Novel Single Chain cAMP Sensors for Receptor-induced Signal Propagation*

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cAMP is a universal second messenger of many G-protein-coupled receptors and regulates a wide variety of cellular events. cAMP exerts its effects via cAMP-dependent protein kinase (PKA), cAMP-gated ion channels, and two isoforms of exchange protein directly activated by cAMP (Epac). Here we report the development of novel fluorescent indicators for cAMP based on the cAMP-binding domains of Epac and PKA. Fluorescence resonance energy transfer between variants of green fluorescent protein (enhanced cyan fluorescent protein and enhanced yellow fluorescent protein) fused directly to the cAMP-binding domains was used to analyze spatial and temporal aspects of cAMP-signaling in different cells. In contrast to previously developed PKA-based indicators, these probes are comprised of only a single binding site lacking cooperativity, catalytic properties, and interactions with other proteins and thereby allow us to easily image free intracellular cAMP and rapid signaling events. Rapid β-adrenergic receptor-induced cAMP signals were observed to travel with high speed (~40 μm/s) throughout the entire cell body of hippocampal neurons and peritoneal macrophages. The developed indicators could be ubiquitously applied to studying cAMP, its physiological role and spatiotemporal regulation.

The second messenger cAMP was discovered in the early 1950s, and since then its cellular function and regulation have been gaining increased interest (1). cAMP is a ubiquitous second messenger and regulates a wide variety of cellular events and biological processes from metabolism and gene expression (2), cell division (3) and migration (4), exocytosis (5) and insulin secretion (6) to immune defense (7), memory formation (8), and cell division (3) and migration (4), exocytosis (5) and insulin secretion (6) to immune defense (7), memory formation (8), and cardiac myocytes (15). Because PKA has a complex mechanism of activation (cooperative cAMP binding to four interconnected sites of different affinities) (16) as well as potent regulatory functions in cells, we sought to develop simpler sensors based on individual cAMP-binding domains lacking catalytic properties and cooperative binding. Fusing single binding domains of PKA and Epac with GFP variants we obtained several highly sensitive cAMP sensors, which give new insights into spatio-temporal and regulatory patterns of receptor-mediated responses of cAMP.

EXPERIMENTAL PROCEDURES

Construction of Fluorescent cAMP Indicators—The DNA constructs encoding for cAMP-sensing proteins were generated by PCR using human Epac1 (GenBank™ accession number AF103905), murine Epac2 (AF115480), or murine PKA regulatory type IIβ subunit (M12492) cDNA as a template. GFP variants were amplified with standard primers from pEYFP and pECFP (Clontech) and cloned into pcDNA3 vector (Invitrogen) for transient expression in mammalian cells. For purification, the Epac2-cams (for cAMP sensor) cDNA was cloned into pVL1393 vector (Invitrogen), and a sequence encoding for a hexa-histidine tag was inserted directly on the N terminus. The protein was expressed in Sf9 cells (BaculoGold, Pharmingen) and purified using a nickel-agarose method (Qiagen).

Cell Culture—CHO cells stably expressing adenosine A2A receptors and TsA201 and HEK293 cells stably expressing β1 or β2-adrenergic receptors were maintained in Dulbecco’s modified Eagle’s medium/F-12 (37 °C, 5% CO2) or Dulbecco’s modified Eagle’s medium (37 °C, 7% CO2) medium, respectively, plated onto 24-mm glass coverslips for imaging experiments or 90-mm Petri dishes for cuvette fluorometric measurements, and transfected with 3 or 30 μg, respectively, of DNA for each construct using the calcium phosphate method. Peritoneal macrophages were isolated from 10–12-week-old FVB/N mice as described (17), resuspended in phosphate-buffered saline, and immediately transfected by electroporation using Gene Pulser (Bio-Rad) at 250 V, 500 microfarads, seeded onto 24-mm glass coverslips, and maintained in RPMI 1640 medium (37 °C, 5% CO2). Primary neurons were transiently transfected with the Nucleofector system (Amaxa, Köln, Germany). Briefly, 3–4 × 106 freshly dissociated cells were electroporated with 3–5 μg of plasmid DNA, and cells were plated on poly-L-lysine and Matrigel-coated (BD Biosciences, Heidelberg, Germany) glass slides in Neurobasal-A medium for 3–5 days. 18 h after transfection, 5 μM cytosine arabinoside was added to prevent proliferation of glial cells. All animal
procedures were approved by the responsible government authority (protocol number 201-2531.01-1098).

**FRET Measurements and Cell Imaging**—Fluorescent microscopy was done as described (19) with adherent cells using a CoolSNAP-HQ CCD camera and a beam splitter 505 DXKR. The imaging data were analyzed with Meta Morph 5.0 (Visirion Systems) and Origin (Microcal, Amherst, MA) software. To study agonist-induced changes in FRET, cells were continuously superfused with buffer A plus agonists. For local stimulation of a cell, patch pipettes (Harvard Apparatus, Edenbridge, UK) filled with the respective agonists were accurately positioned onto the plasma membrane by a manipulator (Patchman, Eppendorf, Germany). To avoid free agonist diffusion along the membrane, cells were continuously superfused with laminar flow of buffer in the direction opposite to the pipette.

**Fluorescence Measurements in Vitro**—TsA201 cells 24 h post-transfection were washed thrice with chilled phosphate-buffered saline, scraped from the plate, and resuspended in 5 mM Tris-HCl, 2 mM EDTA (pH 7.3). After disruption with an Ultraturrax device for 40 s on ice and 20-min centrifugation at 80,000 rpm, fluorescence emission spectra of the supernatant (excitation at 436 nm, emission range 460–550 nm) were measured with a fluorescence spectrometer LS50B (PerkinElmer Life Sciences) before and after adding various concentrations of cAMP, cGMP, AMP, or ATP (Sigma). Purified proteins from Sf9 cells were diluted prior to measurements in the same buffer to 40 nM final concentration. CAMP saturation curves were plotted using KaleidaGraph 3.0.5 software (Abelbeck).

**RESULTS**

**Development of Novel Fluorescent cAMP Indicators**—Several fusion proteins were generated that contained a single or both cAMP-binding domains of Epac1, Epac2, or PKA regulatory βI-subunit fused to GFP variants at different positions (Fig. 1A). Based on structural data for cAMP binding to Epac2 (20), we hypothesized that positioning of YFP and GFP directly on α-helices H4 and O6: B, encompassing the cAMP-binding domain B of Epac2, might lead to a change in the distance between the fluorophores due to a cAMP-induced conformational switch (Fig. 1B). We obtained a fusion protein termed Epac2-camps exhibiting FRET that decreased upon addition of cAMP (Fig. 1C), compatible with an increased distance of the CFP and YFP moieties as deduced from structural predictions of the cAMP-induced conformational change (see arrow in Fig. 1B). This decrease in FRET in response to CAMP was specific, since fluorometric measurements in vitro using Epac2-camps purified from Sf9 cells further demonstrated that other nucleotides were recognized only weakly (e.g., AMP, >10 mM; ATP, 2.5 ± 0.4 mM; cGMP, 10.6 ± 0.4 μM). Using this experimental system we sought to compare the novel system with the previously developed PKA-based approach (12). Measuring activation of purified proteins in vitro, we observed a lower speed of cAMP-induced PKA complex dissociation, which was even slower in the presence of lower CAMP concentrations, whereas the Epac2-camps was switched on much more rapidly at all CAMP concentrations with kinetics faster than our experimental setup allowed to resolve (0.5 Hz sampling rate; Fig. 1, D and E). This demonstrates higher temporal resolution of the new single-domain sensor.

**FRET Measurements in Living Cells**—To monitor agonist-induced CAMP accumulation in living cells (CHO, COS7, HEK293) we transfected them with Epac2-camps. FRET between the CFP and YFP moieties was confirmed both by ratio-metric measurements (Fig. 2A) and by acceptor photobleaching resulting in 15.5 ± 1.6% donor quenching (n = 4, data not shown). Stimulation of adenyl cyclase with adenosine in CHO cells stably expressing adenosine A2B receptors (CHO-A2B) and transiently transfected with Epac2-camps led to a decrease in the FRET signal, reflecting a rise in intracellular cAMP (Fig. 2A). A direct comparison of Epac2-camps with the tetrameric PKA system (12) uncovered that Epac2-camps reacted to adenyl cyclase stimulation much more rapidly (Fig. 2B) in accordance with the faster activation kinetics of the new indicator observed in vitro (Fig. 1, C and D). Comparison of the in vitro kinetics and the kinetics in intact cells reveals that the response of the Epac-based sensor may be limited by the kinetics of CAMP production, whereas the tetrameric PKA sensor is limited primarily by activation kinetics of the sensor itself (binding of four cAMPS and subsequent subunit dissociation).

Having shown that a single CAMP-binding domain is sufficient to generate a conformational change detectable by FRET, we optimized the position of fluorophore insertion using again the CHO-A2B cells and stimulation with adenosine (Figs. 1A and 2C). We next generated similar sensors using the cAMP-binding domains of Epac1 and PKA regulatory subunit (with ligand, left) and Epac2 (without CAMP, right). The black arrow indicates the proposed cAMP-induced conformational change. The positions of fluorophore (CFP and YFP) insertion are labeled. C, fluorescence emission spectra of Epac2-camps. A cAMP-dependent decrease in FRET is demonstrated. Excitation of the CFP in Epac2-camps at a wavelength of 436 nm led not only to CFP emission at ~480 nm but also to YFP emission at ~525 nm. Addition of increasing concentrations of cAMP caused a progressive reduction of the YFP peak and a smaller increase in the CFP emission, indicative of a loss in FRET. D and E, differences in activation speed of tetrameric PKA (12) and Epac-based cAMP sensors. Ratiometric measurements were performed in vitro using proteins expressed Sf9 cells. cAMP in various concentrations was added at time point 0. One representative experiment for each concentration is shown. Epac-camps is switched on faster than resolved by the instrument (<2 s), whereas dissociation of PKA complex is much slower, particularly at low cAMP concentrations. BFP, enhanced blue fluorescent protein.
receptors (HEK-βAR) further demonstrated similar activation properties of Epac1-, Epac2-, and PKA-camps (Fig. 2E) but revealed a significantly larger signal amplitude and activation speed for Epac1-camps, which was, therefore, used in all further assays.

Imaging experiments with Epac1-camps were then done to view the spatial properties of intracellular cAMP signals. Expression of Epac1-camps in individual CHO-A2B cells (Fig. 2F) and other cells showed uniform distribution throughout the cytosol (as judged from simple YFP fluorescence), which is presumably due to the fact that the sensor contains only the cAMP-binding domain of Epac. Ratiometric imaging allowed the determination of FRET, and stimulation of A2B receptors with adenosine caused a decrease in the FRET signal throughout the cells (Fig. 2F).

**Rapid Gradients of cAMP**—It has been argued over decades whether cAMP acts in cells as a freely diffusing second messenger or whether such signals are more localized (22). PKA-dependent β2-adrenergic receptor-mediated stimulation of L-type calcium channels in primary hippocampal neurons (23) as well as PKA activation in cardiac myocytes (15) has been suggested to occur in spatially restricted signaling complexes. To assess the spatio-temporal aspects of cAMP-induced Epac signaling, we transfected primary hippocampal neurons with Epac1-camps. The Epac1-camps-expressing cells were then stimulated with the β2-adrenergic agonist isoprenaline (50 nM) delivered locally with a patch pipette. This local stimulation resulted in a rapidly spreading change in the FRET signal, reflecting a rise in cAMP propagating from the site of stimulation through the whole neuron on the scale of a few hundred milliseconds (Fig. 3A; supplemental Movie 1). Rapidly spreading cAMP signals were also observed with Epac1-camps in other cells, including mouse peritoneal macrophages stimulated locally with isoprenaline (Fig. 3B). In CHO-A2B cells, as well as in PC12 cells with endogenous adenosine A2B receptors (24), similar spatial patterns of cAMP responses to adenosine were observed (not shown).

Measuring these rapidly propagating FRET changes in many cells enabled us to calculate the speed of the cAMP gradient in neurons. To do so, we fitted the FRET signals in different regions of the cell (Fig. 3A) to a first-order exponential function and defined the intersection of the fit with the baseline as the time of activation. The speed of the cAMP gradients after local stimulation with 50 nM isoprenaline was calculated...
at almost 40 μm/s, from which a diffusion coefficient at 487 ± 23 μm²/s was derived. This is much faster than previously described for cells stimulated with neurotransmitters (14). For example, in lobster somatogastric neurons microinjected with fluorescently labeled tetrameric PKA, receptor-mediated cAMP gradients took many seconds to reach distant parts of the cell (14). These differences are due to the different sensor speeds (see Figs. 1, D and E, and 2B), since transfecting hippocampal neurons with the tetrameric PKA sensor (12, 15) revealed a speed of cAMP propagation (even at 1 μm isoprenaline, Fig. 3C) that was ~20-fold slower than that recorded with Epac1-camps (Fig. 3D). The speed of cAMP propagation measured with Epac1-camps corresponds well with that estimated for cAMP using patch-clamp recording of cyclic nucleotide-gated channels (270 μm²/s) (25) and with the diffusion coefficients calculated for cGMP (500 μm²/s) (26) and microinjected cAMP (780 μm²/s) (13).

**DISCUSSION**

Our initial aim was to develop a novel cAMP fluorescent indicator, lacking catalytic activity and cooperative binding typical for PKA and containing only one ligand-binding moiety fused to both GFP variants to provide a robust monomolecular change in FRET in response to elevating intracellular cAMP. Having four binding sites with different affinities the tetrameric PKA-based indicator demonstrated relatively slow activation kinetics (Figs. 1, D and E, 2B, and 3, C and D), reflecting subunit dissociation. In contrast, the new indicators presented in this paper are based on a single binding domain and reveal a fast speed of activation and are, therefore, more suitable for measuring cAMP with high temporal resolution. Taking only one binding domain of different cAMP-binding proteins made it possible to achieve not only a higher temporal resolution but also an equal level of YFP/CFP expression. Short cAMP-binding sequences in our indicators do not contain any catalytic or targeting domains that might interfere with intracellular regulatory processes. Transfected cells demonstrated uniform, stably reproducible, and rapid cAMP signals following agonist stimulation.

Using constructs based on different cAMP-binding proteins in our imaging (Fig. 2E) and fluorometric experiments (Fig. 2D), we show that all three, Epac1, Epac2, and PKA regulatory subunit, seem to have a common mechanism of ligand-induced rearrangement in the cyclic nucleotide-binding domain suggesting a high functional homology.

The fluorescent indicator allowed us to gain insight into the spatio-temporal organization of cAMP signaling in cells. The present study demonstrates rapid gradients of CAMP, propagating from the site of receptor activation through the whole living cell on the scale of a few hundred milliseconds, which is much faster than described previously for cells stimulated with neurotransmitters (14). cAMP signals recorded with Epac1-camps were uniformly distributed throughout hippocampal neurons, whereas PKA-mediated signals such as β-adrenergic stimulation of L-type calcium channels have been reported to be locally restricted due to formation of signaling complexes including receptors, G-proteins, adenylyl cyclases, effectors, and phosphatases (23). The tetrameric PKA sensor used in cardiomyocytes (15) is spatially restricted by binding to protein kinase A anchoring proteins, whereas Epac-camps (and the analogous sensors) appears to be uniformly distributed throughout the cytosol and to provide a novel sensitive method to image free cAMP concentrations in living cells.

Interestingly, the speed of CAMP signals we measured in primary neurons or macrophages (Fig. 3, A and B) was remarkably higher than that in CHO or HEK293 cells (Fig. 2). This fact demonstrates that it is particularly important to study rapid signaling events in more physiological systems to fully understand their spatio-temporal properties. On the other hand, such physiological systems allow us to further study kinetic differences between distinct CAMP signaling pathways. Here we show that Epac signals are faster than PKA signals, suggesting that Epac may be a CAMP target physiologically developed to regulate more rapid receptor-mediated intracellular events.

The binding domain of Epac, which is highly expressed in many different tissues (10, 27), provides an excellent backbone for fluorescent probes to monitor the dynamics of CAMP in neurons and other cells. Epac-based fluorescent indicators presented here are capable of measuring CAMP in the physiological range with high temporal and spatial resolution and could therefore be ubiquitously applied to study regulation and biological function of CAMP in living cells.

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