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RESEARCH ARTICLE

Label-free optical biosensor for probing integrative role of adenylyl cyclase in G protein-coupled receptor signaling

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Abstract

Adenylyl cyclase is considered as an integrator for receptor signaling. However, its integrative role in receptor signaling is largely studied at the level of point of contacts in complex pathways. Here we used forskolin as a pharmacological probe and the resonant waveguide grating (RWG) biosensor to examine the signal integration of G protein-coupled receptors (GPCRs) at the cyclase-cyclic AMP-PKA module. The biosensor is a refractive index sensitive optical biosensor that is capable of detecting ligand-induced dynamic mass redistribution in cells without labels and cellular manipulations. Stimulation of seven cell lines with forskolin led to distinct optical responses, indicative of distinct expressions and/or organization of cyclase isoforms. The forskolin response in A431 was sensitive to the activities of protein kinase A, Rho kinase, and MAP kinases. Desensitization assays showed that the forskolin pretreatment heterologously desensitized G_s signaling, partially attenuated G_q signaling, but had complicate impacts on G_i signaling. This study documents the integrative role of adenylyl cyclase in GPCR signaling and the power of forskolin as a pharmacological probe to differentiate receptor signaling using the label-free biosensor cellular assays.

KeyWords: *Optical biosensor; resonant waveguide grating biosensor; G protein-coupled receptor; adenylyl cyclase; signal integration; forskolin*

Introduction

The past several decades have witnessed dramatic evolution in the concept of receptor signaling from a single and linear cascade to signaling network and interactions mediated through the activation of a receptor (1). The cell relies on a myriad of signaling transduction systems in its decision-making processes, and the spatial-temporal integration of various signaling systems demands that such processes communicate with each other in feedforward and feedback regulatory loops (2). A common means of achieving this is for key components in one signaling system to be expressed as isoforms that differ in their ability to couple to other regulatory signaling systems, as exampled in adenylyl cyclase (AC). Adenylyl cyclase is a lyase enzyme whose activation leads to production of cyclic adenosine 3′,5′-monophosphate (cAMP). cAMP is a prototypical second messenger to relay extracellular signals to intracellular effectors including protein kinase A (PKA). There are nine membrane-bound isoforms of adenylyl cyclase with distinct tissue distribution (3) and modes of regulation by G protein subunits, by Ca^{2+} and by phosphorylation (4,5). Such differential sensitivity to regulation, together with spatiotemporal organization of cAMP signals generated, permit diverse interplay of the AC-cAMP module with a range of signaling pathways.

Parallel is the radical change in approaches to study cell signaling and its network interactions. Approaches based on a single readout in a signaling cascade have been, and will be, valuable to provide insights into cell signaling. However, new methods and technologies

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that enable systematic study of complex interactions in cells or cell systems have emerged and started gaining attraction in both fundamental research and industrial applications (6). Recently, label-free optical biosensors including resonant waveguide grating (RWG) that have been routinely used for biomolecular interaction analysis have found applications in whole cell sensing (7–13). Instead of measuring single point of contacts within complex signaling pathways, RWG biosensor can follow the evolution of receptor signaling in time and space, leading to a kinetic and integrative response of cells, termed dynamic mass redistribution (DMR) (8). The DMR measures enable systems cell biology studies of receptor signaling (9,10). These biosensor cellular assays are pathway-unbiased but pathway-sensitive (11,12). However, a common drawback to all label-free biosensor cellular assays is the apparent "nonspecificity" of the biosensor responses relative to conventional cellular assays, due to the complexity and cellular context dependency of receptor signaling, and the pathway-biased agonism of many ligands (12,14). Thus, a chemical biology tool to differentiate receptor signaling would be greatly beneficial to recapitulate the complexity of biology in a system that is amenable to mechanistic deconvolution.

Here we used the well-known AC activator forskolin as a pharmacological probe and applied label-free biosensor cellular assays to study the signal integration of endogenous G protein-coupled receptors (GPCRs) at the AC-cAMP-PKA module. Forskolin differentially modulated the DMR signals mediated through the activation of different classes of GPCRs, suggesting the crucial roles of adenylyl cyclases in the integration of receptor signaling.

Materials and methods

Materials

Forskolin was obtained from Tocris (St. Louis, MO). SFLLR-amide and vasoactive intestinal peptide (VIP) were obtained from Bachem (King of Prussia, PA). Adenosine 5′-triphosphate (ATP), dopamine, epinephrine, histamine, NECA, fatty acid-free bovine serum albumin (BSA), dioctanoyl-L-α-phosphatidic acid (DPA), 1-oleoyl-L-α-lysophosphatidic acid (LPA), and nicotinic acid were obtained from Sigma (St. Louis, MO). Both DPA and LPA were dissolved in chloroform-methanol and then resuspended in buffer containing 0.1% BSA. The rest of the compounds were dissolved in dimethyl sulfoxide (DMSO). The kinase inhibitor library containing 80 kinase inhibitors dissolved in DMSO was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Cell culture-compatible Epic® 384well RWG biosensor microplates were obtained from Corning Inc (Corning, NY) and used directly for cell culture.

Cell culture

All cell lines were obtained from American Type Cell Culture (Manassas, VA). The cell culture medium used was 1) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4.5g/L glucose, 2 mM glutamine, and antibiotics for human epidermoid carcinoma A431, human lung carcinoma A549, and human hepatocellular carcinoma HepG2C3A; 2) Kaighn's modification of Ham's F12 medium (F-12K) supplemented with 10% FBS, 2 mM L-glutamine, 3 mg/mL sodium bicarbonate, and antibiotics for Chinese hamster ovary (CHO-K1); 3) McCoy's 5a medium modified supplemented with 10% FBS, 4.5g/L glucose, 2 mM glutamine, and antibiotics for human colorectal adenocarcinoma HT29; and 4) RPMI-1640 medium supplemented with 10% FBS, 4.5 g/L glucose, 2 mM glutamine, and antibiotics for human rhabdomyosarcoma RMS 13; and 5) minimum esential medium (MEM) with 2 mM glutamine, 4.5 g/L glucose, 10% FBS, and antibiotics for human embryonic kidney HEK293.

Cells were typically grown by using ~1–2 × 10^4 cells per well at passage 3–15 suspended in 50 μL of the corresponding culture medium in the biosensor microplate and were cultured at 37°C under air/5% CO_2 for ~1 day. Except for A431, which was subject to ~20-hr starvation through continuously culture in the serum-free DMEM, the other cell types were directly assayed without starvation. The confluency for all cells at the time of assays was ~95%–100%.

Optical biosensor system and cell assays

Corning® Epic® wavelength interrogation system was used for whole cell sensing. This system consists of a temperature control unit, an optical detection unit, and an onboard liquid handling unit with robotics. The detection unit is centered on integrated fiber optics and enables kinetic measures of cellular responses with a time interval of ~15 sec.

The RWG biosensor is capable of detecting minute changes in local index of refraction near the sensor surface. Because the local index of refraction within a cell is a function of density and its distribution of biomass (e.g., proteins and molecular complexes), the biosensor exploits its evanescent wave to noninvasively detect ligand-induced dynamic mass redistribution in native cells (8). The evanescent wave extends into the cells and exponentially decays over distance, leading to a characteristic sensing volume of ~150 nm, implying that any optical response mediated through the receptor activation only represents an average over the portion of the

cell that the evanescent wave is sampling. The aggregation of many cellular events downstream the receptor activation determines the kinetics and amplitudes of a ligand-induced DMR (8).

For biosensor cellular assays, compound solutions were made by diluting the stored concentrated solutions with the HBSS (1 × Hank's balanced salt solution, plus 20 mM Hepes, pH 7.1) and transferring it into a 384well polypropylene compound storage plate to prepare a compound source plate. Two compound source plates were made separately when a two-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 ~1 hr 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 and 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 by using the onboard liquid handler.

To study the influence of chemical interventions on a ligand-induced response, a second stimulation with the ligand 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 second baseline, right before the second stimulation. The two stimulations were usually separated by ~1 hr.

For probing the mechanisms of receptor heterologous desensitization, the cells were preincubated with well-known pathway modulators, each at 10 μM, for about 1 hr. Afterward, a 2-min baseline was established, followed by two subsequent stimulations: the first with forskolin at 500 nM and the second with epinephrine at 2 nM. The two stimulations were separated by 1 hr. Throughout the assays, the pathway modulators were presented at all time. The cellular biosensor responses were continuously monitored throughout the assays.

All studies were carried out at a controlled temperature (28°C). At least two independent sets of experiments, each with at least three replicates, were performed. The assay coefficient of variation was found to be < 10%. All dose-dependent responses were analyzed by using nonlinear regression method with the Prism software (Graph Pad).

Results

The forskolin DMR signals are cellular context-dependent

The diterpene forskolin is an effective activator of AC isoforms 1–8 but not of AC9 and is able to raise cAMP levels in a wide variety of cell types (15). Thus, we were first interested in forskolin-induced DMR responses in seven different cell lines. Forskolin triggered dose-dependent and saturable responses in each type of cells examined, but with distinct response profiles as well as potencies. Figure1 showed the DMR signals of seven cell lines induced by forskolin at a saturated concentration. For A431, HepG2C3A, HT29, A549, and HEK293 cells, forskolin led to a G_s-like DMR signal (11,12) but with different characteristics (i.e., amplitudes and kinetics). The DMR signals generally consist of an initial negative-DMR (N-DMR) event, followed by a positive-DMR (P-DMR) signal. At the saturating doses, the transition time from the N-DMR to the P-DMR was found to be cell line-dependent, decreasing in the following order: HT29 (260 ± 25 sec) > HEK293 ~ HepG2C3A (210 ± 18 sec) > A431 (160 ± 20 sec) > A549 (60 ± 15sec) (n = 16). Nonetheless, all dose-dependent responses were saturable and seemed fit well with nonlinear regression, yielding single apparent EC_{50} values (exampled in Figure. 2). Based on the P-DMR amplitudes, the potency of forskolin was found

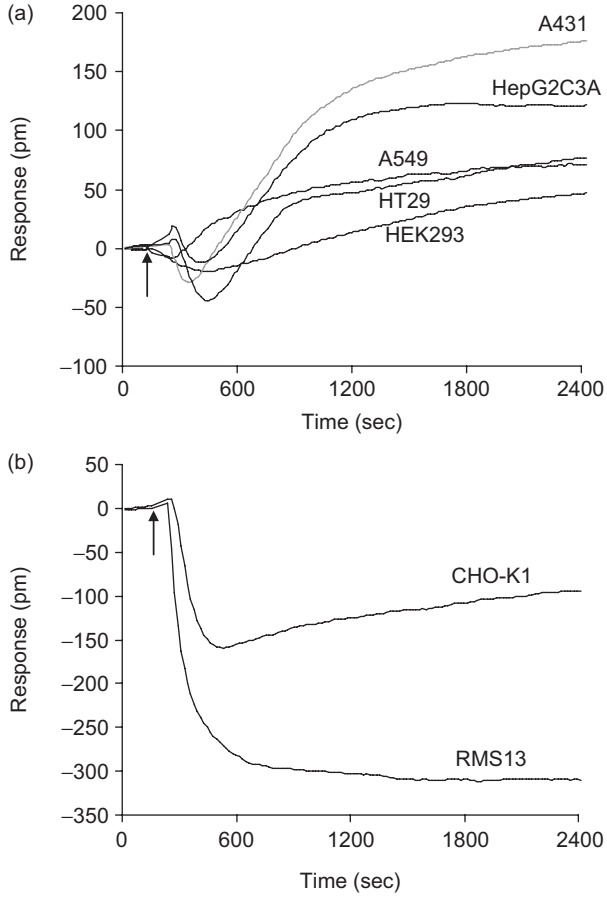

Figure 1. The forskolin mediated DMR signals in seven cell lines. (a) A431, HepG2C3A, HT29 cells, A549, and HEK293 cells. (b) CHO-K1 and RMS13 cells. Except for A549 cells, wherein 16 μM forskolin was used, the forskolin concentration was 2 μM. The black arrows indicated the time when forskolin was introduced.

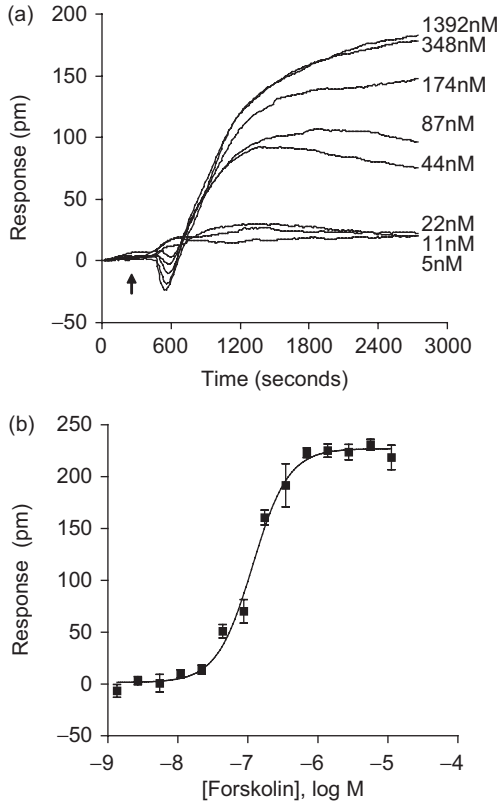

Figure 2. The forskolin DMR signal in quiescent A431 cells. (a) Real-time kinetic response. (b) The amplitude of the P-DMR event as a function of forskolin concentration. The black arrow indicated the time when the agonist was introduced.

to be 421 ± 50 nM, 461 ± 21 nM, 117 ± 11 nM, 430 ± 27 nM, and 9.0 ± 1.2 μM (n = 3) for HT29, HepG2C3A, A431, HEK293, and A549 cells, respectively.

In CHO-K1 and RMS13, forskolin led to a DMR signal that only consists of a rapid N-DMR event. Based on the N-DMR amplitudes, the potency of forskolin was found to be 240 ± 57 nM and 586 ± 43 nM (n = 3) for CHO-K1 and RMS13 cells, respectively. At the high doses, forskolin led to a biphasic DMR signal in CHO-K1 cells—a rapid and initial N-DMR followed by a small P-DMR (data not shown). Nonetheless, the forskolin DMR signals were strongly cellular context-dependent, possibly due to the differential expression and/or organization patterns of adenylyl cyclase isoforms in the cell lines examined.

The forskolin DMR signal in A431 is sensitive to kinase modulation

Given the integrative nature of ligand-induced DMR signals (8–13), we were interested in the cellular mechanisms accounted for the forskolin response. Previously, the chemical biology approach, based on the modulation profiles of a ligand-induced DMR signal by panels of modulators acting on distinct cellular targets, had shown to be very useful to map out the signaling pathways involved (9,10). Here we used the Biomol kinase inhibitor library to study the forskolin-mediated signaling in quiescent A431 cells. Figure 3 showed the P-DMR amplitude of the forskolin response as a function of compounds. Here the quiescent cells were pretreated with compounds and were then stimulated with forskolin at 500 nM. Results showed that the assay was robust

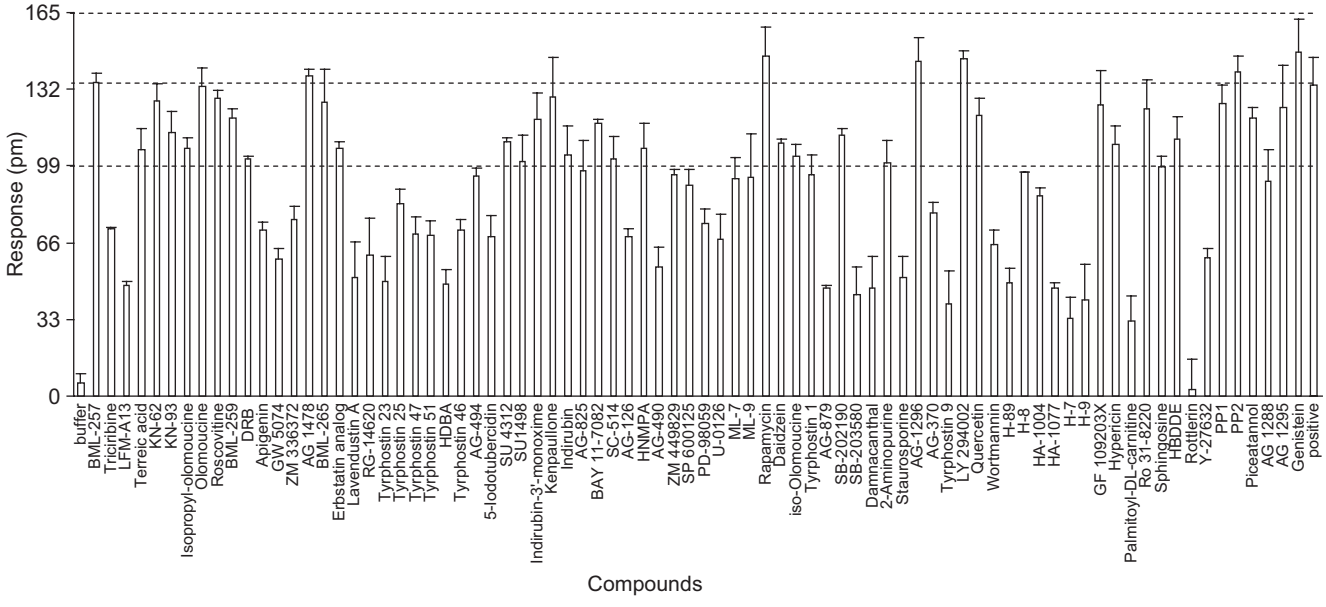

Figure 3. The kinase inhibitor modulation profiles of the forskolin response in quiescent A431 cells. The amplitude of the P-DMR event was plotted as a function of kinase inhibitors. Both the negative controls (the buffer only) and the positive controls (forskolin only) were also included. The forskolin concentration was 500 nM, whereas the concentrations of all kinase inhibitors were 10 μM.

with a Z′ factor of 0.75, a positive control response of 134 ± 12 pm (n = 32) and a negative control response of 6 ± 4 pm (n = 32).

Using 3σ of the positive controls, several kinase inhibitors that attenuated the forskolin response were identified. Results showed that all six PKA kinase inhibitors (H-89, H-8, HA-1004, HA-1077, H-7, and H-9) in the library partially attenuated the forskolin response, indicating that the activation of ACs by forskolin indeed mediates signaling through PKA. Furthermore, the Rho kinase inhibitor Y27632 also partially attenuated the forskolin response, consistent with the ability of the cAMP-PKA pathway to cause cytoskeletal remodeling (16).

It is known that the activation of AC-cAMP-PKA pathway cross-talks with the mitogen-activated protein kinase (MAPK) pathway (17). As expected, several inhibitors targeting this pathway also partially attenuated the forskolin response. These inhibitors included two c-Raf inhibitors GW 5074 and ZM 336372, an ERK2 inhibitor 5-Iodotubercidin, two MEK inhibitors PD-98059 and U-0126, a p38MAPK inhibitor SB-203580, and the broad spectrum inhibitor staurosporine.

Agreed with the ability of cAMP to activate Akt/PKB (18) is that the Akt signaling pathway inhibitor triciribine also partially attenuated the forskolin response. Similarly, several inhibitors that modulate the activity of phosphoinositide 3-kinase (PI3K) pathway also resulted in partially attenuation of the forskolin response. These inhibitors are two BTK inhibitors LFM-A13 and terreic acid, a PI3K inhibitor wortmannin, and two PKC inhibitors palmitoyl-DL-carnitine and rottlerin. PI3K was shown to be downstream of cAMP but upstream of Akt/PKB pathway in endocrine cells (19).

It is interesting that almost all tyrphostins in the library also attenuated the forskolin response. Among them, tyrphostins that attenuated the forskolin response by 40%–70% included tyrphostins 9, tyrphostin 23, tyrphostin 25, tyrphostin 46, tyrphostin 47, tyrphostin 51, AG126, AG370, AG490, and AG879, whereas the other four tyrphostins including AG494, AG825, tyrphostin 1, and AG1288 only led to ~30% suppression. These tyrphostins are inhibitors for tyrosine kinases including EGF receptor kinase. Consistent with previous studies done by others concerning a small set of these tyrphostins (20), our follow-up studies suggested that these tyrphostins are also inhibitors for phosphodiesterases (PDEs) including PDE4s (unpublished data). PDE4s are crucial to shape the cAMP gradient and thus to direct the cAMP-mediated signaling (4). However, the possible role of tyrosine kinases cannot be completely ruled out, because several nontyrphostin inhibitors for EGFR (lavendustin A, RG-14620, and HDBA) and p56ICK (damnacanthal) also attenuated the forskolin response. Taken together, these results indicated that the forskolin DMR response in A431 is complicated in nature and is mostly downstream of PKA and is involved with Rho kinase, PI3K-Akt, and MAP kinase pathways.

Forskolin heterologously desensitizes G_s-coupled receptor DMR signals

GPCRs can be desensitized in two distinct ways: 1) homologous desensitization wherein the activated GPCR is down-regulated and 2) heterologous desensitization wherein the activation of second messenger-dependent protein kinases or of a different receptor causes down-regulation of a GPCR (21,22). Because forskolin directly activates AC, which is a downstream target of G_s-coupled receptors, we were interested in the heterologous desensitization of G_s signaling using a two-step biosensor desensitization assay, in which the cells are repeatedly stimulated with two stimuli and the two stimulations are separated by about 1 hr (23). Figure 4a showed the forskolin effect on the epinephrine response in quiescent A431 cells. The forskolin pretreatment dose-dependently attenuated the DMR signal of 2 nM epinephrine, yielding an IC_{50} of 188 ± 37 nM (n = 32), which is close to the EC_{50} of forskolin obtained. At the high doses, forskolin completely inhibited the epinephrine response. Similar effects were observed on the DMR signals induced by forskolin, dopamine, NECA, and VIP, with an IC_{50} of 156 ± 28, 164 ± 39, 143 ± 19, and 147 ± 21 nM (n = 4), respectively (Figure 4b). All these ligands led to similar G_s-type DMR signals in A431 (11; data not shown). Previously, we showed that both epinephrine and dopamine responses in A431 are specific to β_2-adrenoceptor (β_2AR) in A431, whereas NECA activates endogenous adenosine A_2 receptors in A431 (11,12). Similarly, the pretreatment of quiescent A431 cells with these ligands also dose-dependently desensitized the forskolin response with an apparent IC_{50} being close to their corresponding EC_{50} (data not shown). These results suggest that the heterologous desensitization between forskolin- and G_s-mediating signaling is quite universal, at least in A431 cells.

Because desensitization is initiated by receptor phosphorylation and is involved with multiple mechanisms (22), we further examined how forskolin causes receptor desensitization. Here a three-step assay was used: cells were preincubated with different PKA inhibitors, followed by stimulation with forskolin, and epinephrine; each stimulation was about 1 hr. Such three-step assay is made possible due to the noninvasive nature of the biosensor. Results showed that inhibiting PKA activity by several well-known PKA inhibitors partially attenuated the forskolin response; and different PKA inhibitors, each at 10 μM, led to different degrees of suppression of the forskolin response with the following order: H-7 > H-9 > HA-1004 (Figure 3 and Figure 4c). It is interesting that quiescent A431 cells still responded to

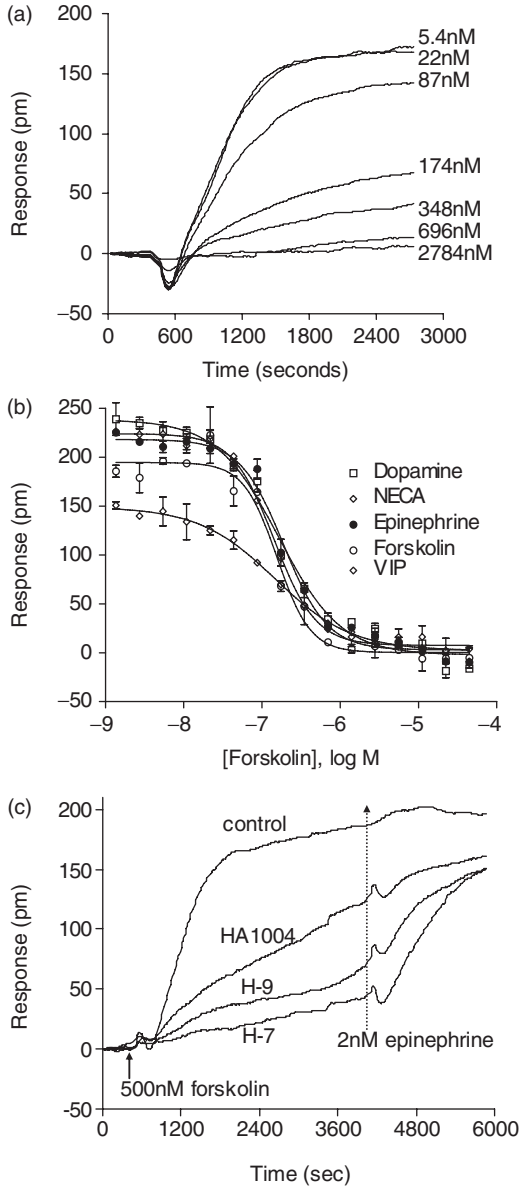

Figure 4. The heterologous desensitization between the forskolin- and G_s-mediated signaling in quiescent A431 cells. (a) Real-time kinetic responses of forskolin-pretreated A431 upon stimulation with 2 nM epinephrine. (b) The amplitudes of the P-DMR events of the DMR signals induced by 1 μM forskolin, as well as G_s-coupled receptor agonists as a function of forskolin concentration. The concentration was 2 nM, 5 μM, 100 nM, and 100 nM for epinephrine, dopamine, NECA, and VIP, respectively. The concentration was close to its corresponding $EC_{80\text{-}100}$ for each agonist. (c) Desensitization of A431 to 2 nM epinephrine. The cells were subject to preceding treatments with a PKA inhibitor (H-7, H-9, or HA1004) at 10 μM, followed by 500 nM forskolin, and then by 2 nM epinephrine. The cells that were pretreated with forskolin only were used as the control. The arrows indicated that time when the compound indicated was introduced.

2 nM epinephrine after the sequential preceding treatments with the PKA inhibitor and forskolin (Figure 4c). The epinephrine response was inverse proportional to the forskolin response. The greater suppression of the forskolin response by a PKA inhibitor is, the greater the epinephrine response is. Conversely, the preceding treatment only with 500 nM forskolin caused complete desensitization of cells to the epinephrine stimulation (Figure 4c). Taken together, these results suggest that the heterologous desensitization of β_2AR signaling by forskolin is at least partially mediated through PKA. Further studies are ongoing for deciphering the detailed cellular mechanism(s) accounted for the heterologous desensitization of G_s-coupled receptors by forskolin.

Forskolin partially desensitizes G_q-coupled receptor DMR signals

Because several AC isoforms are regulated by the ubiquitous second messenger Ca^{2+} (4,5), we were interested in the role of ACs in the DMR signals induced by the activation of G_q-coupled receptors. Previously, we detected G_q-type DMR signals in A431 cells for several G_q-coupled receptors, including P2Y receptor agonist ATP, protease activated receptor PAR_1 agonist SFLLR-amide (11,23), and histamine receptor agonist histamine (Figure 5). Forskolin dose-dependently attenuated the histamine response in quiescent A431 cells (Figure 5a); a similar effect was observed for both ATP and SFLLR-amide responses. The apparent IC_{50} values observed were 148 ± 45 nM, 317 ± 78 nM, and 150 ± 32 nM for ATP, SFFLR-amide, and histamine, respectively (Figure 5b). In contrast to G_s-coupled receptors, the preceding stimulation of cells with either agonist had little impact on the forskolin response (data not shown). These results suggest that the activation of G_q-coupled receptors cross-talks with the cAMP pathway, probably via Ca^{2+}-sensitive AC isoforms.

Forskolin has complicated impacts on G_i-coupled receptor DMR signals

Acute activation of $G_{i/o}$-coupled receptors inhibits cAMP accumulation, whereas the persistent activation of many $G_{i/o}$-coupled receptors in many cell lines causes heterologous sensitization of AC activity (24). The development of heterologous sensitization occurs rapidly, within tens of minutes (25). Thus, we were interested in the effect of forskolin on the DMR signals mediated through G_i-coupled receptor. A431 is known to endogenously express LPA_1, LPA_2, and LPA_3 receptors. LPA_1 is mainly a G_i-coupled receptor, where the other two are dominantly coupled to G_q. Previously, we found that LPA led to a unique dose-dependent switching of DMR signals (11). Because LPA is a nonselective agonist to LPA receptor family, the DMR signals observed in the partially quiescent A431 may reflect the signal integration of all LPA receptors being activated. Here, we examined the DMR signal induced by a LPA_1-specific agonist DPA. DPA led to

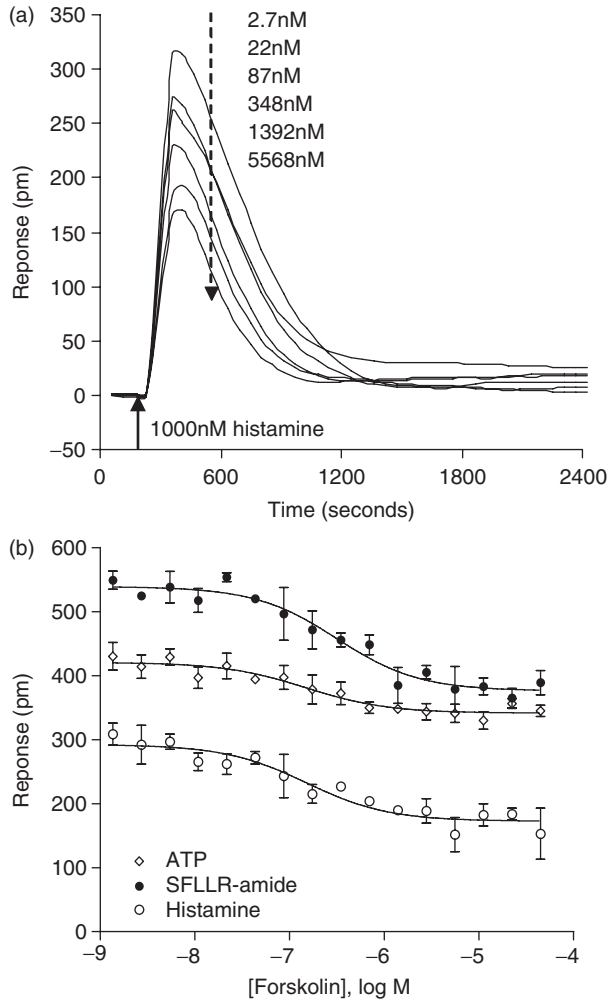

Figure 5. The cross-talks between the forskolin- and G_q-coupled receptor agonist-mediated signaling. (a) Real-time kinetic responses of A431 upon stimulation with 1 μM histamine. (b) The dose-dependent attenuation of the P-DMR amplitudes of the G_q-coupled receptor agonist-induced DMR signal by forskolin. ATP, 1 μM; SFLLR-amide, 2 μM; and histamine, 1 μM. The cells were pretreated with forskolin at different doses. The arrow indicated the time when histamine was introduced.

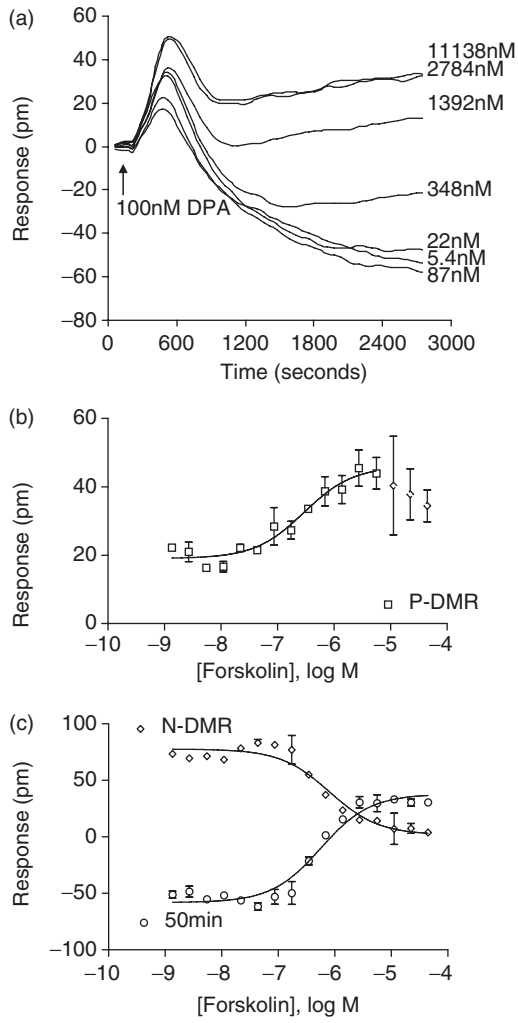

Figure 6. The cross-talks between the forskolin- and G_i-coupled receptor agonist-mediated signaling. (a) Real-time kinetic responses of A431 upon stimulation with 100 nM DPA. (b,c) The amplitudes of the DPA responses as a function of forskolin: the P-DMR event (b), and the N-DMR event and the endpoint response at 50 min after DPA stimulation (c). The cells were pretreated with forskolin at different doses. The arrow indicated the time when DPA was introduced.

a dose-dependent DMR signal, which consists of a small P-DMR followed by a larger N-DMR event (Figure 6a). In contrast to G_s- and G_q-coupled receptors, forskolin had a complicated impact on the DPA response. Forskolin not only dose-dependently increased the P-DMR event with an EC_{50} of 300 ± 73 nM but also increased the endpoint response (e.g., 50 min after DPA stimulation) with an EC_{50} of 564 ± 121 nM. Conversely, forskolin dose-dependently attenuated the N-DMR event with an IC_{50} of 810 ± 271 nM (n = 3) (Figure 6b and c). A Similar effect was observed for another endogenously expressed G_i-coupled receptor GPR109A (unpublished data). In contrast, forskolin had little impact on the DMR signal induced by LPA at 100, 500, or 2000 nM in the fully quiescent A431 cells (data not shown). It is interesting that the activation of GPR109A by nicotinic acid potentiated the N-DMR event significantly but had little effect on the P-DMR event of the forskolin response (unpublished data). These results suggest that the activation of G_i-coupled receptors has complicated effect on the adenylyl cyclase-cAMP pathway.

Discussion

Adenylyl cyclase consists of nine membrane-bound isoforms and one soluble isoform and displays distinct tissue distribution and basal activity (4,5). For example, HEK293 cells express three major isoforms AC3, AC6, and AC9 (3), whereas CHO-K1 cells express mainly AC6 and AC7 (26). As a result, basal cAMP levels differ

greatly in distinct cell types (5). Furthermore, some AC isoforms may predominantly locate in specific domains of the cell surface (27). Recent studies showed that all of the Ca^{2+}-sensitive ACs (AC1/3/5/6/8) are located in lipid rafts from various sources, whereas the Ca^{2+}-insensitive AC2/4/7 isoforms are excluded (4). Consistent with the distinct distribution and organization of AC isoforms is the distinct types of forskolin-induced DMR signals in seven cell lines detected by the biosensor cellular assays.

Forskolin is an effective activator of ACs, leading to cAMP production. The cAMP signaling pathway controls a diverse range of cellular processes and is known to cross-talk with extracellular signal regulated kinase (ERK) through multiple mechanisms, primarily via Raf-MEK pathway (17,28). In A431 cells, forskolin triggered a G_s-type DMR signal, similar to the epinephrine DMR signal of β_2AR, a prototypical G_s-coupled receptor (11,12). Kinase modulation analysis identified several important downstream targets in the forskolin-mediated signaling, including PKA, Rho kinase, PI3K-Akt, and MAP kinase pathways. Such modulation profiles indicated the complexity in origin of the forskolin DMR signal and strengthened our previous assessment, based on theoretical, numerical, and experimental analysis, that a ligand-induced DMR is an integrated cellular response (8–10). It is interesting that the biosensor cellular assays also documented that the tyrphostins in the Biomol kinase inhibitor library act as inhibitors for phosphodiesterases, particularly PDE4s, a family of enzymes that shape the dynamics of cAMP signaling (4). Furthermore, several G_s-coupled receptor agonists caused complete desensitization of A431 cells to the forskolin stimulation. Together, these results suggest that the forskolin DMR signal is almost entirely due to the activation of ACs and its subsequent signaling.

Cross-talk between signaling pathways is necessary to effect efficient and fine-tuned regulatory control over physiology and pathophysiology. Adenylyl cyclase, whose activation produces cAMP, is considered as an integrator for many cellular processes, including receptor signaling (29,30). This is due to the diverse regulatory properties of ACs by G proteins (Gα and Gβγ), Ca^{2+}, and phosphorylation, together with the tissue-specific distribution and organization. Forskolin is widely used as a versatile pharmacological probe in studying AC function, as well as drug screening, particularly for G_i-coupled receptors (13). Using label-free and noninvasive biosensor cellular assays, we have examined the cross-talks between the forskolin-AC-cAMP signaling and GPCR signaling. Results showed that there was a heterologous desensitization between forskolin and G_s-coupled receptor signaling: The forskolin-treated cells lost their responsiveness to G_s-coupled receptor agonists, such as epinephrine, dopamine, VIP, and NECA; vice versa, these G_s-coupled receptor agonists caused the desensitization to succeeding forskolin stimulation. The heterologous desensitization was found to occur at least partially via PKA. Such heterologous desensitization appears quite universal, because similar phenomena were observed for some G_s-coupled receptors in different cell lines (31; data not shown).

Forskolin also dose-dependently attenuated but was unable to completely inhibit several G_q-coupled receptor-mediated DMR signals. It has been reported that the activation of G_q-coupled receptor can directly and indirectly influence cAMP signaling (4). Both Ca^{2+} and PKC can directly modulate the activity of ACs. Conversely, the Ca^{2+} release can modulates proteins, including Src and PYK2 upstream to cAMP signaling, which ultimately enhances the activation of Raf1-MEK-ERK pathway. The present biosensor cellular assays clearly suggested that there are cross-talks of G_q-mediated signaling with the cAMP pathway. It is interesting that these G_q-coupled receptor agonists had little impact on the forskolin response, suggesting that the activation of these G_q-coupled receptors cannot fully activate the adenylyl cyclase-cAMP-PKA module.

Conversely, forskolin had complicated impact on the DMR signals mediated through the activation of endogenous G_i-coupled receptors in A431, including LPA_1 and GPR109A. It is more interesting that the activation of GPR109A seems to potentiate the N-DMR event, but not the P-DMR, of the forskolin response (unpublished data), suggesting the heterologous sensitization of G_i-signaling on the cAMP pathway (24,25). However, there are still many unanswered questions: What are the cellular mechanisms behind the heterologous sensitization of G_i signaling on the cAMP pathway. This warrants further studies?

In summary, we have used noninvasive optical biosensor to study the signal integration of endogenous GPCRs in A431 cells at the AC-cAMP-PKA module. Surveying seven different cell lines recorded cellular context-dependent DMR signals upon stimulation with forskolin. Kinase modulation profiles linked the forskolin response in A431 to several signaling pathways, including PKA, PI3K-Akt, Rho kinase, and Raf-MEK-ERK pathway. Desensitization assays uncovered the PKA-mediated heterologous desensitization between forskolin and several G_s-coupled receptors. Furthermore, forskolin can be used as an attractive chemical biology approach to differentiate receptor signaling and ligand pharmacology by using label-free biosensor cellular assays. Amenable to mechanistically deconvolute an apparently "nonspecific" response is crucial to the successful adoption of these biosensor cellular assays for drug discovery and development, where reducing attrition rates is critical. The present study clearly demonstrates the integrative role of adenylyl cyclases in GPCR

signaling and illustrates the power of label-free biosensor cellular assays for probing receptor biology.

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