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Parallel tracking of cAMP and PKA signaling dynamics in living cells with FRET-based fluorescent biosensors†

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Abstract

Proper regulation of cellular functions relies upon a network of intricately interwoven signaling cascades in which multiple components must be tightly coordinated both spatially and temporally. To better understand how this network operates within the cellular environment, it is important to define the parameters of various signaling activities and to reveal the characteristic activity structure of the signaling cascades. This task calls for molecular tools capable of parallelly tracking multiple activities in cellular time and space with high sensitivity and specificity. Here, we present new biosensors developed based on two conveniently co-imageable FRET pairs consisting of CFP–RFP and YFP–RFP, specifically Cerulean–mCherry and mVenus–mCherry, for parallel monitoring of PKA activity and cAMP dynamics in living cells. These biosensors provide orthogonal readouts in co-imaging experiments and display a comparable dynamic range to their cyan–yellow counterparts. Characterization of signaling responses induced by a panel of pathway activators using this co-imaging approach reveals distinct activity and kinetic patterns of cAMP and PKA dynamics arising from differential signal activation and processing. This technique is therefore useful for parallel monitoring of multiple signaling dynamics in single living cells and represents a promising approach towards a more precise characterization of the activity structure of the dynamic cellular signaling network.

Introduction

Living cells have an amazing capability of deciphering various environmental cues to generate appropriate responses. For any given signal, a set of signaling molecules are involved in controlling the information flow within the cellular signaling network via intricate cross-regulation and tight coordination to generate specific cellular responses. The activities of these key signaling molecules are therefore inherently linked, both spatially and

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temporally. Elucidation of such spatiotemporal correlation and interrelationship of these signaling molecules is believed to be critical in understanding the logic and functioning of signal transduction and should help unravel the characteristic activity structure of an extremely dynamic cellular signaling network.¹ While there exist several methods for analyzing multiple signaling molecules from cells or tissue samples such as protein expression profiling,^{2,3} mass spectrometry or protein array-based detection of posttranslational modifications,⁴ flow cytometry-based signal profiling^{5,6} and small molecule probe-based activity profiling,⁷ these methods lack the ability to continuously monitor the dynamic interplay between signaling molecules in their biological context. Therefore, there is a compelling need to develop methods to parallelly track the activities of multiple signaling molecules in their native environment, the living cell.^{8,9}

An emerging technique suitable for developing methods to parallelly track multiple activity parameters in living cells involves genetically-encoded biosensors based on fluorescence resonance energy transfer (FRET).^{10–12} These biosensors are typically constructed with a molecular switch sensitive to the signaling event of interest, and a fluorescent protein FRET pair as the “reporting unit”, in which a signal-induced conformational change in the molecular switch can be transduced into a change in FRET. This general design has yielded FRET-based biosensors for various signaling molecules, including second messengers and protein kinases.^{13,14} For instance, we have previously developed two FRET-based biosensors, Indicator for cAMP Using Epac (ICUE) and A-Kinase Activity Reporter (AKAR), for visualizing dynamic activities in the signaling pathway involving cAMP and cAMP-dependent protein kinase (PKA).^{15–20} The cAMP biosensor, ICUE, has been developed in such a way that cAMP binding changes the conformation of a truncated version of exchange proteins activated by cAMP-1 (Epac1), resulting in a decrease in FRET between the two flanking fluorescent proteins.¹⁵ In the case of the PKA activity reporter, AKAR, a PKA-dependent molecular switch, consisting of a phospho-threonine binding FHA1 domain and a PKA-substrate sequence, was sandwiched between the two fluorescent proteins serving as a FRET pair.¹⁸ Upon phosphorylation by PKA, the FHA1 domain binds to the phosphorylated PKA substrate resulting in a conformational change and a concomitant increase in FRET.

The cAMP/PKA pathway governs many fundamental cellular functions, including metabolic, electrical, cytoskeletal, and transcriptional processes. PKA, a major mediator of cAMP signaling,²¹ not only controls downstream signaling events at various subcellular locations, but also directly regulates cAMP dynamics through upstream components that are responsible for cAMP production and degradation, such as G-protein coupled receptors (GPCR),²² adenylyl cyclases (AC)²³ and phosphodiesterases (PDE),²⁴ thereby achieving tight spatiotemporal controls *via* complex feedback regulatory mechanisms. ICUE and AKAR have served as useful tools for tracking spatiotemporal dynamics of cAMP²⁵ and PKA signaling^{26,27} in living systems, respectively. It is therefore expected that development of parallel imaging methods based on these biosensors will enable us to precisely correlate the interlinked cAMP and PKA signals and to better characterize their interplay in the native context, thereby further extending our understanding of the complex signaling controls mediated through these signaling molecules.

Results

Developing co-imageable FRET pairs with a common acceptor

In general, parallel tracking of two signaling events in the same cellular environment requires readouts that are both orthogonal and sensitive. In the case of FRET-based biosensors, a typical approach is to utilize two spectrally distinct FRET donors paired with appropriate acceptors. While the ever growing list of new fluorescent proteins^{28,29} offers FRET pairs that meet this spectral requirement,^{30–35} the challenge remains in identifying two co-imageable FRET pairs that offer a sufficient dynamic range—a parameter that has proven critical in detecting physiologically relevant signals^{26,36} as well as in broadening the scope of their applications such as in high throughput detection of signaling activities using FRET-based biosensors.³⁷

Among different color variants of fluorescent proteins, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) have distinct excitations, bearing little cross excitation of YFP at the optimal CFP excitation wavelength and minimal CFP excitation at the YFP excitation wavelength. Importantly, they can potentially serve as spectrally distinct donors for the same red-shifted fluorescent protein, such as red fluorescent protein (RFP), to yield two FRET pairs that satisfy the spectral requirements of orthogonal pairs (Fig. S1, ESI[†]). In addition, utilization of CFP–RFP and YFP–RFP FRET pairs for co-imaging offers the convenience of using existing imaging setups for CFP–YFP ratiometric imaging^{38,39} with the minimal addition of an RFP emission filter. However, the critical question is whether these pairs can undergo FRET efficiently. Previously, we tested a FRET circuit containing Cerulean (a CFP variant), mVenus (a YFP variant), and mCherry (an RFP variant) for engineering new fluorescent biosensors.⁴⁰ During this study, not only did we find that mVenus and mCherry acted as a well-behaved FRET pair but we also found that most of the energy transferred to mCherry from excited Cerulean occurred directly, suggesting that Cerulean and mCherry can undergo FRET efficiently. Thus, Cerulean–mCherry and mVenus–mCherry emerged as potentially useful FRET pairs for co-imaging.

These new FRET pairs were then tested in the context of ICUE and AKAR. For the Cerulean–mCherry pair, we generated a Cyan-Red-AKAR (CR-AKAR), in which the PKA-dependent molecular switch is sandwiched between Cerulean and mCherry (Fig. 1A). As shown in Fig. 1B, CR-AKAR responded to stimulation by the AC-agonist forskolin (Fsk) in HEK293T cells, yielding an average increase in red/cyan emission ratio of $36 \pm 7\%$ ($n = 4$) [avg. $\pm$ stdev. (n = number of cells)]. The maximal response observed (dynamic range of the reporter), a more than 40% emission ratio change, is comparable to that of the widely used version of AKAR based on the CFP–YFP pair, AKAR3.¹⁹

For the mVenus–mCherry pair, we generated a Yellow-Red-ICUE (YR-ICUE), in which the cAMP sensing unit, truncated Epac1, is sandwiched between mVenus and mCherry (Fig. 1A). YR-ICUE was also found to generate a robust signal, showing an emission ratio change of $42 \pm 3\%$ ($n = 4$) upon Fsk stimulation (Fig. 1C). Of note, the spectral profiles of YFP and RFP^{28,41} (Fig. S1, ESI[†]) suggested a possible cross-excitation of RFP when YFP is excited, which would affect the reading in the FRET channel. However, this cross excitation did not prevent YR-ICUE from achieving a desirable dynamic range (around 50% emission ratio

change). In addition, based on the observation that the emission ratio change from YR-ICUE was mainly attributed to changes in YFP intensity (Fig. S2, ESI[†]), we expect that the presence of additional RFP from CR-AKAR will not significantly affect the dynamic range of YR-ICUE when they are expressed together in a co-imaging setting.

Next, with Cerulean and mVenus as spectrally distinct donors and mCherry as their common acceptor, the specificity and sensitivity of CR-AKAR and YR-ICUE were tested in a co-imaging setting. As shown by the time courses in Fig. 2A, Fsk-stimulated changes in cAMP and PKA activities were detected in the same cell in parallel and kinetically matched those seen with either CR-AKAR or YR-ICUE alone. After the Fsk-stimulated responses were stabilized, subsequent treatment with PKA inhibitor H89 caused a reversal in the CR-AKAR response with no effect on that of YR-ICUE (Fig. 2A). Conversely, pretreatment of cells with H89 blocked the response of CR-AKAR but did not affect the Fsk-stimulated response of YR-ICUE (Fig. 2B). Taken together, orthogonal detection of cAMP dynamics and PKA activity is achieved with high specificity using YR-ICUE and CR-AKAR. Importantly, the average signals for the PKA and cAMP readouts are $36 \pm 3\%$ ($n = 6$) and $32 \pm 2\%$ ($n = 6$) respectively (Fig. 2A). In the co-imaging setting, the maximal responses were found to be close to 40% for both YR-ICUE and CR-AKAR, largely preserving their individual dynamic ranges as expected (Table S1, ESI[†]). In addition, changes in the relative expression levels of the two reporters do not appear to affect their responses (Fig. S3, ESI[†]). Thus, two FRET pairs based on Cerulean-mCherry and mVenus-mCherry can be used for parallel tracking of the spatiotemporal dynamics of signaling events in real time with orthogonal ratiometric readouts.

Kinetic profiling of cAMP/PKA pathway agonists

Various stimulatory signals, including both hormones and pharmacological ligands, may differentially induce the cAMP/PKA signaling pathway with distinct spatiotemporal profiles by inducing only selected subsets of the pathway's rich repertoire of behaviors and functions. Thus, the ability to parallelly measure cAMP dynamics and PKA activity offers a unique opportunity to characterize agonist-induced responses based on multiple activity parameters that are measured in the native cellular environment. We therefore applied such a co-imaging approach to examine the intrinsic signaling responses induced by a series of pathway activators (Fig. S4, ESI[†]), including various GPCR ligands, in a well-established cell system.^{15,16,42} With parallel tracking of both cAMP and PKA signals, the real-time kinetic profiles of various agonists reveal different characteristics of the agonist-induced signaling responses (Fig. 3).

As shown in Fig. 3A, in HEK293T cells expressing both CR-AKAR and YR-ICUE, Fsk, a potent AC activator, yielded a gradual, large increase in the cAMP signal accompanied by a large PKA response. This gradual response can be accelerated by addition of 3-isobutyl-1-methylxanthine (IBMX), a general PDE inhibitor (Fig. S5, ESI[†]). By contrast, prostaglandin E1 (PGE1), an agonist of the EP receptor, caused a small cAMP signal, which correlated with a large and sustained PKA activation that lasted for more than 20 minutes (Fig. 3B), showing that PKA can maintain high signaling activity at a relatively low level of cAMP, given the activation constants in the nanomolar range and cooperative cAMP binding of

regulatory subunits of PKA.^{43,44} In addition, as shown in Fig. 3C, a general β -adrenergic agonist isoproterenol (ISO) elicited a faster, but more transient, burst of cAMP accumulation, when compared to PGE1. In both cases, PKA activity remained relatively sustained within the same time frame. Lastly, HEK293T cells expressing the reporters were treated with a β 2-adrenergic receptor agonist ritodrine (RITO) (Fig. 3D). In this case, the stimulated cAMP accumulation was below the detection limit of YR-ICUE and thus, less than those stimulated under all prior conditions. However, a gradual increase in PKA activity was clearly detected, which was slower than those elicited from either PGE1 or ISO treatment. The combined profile for RITO-induced cAMP dynamics and PKA activity therefore suggests that even a small elevation in cAMP concentration, albeit below the detection limit of YR-ICUE, can, over time, lead to accumulated PKA activity.

Importantly, these distinct response patterns in terms of cAMP/PKA signals for different agonists, which are consistent with data from single-probe imaging experiments using CR-AKAR and YR-ICUE as well as their CY-counterparts (not shown), can indeed be correlated with their individual functional characteristics. First, sustained cAMP and PKA responses by Fsk, which should lead to potent activation of downstream events such as gene transcription, are consistent with our previous finding that PKA-mediated inhibition of adenylyl cyclases does not play a significant role in this system.²⁵ Second, the different kinetic patterns of PGE1 and ISO responses are consistent with different desensitization characteristics of β -adrenergic and EP receptors⁴²—more rapid desensitization of the β -adrenergic receptor should lead to more transient functional effects compared to the case of the EP receptor. Yet, different patterns of cAMP burst generated by these two agonists elicited maximal and sustained AKAR response, suggesting that attenuation of cAMP and PKA activities could be uncoupled with the latter also depending on phosphatase activities. Third, the relatively low production of cAMP stimulated by RITO, possibly due to its relatively low efficacy, may in fact lead to selective activation of PKA without turning on exchange proteins activated by cAMP (Epac), which are known to be activated by higher concentration of cAMP than PKA.⁴⁵ The null response of YR-ICUE (Fig. 3D) suggests that Epac-1, on which ICUE is based, is not efficiently activated under this condition. Future studies investigating differential responses of Epac and PKA to agonists such as RITO could also benefit from using reporters of Epac activation^{15,46} and PKA activity in such co-imaging experiments to examine different cAMP effectors in the same cellular context.

Discussion

In this study, we demonstrate that a co-imaging method based on Cerulean–mCherry and mVenus–mCherry is useful for parallel tracking of cAMP/PKA signals in single living cells. The two new FRET-based reporters, CR-AKAR and YR-ICUE, are not only well-behaved when utilized individually but also provide orthogonal and large signals in the co-imaging experiments. In addition, this approach may be extended to detecting two other signaling activities in parallel. Depending on the specific reporters, the cross-excitation of RFP upon YFP excitation may affect the dynamic range of the YR-based biosensor. In such cases, the contribution from the cross-excited RFP can be removed by a straightforward correction (Suppl. Method, ESI[†]). Overall, this co-imaging method provides a technically convenient alternative to existing FRET-based multi-parameter co-imaging schemes.^{30–32,34–35} The

feature of a shared acceptor further offers new opportunities for biosensor designs. For example, we recently developed a three-fluorophore single chain dual biosensor, ICUEPID, which can monitor both PKA activity and cAMP level in a localized fashion.³⁶

Using this method, distinct response profiles of various agonists that activate the cAMP signaling pathway are revealed by parallel imaging. In all GPCR-mediated signaling events, the levels of cAMP accumulated depend on the amount of available receptors in individual cells, as well as the AC isoform coupled to a particular receptor⁴⁷ and the efficiency of PDEs present in a particular cellular compartment.⁴² In this regard, these response profiles can be used to characterize signaling specificity of pathway activators based on their individual capabilities in activating cAMP production, PKA activation, receptor desensitization, and subsequent feedback mechanisms.⁴⁸ Given the fast-growing list of FRET-based biosensors for tracking various signaling activities, we expect that this co-imaging technique can be extended to a variety of signaling molecules, for which cross-regulation and interrelationship play an important role. The ability to parallelly track multiple signaling activities and to yield correlation information about their spatiotemporal dynamics, as demonstrated here with a panel of cAMP/PKA pathway activators including GPCR agonists, should lead to a more comprehensive characterization of the signaling activity structure and to a better understanding of dynamic signaling networks.

Experimental

Gene construction

mCherry was PCR amplified to introduce appropriate restriction digest sites for subcloning into either AKAR²⁰ or ICUE¹⁶ constructs. The genes of CR-AKAR, containing Cerulean and mCherry, and YR-ICUE, containing mVenus, which is circularly permuted at position L194, and mCherry, were generated in pRSETB (Invitrogen) and then moved to pcDNA3 (Invitrogen) behind a Kozak sequence for mammalian expression.

Cell culture

HEK293T cells were plated onto sterilized glass coverslips in 35-mm dishes and grown to 50–90% confluence in DMEM (10% FBS) at 37 °C with 5% CO₂. Cells were transfected by calcium phosphate and grown for 15–48 h before imaging.

Imaging

Cells were washed twice with Hanks' balanced salt solution buffer and maintained in the dark at room temperature. Prostaglandin E1 (PGE1; Sigma), ritodrine (RITO; Sigma), isoproterenol (ISO; Sigma), forskolin (Fsk; Calbiochem), and H89 (Sigma) were added as indicated. Cells were imaged on a Zeiss Axiovert 200M microscope with a cooled charge-coupled device camera (MicroMAX BFT512, Roper Scientific) controlled by METAFLUOR 6.2 software (Universal Imaging). Dual red/cyan emission ratio imaging used a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters [475DF40 for CFP and 653DF95 for RFP]. Dual yellow/red emission ratio imaging used a 495DF10 excitation filter, a 515DRLP dichroic mirror, and two emission filters [535DF25 for YFP and 653DF95 for RFP]. These filters were alternated by a filter-changer Lambda

10–2 (Sutter Instruments). Exposure time was 50–500 ms, and images were taken every 30 s. Fluorescence images were background-corrected by subtracting the fluorescence intensity of background with no cells from the emission intensities of cells expressing fluorescent biosensors. The emission ratios (red/cyan or yellow/red) were then calculated at different time points. The ratios from all time points were normalized by dividing each by the last basal value before drug additions. Standard deviation is calculated as the square root of variance at each time point for a particular imaging experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Tracking of cAMP accumulation and PKA activity in living cells using CR-AKAR and YR-ICUE. (A) Schematic representation of the biosensors CR-AKAR and YR-ICUE. (B) Responses of HEK293T cells expressing CR-AKAR to 50 μ M forskolin (Fsk) ($n = 4$, error bars represent standard deviation) and the pseudocolor images showing CR-AKAR response to Fsk in a HEK293T cell. Red fluorescence image (RFP) shows the distribution of the biosensor. (C) Responses of HEK293T cells expressing YR-ICUE to 50 μ M Fsk ($n = 4$) and the pseudocolor images showing YR-ICUE response to Fsk in a HEK293T cell. Red fluorescence image (RFP) shows the distribution of the biosensor. Warmer color indicates an increase in PKA activity or cAMP level and cooler color indicates a decrease. Scale bar, 10 μ m.

Fig. 2.

Parallel tracking of CR-AKAR and YR-ICUE in HEK293T cells. (A) Responses of HEK293T cells co-expressing CR-AKAR and YR-ICUE treated with 50 μ M Fsk followed by treatment with 10 μ M H89 ($n = 6$). Fluorescence images showed distribution of biosensors in a single cell. (B) Responses of HEK293T cells co-expressing CR-AKAR and YR-ICUE treated with 50 μ M Fsk in the presence of 20 μ M H89 ($n = 6$). Fluorescence images showed distribution of biosensors in a single cell. Scale bar, 10 μ m.

Fig. 3. Kinetic profiling of cAMP/PKA pathway agonists by parallel imaging of CR-AKAR and YR-ICUE responses. The response time courses of cAMP and PKA in HEK293T cells in response to (A) 50 μ M Fsk ($n = 9$), (B) 10 μ M prostaglandin E1 (PGE1) ($n = 6$), (C) 1 μ M isoproterenol (ISO) ($n = 6$) and (D) 10 μ M ritodrine (RITO) ($n = 7$). All data are presented as average $\pm$ STD unless otherwise noted.