptor-ligand pair.[18-22] These assays do not require prior knowledge of cell signaling and are pathway unbiased.[21,22] More importantly, the responses recorded are pathway sensitive and do reflect the complexity of receptor signaling[19] and pathway-biased activity of ligands acting on endogenous β_2-adrenergic receptor (β_2AR) in human epidermoid carcinoma A431 cells.[22] Here we developed a duplexed biosensor cellular assay and described its use to screen ligands in Sigma's LOPAC (Library of Pharmaceutically Active Compounds) for 2 endogenous receptors, histamine H_1 receptor (H_1R) and the β_2AR, in A431 cells.

MATERIALS AND METHODS

Materials

The LOPAC, (S)-epinephrine, dopamine, norepinephrine, histamine, (±)-brompheniramine maleate, (±)-chlorpheniramine maleate, clemizole hydrochloride, clemastine fumarate, diphenhydramine hydrochloride, or triprolidine hydrochloride, SKF

Biochemical Technologies, Science and Technology Division, Corning Incorporated, Sullivan Park, Corning, New York.

Received Jun 16, 2008, and in revised form Jul 3, 2008. Accepted for publication Jul 6, 2008.

Journal of Biomolecular Screening 13(10); 2008
DOI: 10.1177/1087057108326141

91488 dimaleate, ranitidine hydrochloride, catechol, and thioperamide maleate were purchased from Sigma Chemical Co. (St. Louis, MO). Epic® 384-well cell assay microplates tissue-culture treated were obtained from Corning Inc. (Corning, NY) and used directly for cell culture.

Cell culture

Human epidermoid carcinoma A431 cells (American Type Cell Culture, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4.5 g/L glucose, 2 mM glutamine, and antibiotics; 1.8×10^4 cells at passages 3 to 15 suspended in 50 µl of the complete DMEM containing 10% FBS were placed in each well of an Epic® 384-well microplate, and were cultured at 37 °C under air/5% CO_2 for 1 day, followed by 20 h of starvation through continuous culture in the serum-free DMEM. The cell confluency at the time of assays was about 95% to 100%.

Optical biosensor system and biosensor cellular assays

Corning® Epic® system[23] was used for GPCR screens. The system uses integrated fiber optics to perform kinetic measurements of ligand-induced cellular responses with a time resolution of about 15 sec. The system is capable of detecting minute changes in the local index of refraction at the biosensor-cell interface, which are manifested as changes in resonant wavelength in picometers (pm). Because the local index of refraction within a cell is a function of the density and distribution of biomass (e.g., proteins, molecular complexes),[24] the optical response measured is related to ligand-induced dynamic mass redistribution (DMR) with the bottom portion of native cells.[18]

For cellular assays, compound solutions were made by diluting the stored stock solutions with HBSS (1× Hanks' balanced salt solution, 20 mM Hepes, pH 7.1) and transferred into a 384-well polypropylene compound storage plate to prepare a compound source plate. Two compound source plates were made separately when a 2-step assay was performed. In parallel, the cells were washed twice with the HBSS and maintained in 30 µl of the HBSS to prepare a cell assay plate. Both the cell assay plate and the compound source plate(s) were then incubated in the hotel of the reader system. After about 1 h of incubation, the baseline wavelengths of all biosensors in the cell assay microplate were recorded and normalized to zero. Afterward, a 2- to 10-min continuous recording was carried out to establish a baseline to ensure that the cells reached a steady state. Cellular responses were then triggered by transferring 10 µl of the compound solutions into the cell assay plate using an on-board liquid handler.

To study the influence of compounds on an agonist-induced response or to screen antagonists, a 2nd stimulation with an agonist at a fixed dose (typically at EC_{80} or EC_{100}) was applied. The resonant wavelengths of all biosensors in the microplate were normalized again to establish a 2nd baseline, right before the 2nd stimulation. The 2 stimulations were separated by about 1 h.

The Sigma-Aldrich LOPAC 1280™ library of compounds received were stored in bar-coded 96-well microplates as 10 mM solutions in neat dimethyl sulfoxide (DMSO). Compound microplates were aliquoted and reconfigured into 384-well compound source microplates. The compounds were diluted with the HBSS containing 1% DMSO. Because DMSO is a solvent having a high index of refraction and is also considered a cytotoxic agent at high doses, the presence of DMSO could interfere with the label-free measurements. Thus, to minimize the DMSO effect, the amount of DMSO between the cell solution and the compound solution was carefully matched when a compound solution contained greater than 0.1% DMSO. Once being matched, DMSO of less than 2% was found to have little impact on the label-free measurements (data not shown).

All studies were carried out at a controlled temperature (28 °C). The assay coefficient of variation was found to be <10%. All dose-dependent responses were analyzed using the nonlinear regression method with Prism software (Graph Pad).

RESULTS

Epinephrine led to G_s-mediated signaling in A431 cells

A431 cells endogenously express large numbers of the β_2AR, but not β_1 or β_3-ARs.[25] Epinephrine mediated a biphasic G_s-type DMR in quiescent A431 cells.[21,22] The epinephrine DMR proceeds with an initial decrease in signal (negative DMR or N-DMR), followed by an increase in signal (positive DMR or P-DMR) (**Fig. 1a**). The epinephrine response was dose dependent and saturable, leading to an apparent EC_{50} of 0.28 ± 0.07 nM ($n = 10$) based on the P-DMR amplitudes (**Fig. 1a** and **c**). The P-DMR amplitudes of the epinephrine responses were calculated using the wavelength shifts between 3 min and 50 min after stimulation. The β-blocker propranolol at 1 µM was found to completely inhibit the DMR induced by 2 nM epinephrine (data not shown), suggesting that the epinephrine response is β_2AR specific.[22]

Histamine led to G_q-mediated signaling in A431 cells

A431 cells also endogenously express the H_1R.[26] Histamine triggered a dose-dependent and saturable G_q-type DMR signal, which consists of an initial P-DMR and a subsequent N-DMR (**Fig. 1b**). The saturation curve, plotted as the P-DMR amplitude as a function of histamine concentration, appeared to fit well with sigmoidal nonlinear regression with variable slope, leading to an apparent EC_{50} of 687 ± 34 nM ($n = 6$) and a Hill coefficient of 1.87 (**Fig. 1c**). The P-DMR amplitudes of the histamine responses were calculated using the wavelength shifts between the baseline and 2 min after stimulation. The very steep activation curve observed suggests that there is positive cooperativity of the receptor through unknown mechanism(s). The pretreatment of A431 cells

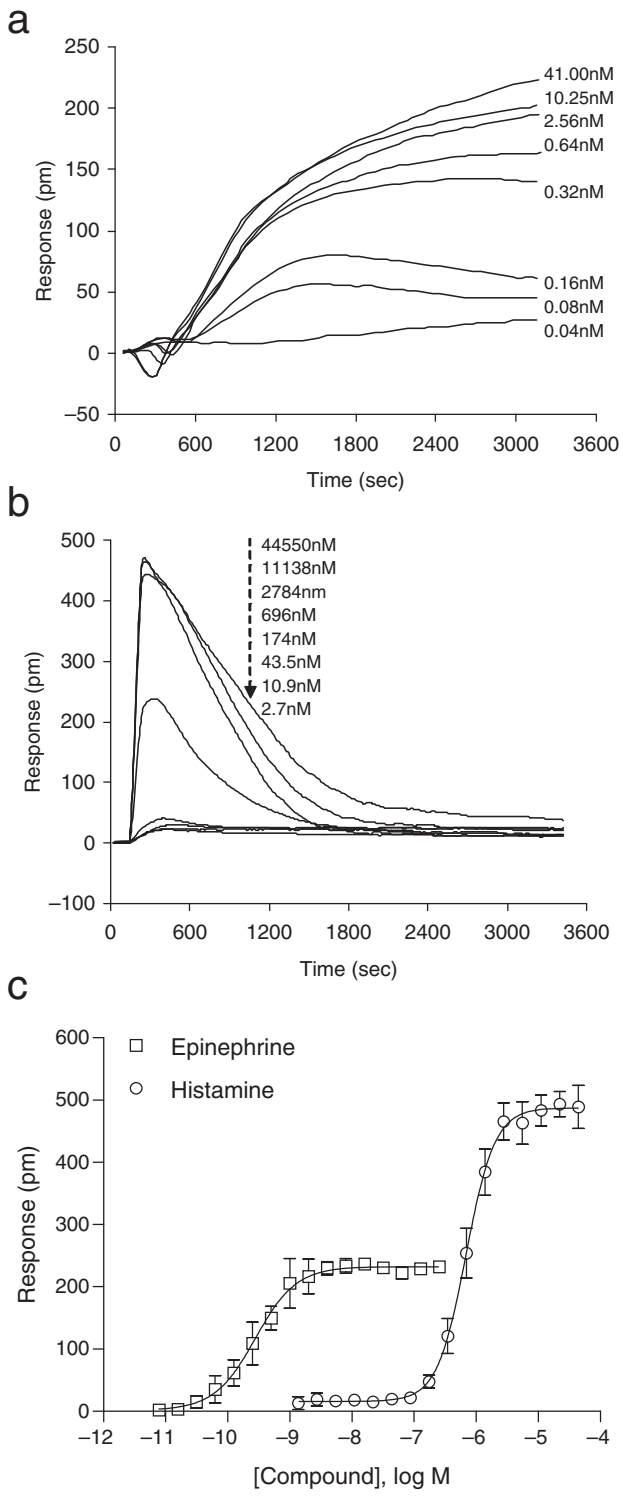

FIG. 1. The epinephrine- and histamine-mediated dynamic mass redistribution (DMR) signals in quiescent A431 cells. (**a**) The dose-dependent DMR of epinephrine. (**b**) The dose-dependent DMR of histamine. (**c**) The saturation curves. The amplitudes of the positive-DMR events for both the epinephrine and histamine responses were plotted as a function of the concentrations of compounds.

with H_1R-specific antagonists brompheniramine, chlorpheniramine, clemizole, clemastine, diphenhydramine, or triprolidine, each at 1 μM, completely inhibited the DMR signal induced by 1 μM histamine (data not shown). Conversely, the H_2 antagonists SKF91488 and ranitidine at 1 μM did not have obvious impact on the histamine response, nor did the H_3-specific antagonist thioperamide (data not shown). These results suggest that the histamine response is largely H_1R specific.

Costimulation with histamine and epinephrine led to a DMR signal additive to the 2 individual DMR signals

Compartmentalized signaling in which unique changes in 2nd messenger occur in both time and space has been established and is known to be central in GPCR signaling.[27,28] Thus, we speculated that the coactivation of 2 receptors coupled to distinct classes of G proteins will lead to a DMR signal that is largely the sum of the 2 individual responses. To test this hypothesis, quiescent A431 cells were stimulated with epinephrine and histamine individually or together. Results showed that indeed costimulation of quiescent A431 cells with a mixture of 1 μM histamine and 2 nM epinephrine led to a DMR signal that is close to the sum (i.e., the calculated signal) of the 2 individual DMR signals (**Fig. 2**). Since the epinephrine N-DMR event is very small relative to the histamine P-DMR, for the costimulation response the early P-DMR event was referred to the histamine response, and the later elevated response was the epinephrine response. Throughout the rest of the study, the histamine responses were referred to the wavelength shifts 2 min after the cosimulation, and the epinephrine responses were the wavelength shifts 50 min after the costimulation, as indicated in **Figure 2**. Nonetheless, this interesting finding strengthened our view that a ligand-induced DMR signal is an integrated cellular response, and is in proximate to globally represent receptor signaling and ligand pharmacology.[18-22] Furthermore, this result provided the 1st evidence that the signaling compartmentalization occurs not only at the 2nd messenger level or at the organization level of specific signaling complexes, but also at the complex signaling pathway level.

However, the costimulation DMR signal is not a simple addition of the 2 individual DMR signals. Although the initial P-DMR was largely identical to the calculated signal, the 2nd decaying phase of the costimulation response exhibited faster kinetics and bigger amplitude than the calculated signal (**Fig. 2**). This difference is possibly due to the crosstalk between the histamine-mediated signaling and the epinephrine-mediated signaling. To test this possibility, a 2-step desensitization assay[29] was performed. A431 cells were first pretreated with either epinephrine or histamine at different doses, and then subject to costimulation with a solution containing both 2 nM epinephrine and 1 μM histamine. Results showed that epinephrine dose-dependently attenuated both the histamine response and the epinephrine response (**Fig. 3a**), with almost identical IC_{50} (0.66 ± 0.20 nM and 0.38 ± 0.09 nM, n = 4,

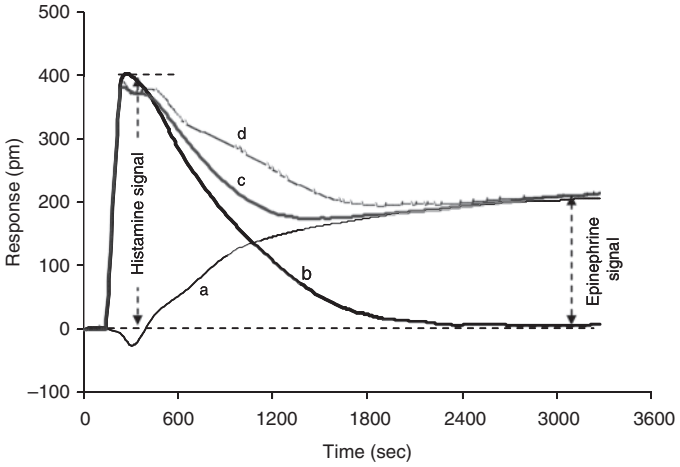

FIG. 2. The costimulation dynamic mass redistribution (DMR) of quiescent A431 cells. (**a**) The epinephrine (2 nM) DMR; (**b**) the histamine (1 μM) DMR; (**c**) the costimulation DMR (2 nM epinephrine and 1 μM histamine); and (**d**) the calculated DMR based on the simple addition of the 2 individual DMR signals.

respectively; **Fig. 3b**). Epinephrine at high doses completely inhibited the epinephrine response, but only partially attenuated the histamine response. Furthermore, the preceding histamine treatment completely inhibited the histamine response with an apparent IC_{50} of 1.6 ± 0.4 μM ($n = 4$), but only slightly attenuated the epinephrine response (**Fig. 3c** and **d**). Similar desensitization patterns were observed for either agonist when performed individually (data not shown). Together, these results suggest that there are crosstalks between the G_q- and G_s-mediated signaling.

Agonist screening with the biosensor cellular assays

The Sigma-Aldrich LOPAC 1280™ library of compounds includes 1280 bioactive small organic molecules against many major target classes including several GPCRs. Thus, the LOPAC was chosen to validate screening using the biosensor cellular assays. Because a ligand-induced DMR signal is an integrated response and many ligands often exhibit cross-activity to more than 1 target in the cells at high doses, the library was diluted with the HBSS containing 1% DMSO to obtain a final concentration of 1 μM to minimize the off-target effect(s). The DMSO was matched between the compound solution and the cell solution. Furthermore, a ligand-induced DMR is a real-time kinetic response and offers high-information content (e.g., phases, durations, amplitudes, and kinetics) for analyzing ligand pharmacology.[20,22] Thus, we were primarily interested in screening agonists for endogenous receptors in A431 cells using real-time kinetic measurements. **Figure 4** summarizes 4 major classes of DMR signals obtained with the library. Histamine led to an expected biphasic G_q-type DMR signal (**Fig. 4a**). Similar to epinephrine, isoproterenol led to a typical G_s-type DMR signal (**Fig. 4b**). CGP12177, a β_2AR partial agonist, led to a G_s-like DMR without the initial N-DMR (**Fig. 4c**). Similar to many compounds in the library, the potent β-blocker propranolol led to a net zero-DMR (i.e., no response) (**Fig. 4d**).

Based on the kinetic profiles of both the epinephrine and the histamine responses, 3 types of end-point measurements were chosen to determine the compound responses. First, the shift in wavelength between the baseline and 2 min after stimulation was calculated for each compound. Results showed that this measurement only identified 1 hit, histamine, from the library (data not shown). This is expected, because the histamine response is rapid and quite large, but the G_s-DMR signals obtained in A431 cells are slow and quite small initially.

Second, the shift in wavelength between before and 50 min after stimulation was calculated for each compound. As expected, this end-point measurement only identified hits for endogenous G_s-coupled receptors, but not for G_q-coupled receptors (data not shown). This is because a G_q-DMR often decays back toward the baseline, whereas a G_s-DMR leads to an elevated level over time.

Third, the shift in wavelength between 2 min and 50 min after stimulation was calculated for each compound and was plotted as a function of compounds. Results showed that this measurement is sufficient and robust to identify agonists for both G_q- and G_s-coupled receptors (**Fig. 5**).

Figure 5 summarizes the hit map for agonist screening. An agonist for the H_1R would lead to a large negative response, whereas a β_2AR agonist would lead to a positive response. A similar hit map was obtained using a 3-point measurement—before, and 2 min and 50 min after stimulation (data not shown).

The assay was robust with an averaged response of 8 ± 7 pm for the negative controls ($n = 128$), 230 ± 12 pm for the epinephrine positive controls ($n = 32$), and a Z' factor of 0.73. For each agonist, there were also 3 replicates of the dose series, leading to consistent EC_{50} values (0.20, 0.45, and 0.24 nM for epinephrine, and 658, 889, and 638 nM for histamine; data not shown).

The LOPAC agonist screen identified only 1 hit (histamine) for the G_q-coupled H_1R. As expected, histamine led to a large negative response. The LOPAC contains the broad spectrum histamine receptor agonist histamine and 2 H_3R-specific agonists, R(–)-α-methyl-histamine and imetit. Similar to the 2 H_3R agonists, P2Y agonists in the library also did not lead to any obvious signal, probably because of their low potencies to activate endogenous P2Y receptors in A431 cells.[21]

In contrast, there were many hits that lead to positive responses with distinct amplitudes. Based on the amplitudes of the epinephrine-positive controls (230 ± 12 pm), hits that led to a response from 170 to 280 pm were considered as full or strong partial agonists. Among 63 hits as full or strong partial agonists, there were 12 adenosine receptor agonists, 27 adrenoceptor agonists, and 7 dopamine receptor agonists. In addition, hits that led

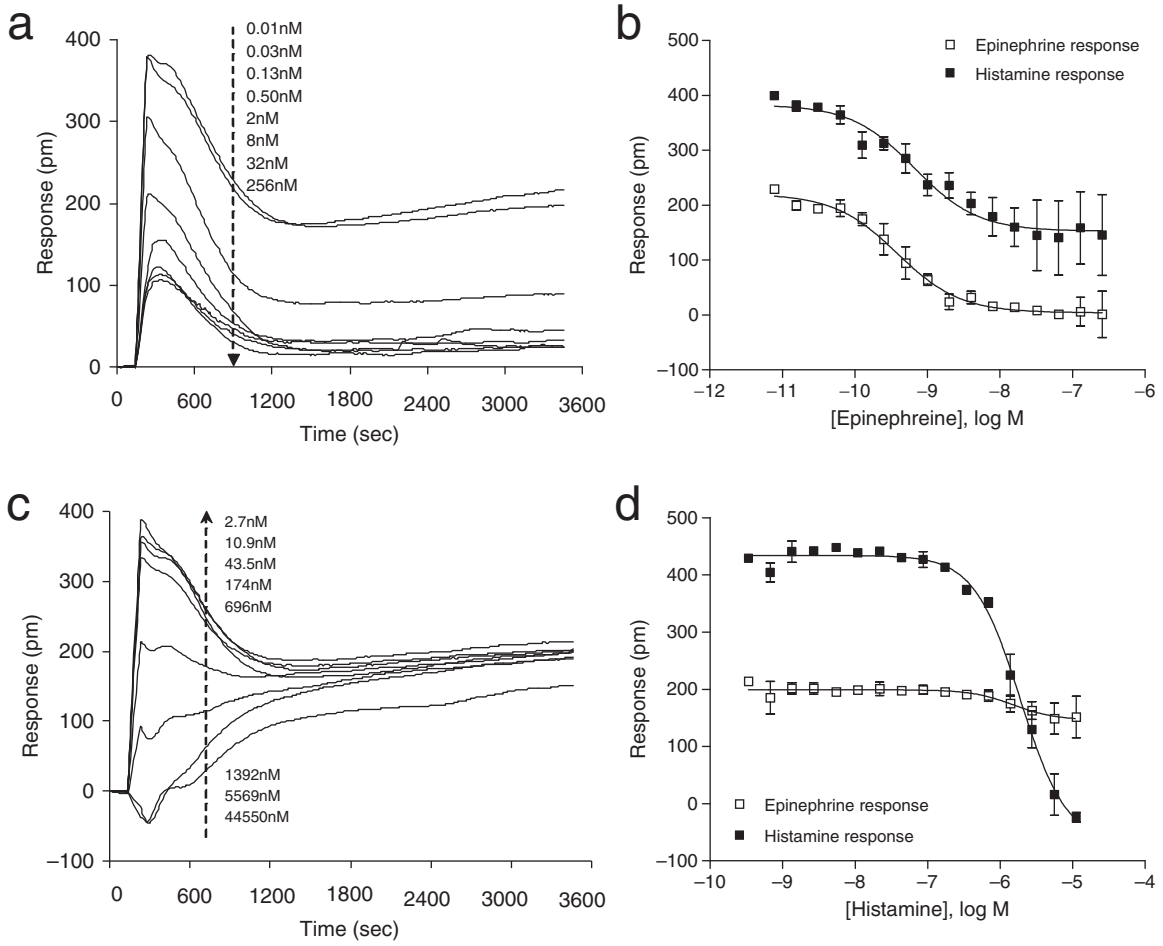

FIG. 3. Crosstalks between the histamine and epinephrine responses. (**a**, **b**) The impacts of epinephrine at different doses on the succeeding costimulation dynamic mass redistribution (DMR) signal. (**c**, **d**) The impacts of histamine at different doses on the succeeding costimulation DMR signal. The costimulation was carried out using 2 nM epinephrine and 1 μM histamine.

to a response from 40 to 170 pm were considered to be partial or weak partial agonists; 51 hits fall into this category, including 6 adenosine agonists, 6 adrenoceptor agonists, and 5 dopamine agonists. The high hit rate obtained reflects the fact that A431 cells also endogenously express adenosine receptors whose activation also led to an epinephrine-like DMR,[21] and that many dopamine agonists are known to activate the β_2AR.[22,30,31] Except for the 2 less potent β-AR agonists (±)-ephedrine and amiodarone, all other β-AR agonists in the library were correctly identified, indicating low false-negative rate.

Many GPCR ligands exhibit pathway-biased efficacies.[12,13] Previously we showed, using the biosensor cellular assays, that some β_2AR ligands display functional selectivity.[22] Consistent with these findings was that all β_2AR agonists identified led to similar DMR signals but with distinct characteristics. The β_2AR ligand-induced DMR can be classified into 3 categories—the isoproterenol DMR, the CGP12177 DMR, and the propranolol DMR (**Fig. 3b–d**). The ligands that caused an isoproterenol-like DMR include R(–)-isoproterenol, S(+)-isoproterenol, (–)-isoproterenol, (±)-isoproterenol, (–)-epinephrine, (±)-epinephrine, L(–)-norepinephrine, (±)-norepinephrine, (–)-α-methylnorepinephrine, albuterol, BRL 37344, R(–)-denopamine, dobutamine, dopamine, N-methyldopamine, fenoterol, formoterol, 4-hydroxyphenethylamine, isotharine, metaproterenol, nylidrin, phenylephrine, ritodrine, salbutamol, salmeterol, (±)-synephrine, terbutaline, and tulobuterol. It is worth noting that these DMR signals also differ in fine features, such as the amplitudes of both N- and P-DMR events and the kinetics of the P-DMR events (data not shown). For example, the known partial agonist dopamine or dobutamine led to a DMR with a smaller N-DMR and a faster P-DMR than the full agonist R(-)-isoproterenol. Ligands that induced a CGP12177-like DMR include alprenolol, (±)CGP12177A, CL316243, 6-fluoronorepinephrine, labetalol, (±)octopamine, pindolol, S(–)-pindolol, pinodolol,

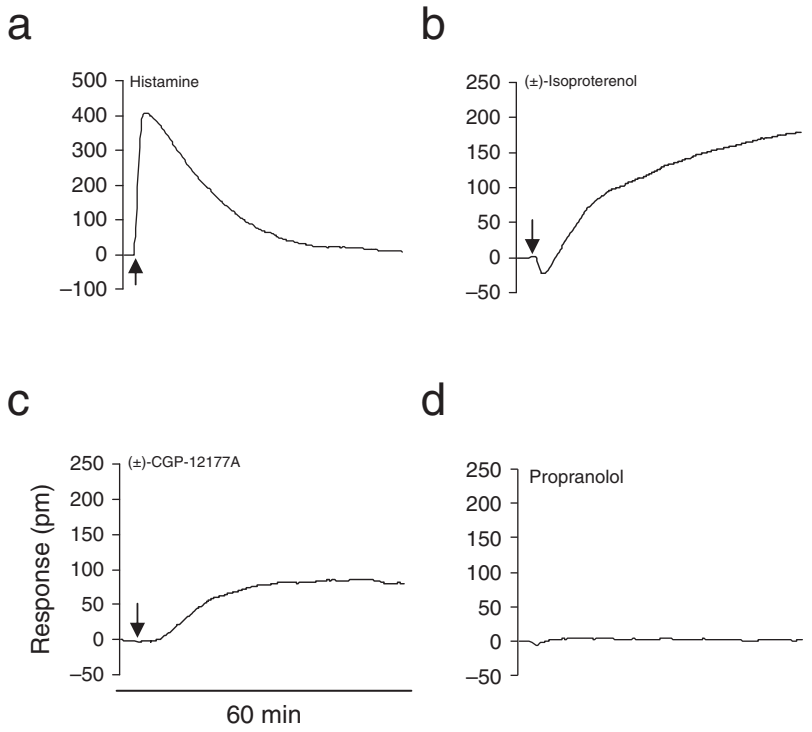

FIG. 4. Representative dynamic mass redistribution (DMR) signals of the LOPAC compounds in the agonist screen. (**a**) Histamine; (**b**) isoproterenol, (**c**) CGP12177A, and (**d**) propranolol, each at 1 μM. The black arrows indicate the time when the compound indicated was introduced. LOPAC = Sigma Library of Pharmaceutically Active Compounds.

and xamterol. Similar to propranolol, other β-antagonists led to almost net-zero DMR. Combined with our previous multiparameter analysis concerning a small set of β_2AR agonists,[22] these results suggest that the biosensor cellular assays are suited for screening functional selective agonists for endogenous receptors and lead to low false negatives due to its pathway-unbiased nature.

Duplexed antagonist screening with the biosensor cellular assays

Inasmuch as the histamine and epinephrine costimulation DMR contains characteristics of both histamine- and epinephrine-mediated signaling, we examined the possibility of duplexed antagonist screening in a single assay. This assay proceeded with the initial agonist screen for about 1 h, followed by a costimulation with histamine and epinephrine. The impacts of compounds on both the histamine response and the epinephrine response were examined (**Fig. 6**). For the epinephrine response, there were 77 apparent antagonists with a suppressed response of 20 ± 30 pm, 57 partial inhibitors with a partially attenuated response of 90 ± 40 pm, and 1146 noninhibitors with a response of 190 ± 60 pm. For the histamine response, there were 51 apparent antagonists with a suppressed response of 15 ± 50 pm, 79 partial inhibitors with a partially attenuated response of 165 ± 100 pm, and 1160 noninhibitors with a response of 370 ± 110 pm.

Correlation analysis between the agonist screen and the antagonist screen provided further clarification about the action of positive hits acting on the H_1R or β_2AR (**Fig. 7**). Histamine led to a G_q-type DMR and also caused the complete desensitization of cells to the 2nd stimulation with histamine. All known H_1R antagonists in the library caused no obvious DMR in the agonist screen, but completely or almost completely attenuated the histamine response in the antagonist screen. These ligands include (±)-brompheniramine, (±)-chlorpheniramine, (+)-brompheniramine, (+)-chlorpheniramine, clemizole, clemastine, diphenhydramine, fexofenadine, doxylamine, methapyrilene, promethazine, pyrilamine, terfenadine, ketotifen, loratadine, pheniramine, and triprolidine. In contrast, neither H_2R- nor H_3R-specific antagonists caused any significant inhibition on the histamine response.

The ligands that led to G_s-like DMR signals in the agonist screen also suppressed significantly the epinephrine response; and the desensitization is well correlated with the DMR amplitude of the ligand in the agonist screen. The bigger the response of the ligand in the agonist screen, the greater desensitization it causes. However, these ligands greatly differ in their ability to attenuate the histamine response.

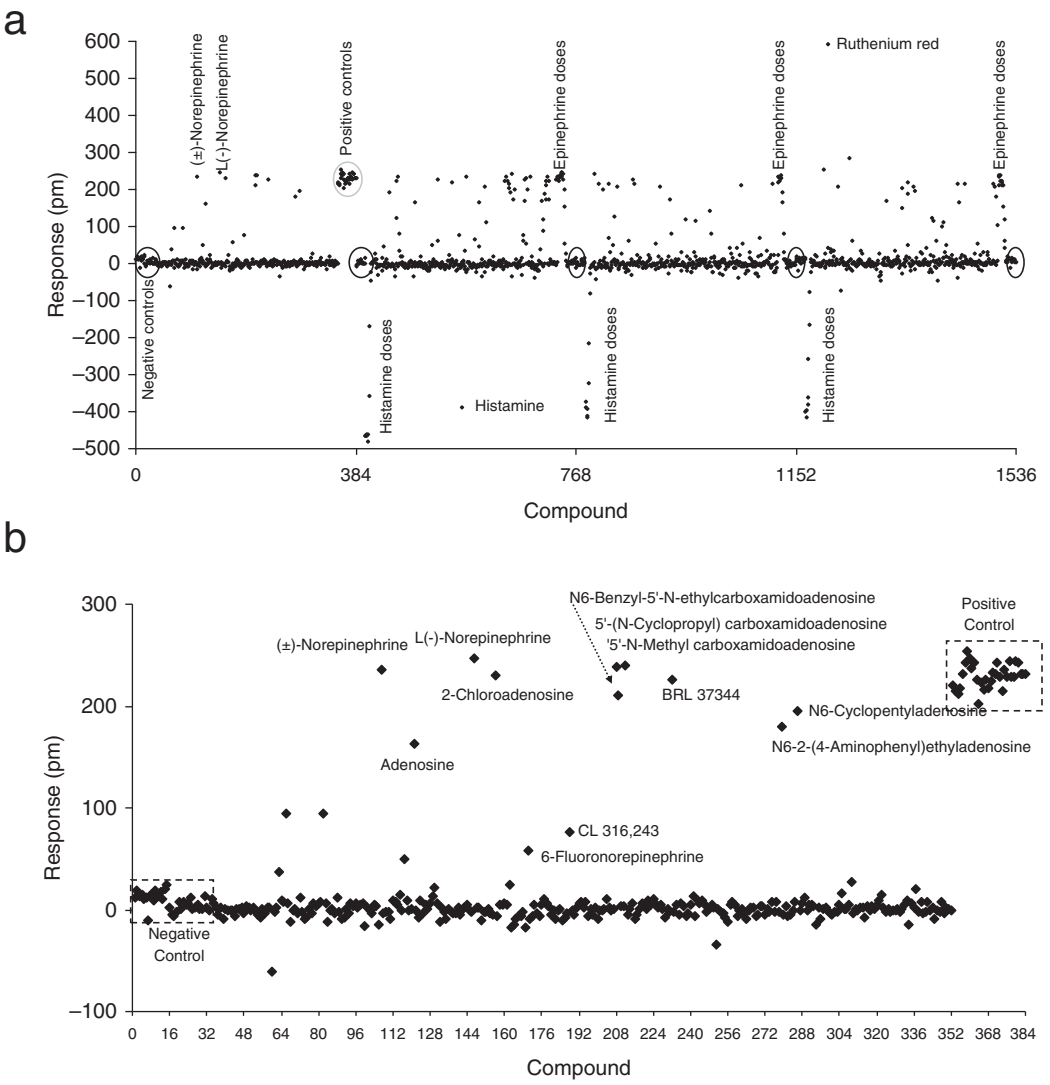

FIG. 5. The hit map of the LOPAC compound agonist screen. (**a**) The responses, the wavelength shifts between 2 min and 50 min after stimulation, were plotted as a function of compounds. In this set of the LOPAC screen, plate 1 contained 2 columns of the negative controls (i.e., the HBSS only), and 2 columns of positive controls for 2 nM epinephrine. For the other 3 plates, there were also 2 columns of the negative controls (columns 1 and 24), a concentration series of epinephrine (column 23) and of histamine (column 2). (**b**) The representative hit map shown in plate 1. The positive hits for both the β_2AR and the adenosine receptors are indicated in the hit map. LOPAC = Sigma Library of Pharmaceutically Active Compounds; HBSS = 1× Hanks' balanced salt solution, 20 mM Hepes, pH 7.1.

The antagonist screen also identified 9 β-blockers that did not result in any DMR signals in the agonist screen, but acted as antagonists to inhibit the epinephrine response. These antagonists were betaxolol, S(–)-timolol, (S)-(–)-propafenone, (S)-propranolol, (±)-metoprolol, SR 59230A, (±)-propranolol, ICI 118,551, and (±)-sotalol. These β-blockers had little impact on the histamine response.

The biosensor screen also discovered several interesting hits. The 2 H_2R antagonists famotidine and SKF95282 led to a CGP12177-like DMR in the agonist screen, and also antagonized the epinephrine response but not the histamine response in the antagonist screen, suggesting that these ligands are able to activate the cAMP-PKA pathway through unknown mechanism(s). In addition, several adrenoceptor uptake/reuptake inhibitors including nortriptyline, amitriptyline, and doxepin did not trigger any DMR in the agonist screen, and had little effect on the epinephrine response but almost completely inhibited the histamine response in the

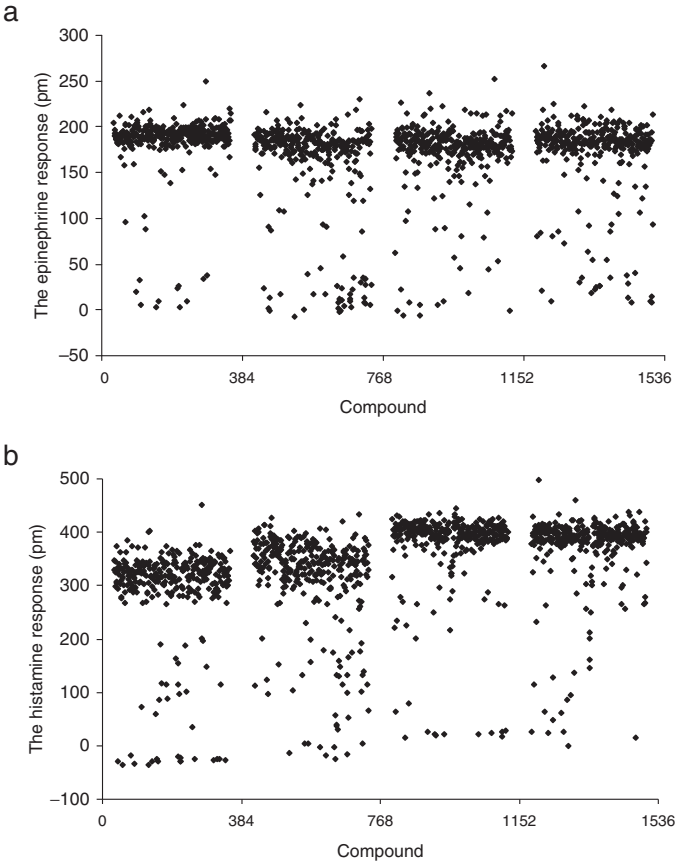

FIG. 6. The hit maps of the LOPAC compound antagonist screen using the duplexed biosensor cellular assays. The epinephrine responses (**a**) or the histamine response (**b**) in the antagonist screen step were plotted as a function of compounds. The cells were treated first with the LOPAC compounds, followed by the costimulation with 2 nM epinephrine and 1 μM histamine. The controls were excluded in these plots. LOPAC = Sigma Library of Pharmaceutically Active Compounds.

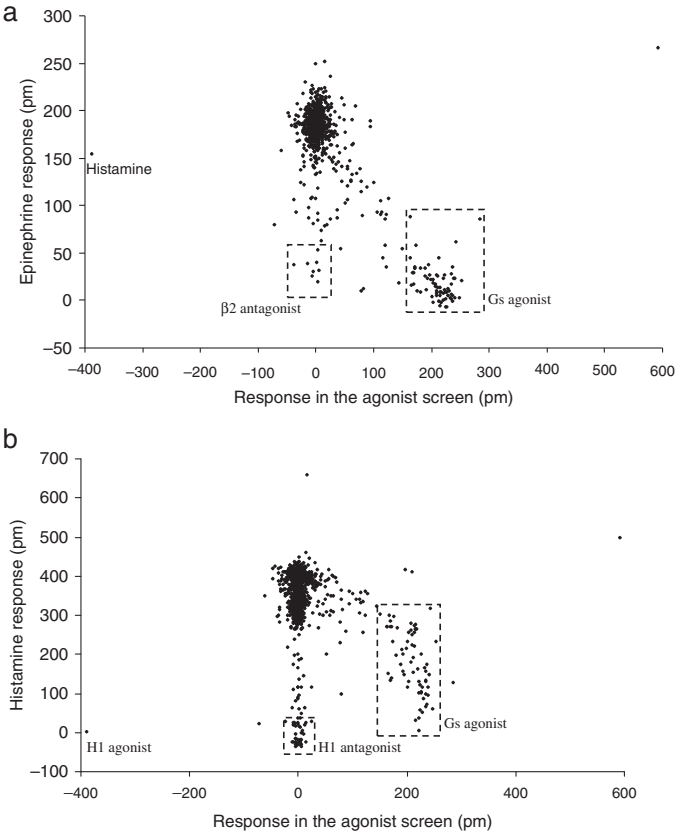

FIG. 7. The correlation analysis between the responses in the initial agonist screen step and the epinephrine responses (**a**) or the histamine responses (**b**) in the succeeding antagonist screen step.

antagonist screen. These findings are interesting and warrant further studies.

DISCUSSION

Compartmentalization in GPCR signaling

The notion of compartmentalization in which unique changes in the 2nd messenger occur in both time and space was established with the advancements in high-resolution single-cell imaging systems and in the design of fluorescent probes.[28] Today, it is widely accepted that GPCR signaling involves a series of highly regulated spatial and temporal events besides the production and regulation of 2nd messengers such as Ca^{2+} and cAMP.[32,33] Recently we found that the RWG biosensor is able to measure an integrated cellular response relating to ligand-induced dynamic mass redistribution in cells within the detection zone of the biosensor.[18] The DMR signals recorded offer a novel readout for receptor signaling and ligand pharmacology. Here we applied the biosensor to study the DMR signal of quiescent A431 cells in response to costimulation with histamine and epinephrine. Histamine mediated signaling through endogenous G_q-coupled H_1R, and epinephrine mediated signaling through endogenous G_s-coupled β_2AR. Results showed that the costimulation led to a DMR signal that is largely the sum of the 2 individual DMR signals, indicating that the cells can respond synergistically to the costimulation. This result is significant, because it implies that G_q- and G_s-mediated signaling largely undertakes distinct routes at the complex pathway level.

Crosstalks in GPCR signaling

Amassing evidence accumulated in recent years suggests that receptor signaling does not work in isolation, and distinct receptors can crosstalk with each other at multiple levels.[34-37] The crosstalk can occur through interaction of intracellular signal transduction pathways, phosphorylation of receptors and regulatory proteins by kinases, physical interaction between

receptors, or effects on 2nd messengers. The crosstalk ensures the exchange of information between the individual signaling pathways and provides the molecular basis for their cooperation. Here using the biosensor cellular assays, we found that histamine slightly attenuated the epinephrine response, and epinephrine partially attenuated the histamine response, both in a dose-dependent manner. Furthermore, the epinephrine and histamine costimulation DMR signal is largely, but not simply, an addition of the 2 individual DMR signals. Taken together, these results indicate that there are crosstalks between the epinephrine- and histamine-mediated signaling. In addition, many adenosine receptor agonists in the library also caused almost complete desensitization of cells to the succeeding epinephrine stimulation, consistent with the well-established heterologous desensitization through the cAMP-PKA pathway for G_s-coupled receptors.[35]

Ligand-directed functional selectivity

The ability of a receptor to couple with more than 1 G protein as well as other regulatory proteins indicates the complexity of receptor signaling.[10,11,38] As a result, GPCRs display rich behaviors in cells. Additionally, many ligands can induce operative bias to favor specific portions of the cell machinery and exhibit pathway-biased efficacies. Our recent study concerning the functional selectivity of a small set of ligands acting on endogenous β_2AR in A431 cells uncovered a strong correlation between the structures of the ligands and the characteristics of their DMR signals.[22] Consistent with these findings was that the β-AR ligands in the LOPAC also resulted in characteristic DMR signals indicative of pathway-biased activity on the β_2AR. These β_2AR agonists suppressed the epinephrine response in the costimulation step; and the epinephrine amplitude is in inverse relation to the respective DMR of a ligand in the initial agonist screen. Interestingly, the 2 H_2R antagonist famotidine and SKF95282 also displayed functional selectivity acting on unknown G_s-coupled receptor(s). These results suggest that the biosensor cellular assays enable screening of pathway-biased ligands.

Multiplexed GPCR screening using the biosensor cellular assays

The biosensor cellular assays are multiplexing in nature.[39] For agonist screening, the biosensor is naturally suited for multiplexed screening agonists for endogenous receptors regardless of their coupled G proteins, because the biosensor cellular assays are pathway unbiased but pathway sensitive. Screening the LOPAC using quiescent A431 cells identified agonists for the G_q-coupled H_1R, and the G_s-coupled adenosine receptors and β_2AR. The agonist screen also identified some dopamine receptor ligands, whose action is mostly mediated through the activation of the β_2AR.[22,30,31] However, the 4 P2Y agonists in the library were not identified as hits, probably because of their low potencies to activate endogenous P2Y receptors.

The biosensor cellular assays are also multiplexing in nature for antagonist screening. This is largely due to heterologous desensitization wherein the activation of 2nd messenger–dependent protein kinases of a different receptor causes down-regulation of a GPCR.[35] As a result, similar to the adenylyl cyclase activator forskolin, the adenosine receptor agonists led to G_s-type DMRs in the agonist screen, and were also detected as antagonists for the β_2AR in the dual receptor antagonist screen. Similarly, the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) led to a small G_q-like DMR in the agonist screen, and was also detected as an antagonist for the H_1R (data not shown).

Label-free biosensor cellular assays for target-specific screening

A ligand-induced DMR signal is an integrated response and consists of contributions from many cellular events downstream the receptor activation. Contributions from these events mediated through a receptor that make the biosensor cellular assays so valuable, however, also render the DMR signal obtained "nonspecific" relative to conventional cellular assays. To exacerbate the situation is the complexity of receptor signaling, the pathway-selective activity and cross-reactivity to other receptors of ligands, and the multiplexing in nature of the biosensor cellular assays. Thus, caution is warranted to analyze ligand pharmacology and to screen ligands for the target receptors using the biosensor cellular assays.

To achieve target-specific screening, several approaches can be applied. For a given cell or cell system, receptor panning should be performed to predetermine how many receptors can be detected using the biosensor cellular assays. Then receptor biology and ligand pharmacology should be systematically studied to evaluate the signaling potentials of the target receptor, and to determine the potential interference of others with the target receptor. When 1 or more endogenous receptors interfere with the target receptor, an antagonist cocktail solution that blocks the activity of these receptors can be used to minimize the "false" positives for the target receptor. Alternatively, a cell engineering approach can also be used either to boost the target-specific DMR signal or to suppress the signal mediated through receptors other than the target. A counter screen between an engineered cell line and its parental cell line, or different cell lines, can also be employed to fish out the positive hits for the target receptor.

Here we described a duplexed biosensor cellular assay for screening agonists for both the β_2AR and the H_1R. In conjunction with the ability to perform both agonist and antagonist screens in a single assay because of the noninvasive nature of the biosensor measurements, correlation analysis can be performed in multiple ways. The correlation analysis between the 2 screens enable sorting of agonists and antagonists for each receptor (**Fig. 6**). The correlation between multiple time point responses differentiates distinct classes of agonists for distinct classes of GPCRs (**Fig. 5**). The correlation between the 2 target receptors identifies pathway modulators (data not shown).

In summary, we have applied noninvasive and label-free biosensor cellular assays to study the signaling induced by the costimulation of quiescent A431 cells with histamine and epinephrine. Based on the costimulation DMR profile that is largely the sum of the 2 individual DMRs, we developed a duplexed biosensor assay to screen both agonists and antagonists for the endogenous β_2AR and H_1R. The costimulation profiles obtained strengthen our previous assessment that a ligand-induced DMR is an integrated cellular response. Screening results using the LOPAC indicate that the biosensor cellular assay is able to robustly identify agonists for multiple receptors, particularly to screen pathway-biased ligands for G_s-coupled receptors. The present study documents the potential advantages and disadvantages of label-free biosensor cellular assays for drug discovery, particularly GPCR screens.

REFERENCES

1. Drews J: Drug discovery: a historical perspective. *Science* 2000;287:1960-1963.
2. Hopkins AL, Groom CR: The druggable genome. *Nat Rev Drug Discov* 2002;1:727-730.
3. Fredriksson R, Schioth HB: The repertoire of G-protein-coupled receptors in fully sequenced genomes. *Mol Pharmacol* 2005;67:1414-1425.
4. Milligan G: High-content assays for ligand regulation of G-protein-coupled receptors. *Drug Discov Today* 2003;8:579-585.
5. William C: cAMP detection methods in HTS: selecting the best from the rest. *Nat Rev Drug Discov* 2004;3:125-135.
6. Butcher EC: Can cell systems biology rescue drug discovery? *Nat Rev Drug Discov* 2005;4:461-467.
7. Leifert WR, Aloia AL, Bucco O, Glatz RV, McMurchie EJ: G-protein-coupled receptors in drug discovery: nanosizing using cell-free technologies and molecular biology approaches. *J Biomol Screen* 2005;10:765-779.
8. Fang Y, Frutos AG, Verkleeren R: Label-free cell-based assays for GPCR screening. *Comb Chem High Throughput Screen* 2008;11:357-369.
9. Mailman RB: Ligand-selective signaling and high content for GPCR drugs. *Trends Pharmacol Sci* 2007;8:390-396.
10. Hermans E: Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacol Ther* 2003;99:25-44.
11. Lefkowitz RJ, Shenoy SK: Transduction of receptor signaling by β-arrestins. *Science* 2005;308:512-517.
12. Kenakin T: New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nat Rev Drug Discov* 2005;4:919-927.
13. Galandrin S, Oligny-Longpre G, Bouvier M: The evasive nature of drug efficacy: implications for drug discovery. *Trends Pharmacol Sci* 2007;8:423-430.
14. Fang Y, Frutos AG, Lahiri J: Membrane protein microarrays. *J Am Chem Soc* 2002;124:2394-2395.
15. Fang Y, Lahiri J, Picard L: G protein-coupled receptor microarrays for drug discovery. *Drug Discov Today* 2003;8:755-761.
16. Cooper MA: Optical biosensors in drug discovery. *Nat Rev Drug Discov* 2002;1:515-528.
17. Tollin G, Salamon Z, Hruby VJ: Techniques: plasmon-waveguide resonance (PWR) spectroscopy as a tool to study ligand-GPCR interactions. *Trends Pharmacol Sci* 2003;24:655-659.
18. Fang Y, Ferrie AM, Fontaine NH, Mauro J, Balakrishnan J: Resonant waveguide grating biosensor for living cell sensing. *Biophys J* 2006;91:1925-1940.
19. Fang Y, Li G, Peng J: Optical biosensor provides insights for bradykinin B_2 receptor signaling in A431 cells. *FEBS Lett* 2005;579:6365-6374.
20. Fang Y, Ferrie AM, Fontaine NH, Yuen PK: Characteristics of dynamic mass redistribution of EGF receptor signaling in living cells measured with label free optical biosensors. *Anal Chem* 2005;77:5720-5725.
21. Fang Y, Li G, Ferrie AM: Non-invasive optical biosensor for assaying endogenous G protein-coupled receptors in adherent cells. *J Pharmacol Toxicol Methods* 2007;55:314-322.
22. Fang Y, Ferrie AM: Label-free optical biosensor for ligand-directed functional selectivity of acting on β_2 adrenoceptor in living cells. *FEBS Lett* 2008;582:558-564.
23. Li G, Ferrie AM, Fang Y: Label-free profiling of endogenous G protein-coupled receptors using a cell-based high throughput screening technology. *J Assoc Lab Automat* 2006;11:181-187.
24. Barer R, Joseph S: Refractometry of living cells. Part I. Basic principles. *Q J Microsc Sci* 1954;95:399-423.
25. Delavier-Klutchko C, Hoebeke J, Strosberg AD: The human carcinoma cell line A431 possesses large numbers of functional β_2-adrenergic receptors. *FEBS Lett* 1984;169:151-155.
26. Tilly BC, Tertoolen LGJ, Remorie R, Ladoux A, Verlaan I, de Laat SW, et al: Histamine as a growth factor and chemoattractant for human carcinoma and melanoma cells: action through calcium-mobilizing H1 receptors. *J Cell Biol* 1990;110:1211-1215.
27. Houslay MD, Milligan G: Tailoring cAMP-signalling responses through isoforms multiplicity. *Trends Biochem Sci* 1997;22:217-244.
28. Willoughby D, Cooper DM: Organization and Ca^{2+} regulation of adenylyl cyclases in cAMP microdomains. *Physiol Rev* 2007;87:965-1010.
29. Fang Y, Ferrie AM: Optical biosensor differentiates signaling of endogenous PAR_1 and PAR_2 in A431 cells. *BMC Cell Biol* 2007;8:e24.
30. Swaminath G, Deupi X, Lee TW, Zhu W, Thian FS, Kobilka TS, et al: Probing the β_2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists. *J Biol Chem* 2005;280:22165-22171.
31. Liapakis G, Chan WC, Papadokostaki M, Javitch JA: Synergistic contributions of the functional groups of epinephrine to its affinity and efficacy at the β_2 adrenergic receptor. *Mol Pharmacol* 2004;65:1181-1190.
32. Jordan JD, Landau EM, Iyengar R: Signaling networks: the origins of cellular multitasking. *Cell* 2000;103:193-200.
33. Kholodenko BN: Four-dimensional organization of protein kinase signaling cascades: the roles of diffusion, endocytosis and molecular motors. *J Exp Biol* 2003;206:2073-2082.
34. Houslay MD, Kolch W: Cell-type specific integration of cross-talk between extracellular signal-regulated kinase and cAMP signaling. *Mol Pharmacol* 2000;58:659-668.
35. Chuang TT, Lacovelli L, Sallese M, de Blasi A: G protein-coupled receptors: heterologous regulation of homologous desensitization and its implications. *Trends Pharmacol Sci* 1996;17:416-421.

36. Watts VJ, Neve KA: Sensitization of adenylate cyclase by $G_{\alpha i/o}$-coupled receptors. *Pharmacol Ther* 2005;106:405-421.
37. Bouvier M: Oligomerization of G-protein-coupled transmitter receptors. *Nat Rev Neurosci* 2001;2:274-286.
38. Sun Y, Huang J, Xiang Y, Bastepe M, Juppner H, Kobilka BK, et al: Dosage-dependent switch from G protein-coupled to G protein-independent signaling by a GPCR. *EMBO J* 2007;26:53-64.
39. Fang Y: Label-free cell-based assays with optical biosensors in drug discovery. *Assays Drug Dev Technol* 2006;4:583-595.

Address correspondence to:
Ye Fang
Biochemical Technologies
Science and Technology Division
Corning Incorporated
Sullivan Park, Corning, NY 14831

E-mail: fangy2@corning.com