EMERGING TECHNOLOGIES

Monitoring of cAMP Synthesis and Degradation in Living Cells

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cAMP is a ubiquitous second messenger that regulates numerous cellular events and complex biological processes (4). For example, cAMP is implicated in memory consolidation (35), immune function (51), regulation of insulin secretion (26), and cardiac frequency (62). cAMP is produced in cells by a family of adenyl cyclases (ACs), enzymes that are localized at the plasma membrane and are capable of converting ATP to cAMP (9). Activity of these enzymes is switched on after activation of G-protein coupled receptors that stimulate Gs proteins (e.g., a2-adrenergic, H2-histamine, and EP4-prostaglandin receptors) and inhibited by receptors coupled to Gi proteins (e.g., a2-adrenergic, M3-muscarinic, and a4-opioid receptors). The action of cAMP is terminated by its hydrolysis by specific phosphodiesterases (PDEs; Refs. 27, 47). cAMP exerts its cellular effects via activation of three different kinds of effectors: cAMP-dependent protein kinases (PKA) (49, 50), cyclic nucleotide-gated channels (CNGC) (6, 16), and exchange proteins directly activated by cAMP (Epac) (7). Activation of all these proteins can be used to monitor changes in intracellular cAMP concentrations.

A classical biochemical approach to measure cAMP is called radiomunoassay (RIA) (8, 24). This is a destructive method that requires a batch of cells to be destroyed to yield a small amount of cytosol where the AMP concentration can be determined using cAMP antibodies and 125I-labeled cAMP. cAMP from the sample competes with the radiolabeled cAMP and thus reduced antibody-coupled radioactivity, whose amount is inversely proportional to the concentration of cAMP in the sample. RIA is a sensitive method that allows detection of total cAMP in cell lysates over a broad range of concentrations. In addition to RIA, several other in vitro techniques for competitive cAMP measurements have been developed and applied for high throughput screening purposes. They are described in detail elsewhere (59). However, all these techniques are destructive and do not allow one to monitor cAMP in living cells with any spatial resolution.

The need for such spatial resolution is dictated by two reasons. First, RIA and other in vitro assays can give information only about total cAMP concentrations calculated from numerous cells, whereas more physiologically relevant are free cAMP concentrations, i.e., second messenger pools that are not sequestered or statically bound to effector proteins. Second, over the last decades, a concept of cAMP compartmentation has emerged that assumes different cAMP concentrations in various cellular compartments (2, 5, 48). Therefore, other methods are needed to resolve changes in cAMP with high spatial resolution at the subcellular level.

During the last two decades, novel microscopic fluorescent techniques have been developed that enable the monitoring of biochemical events and second messengers inside intact cells (52). Such techniques are based on fluorescence resonance energy transfer (FRET), a quantum mechanical phenomenon that occurs between a fluorescence donor and a fluorescence acceptor that are in molecular proximity of each other if the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor (17). Measuring FRET allows one to monitor changes in distance between fluorophores attached to a pair of interacting proteins or a single protein that changes its conformation (54, 63). This methodology could be applied for cAMP measurements based on activation of PKA and Epac, whereas activation of CNGCs could be monitored using electrophysiological techniques or calcium imaging.

Techniques based on PKA dissociation

The first fluorescent method for cAMP imaging was developed based on dissociation of PKA. In the early 1990s, a FRET sensor termed FICrHR (pronounced “flicker”) was reported that consisted of catalytic (C) and regulatory (R) subunits of PKA, chemically labeled with fluorescein (Fl) and rhodamine (Rh), respectively (1). In the absence of cAMP, both subunits form a tetrameric holoenzyme complex, R2C2, in which a high FRET signal between Fl and Rh is observed. When cAMP molecules bind to the regulatory subunits, the catalytic subunits dissociate, thereby eliminating FRET (FIGURE 1A). The change in the fluorescence emission spectrum allowed cAMP concentrations and the activation of the kinase to be nondestructively visualized in single cells microinjected with FICrHR. Calibration curves...
of this sensor for cAMP were comparable to those describing activation of the native PKA (half-maximal constant 90 nM; see also Table 1). FICRhR has been successfully used for several different applications. First, it allowed one to image cAMP in neurons and neuronal networks (3, 19, 25). In Aplysia sensory neurons, which respond to serotonin with an elevation of cAMP, the sensor demonstrated spatial gradients of the second messenger. The authors observed large increases of cAMP in the distal processes and only slight PKA activation in the main body of the neuron. This was the first visual evidence of functional cAMP compartmentation, which was attributed to the structural architecture of these neurons (3). In the intact neural circuit of the stomatogastric ganglion of Panulirus, a distinct pattern of cAMP responses to different neurotransmitters was observed. In this neuronal network, cAMP produced due to synaptic activity in fine neuritis spread into the larger neurites and the cell body to produce a reconfiguration of the neural circuitry (25). In rat brain slices, FICRhR was used to demonstrate that serotonin 5-HT₇ receptors activate cAMP production in intralaminar and midline thalamic neurons, leading to suppression of slow afterhyperpolarization (19, 55). Another study in embryonic spinal neurons demonstrated a reciprocal relationship between spontaneous calcium spikes and cAMP transients (22). In frog ventricular myocytes, FICRhR was combined with patch-clamp recording for the simultaneous measurement of cAMP and L-type calcium channel currents. A brief adrenergic stimulation led to a cAMP transient followed by a slower and longer calcium transient, demonstrating fast PKA-dependent channel gating and high activity of PDEs in these cells (20). FICRhR was also applied to the analysis of cAMP dynamics in mouse oocytes, showing that follicle-stimulating hormone-dependent increases in cAMP in ovarian somatic cells are communicated via gap junctions to the oocytes (58).

**Further studies should demonstrate the correlation of calcium and cAMP oscillations with insulin secretion and investigate how calcium and cAMP interact in other cells with distinct physiological functions.**

However, limitations of FICRhR have hampered its wide applicability. The chemically labeled proteins are rather instable and difficult to produce. They have to be microinjected into cells in micromolar concentrations. This might change the real cAMP kinetics (42) and impair measurements in smaller cells in contrast to large neurons (45). Thus, when the technology of green fluorescent protein (GFP) became available (53), an alternative approach was developed: Zaccolo et al. (60) replaced the organic fluorophores on the PKA by blue and green and subsequently with a more convenient FRET pair, cyan (CFP) and yellow (YFP) fluorescent proteins (61) (FIGURE 1A). These genetically encoded indicators allowed, for the first time, one to visualize microdomains with high cAMP concentrations during the adrenergic stimulation of neonatal cardiac myocytes. Such microdomains were found to be localized to the striated pattern of these cells at the site of PKA targeting by A-kinase anchoring proteins (AKAPs) (59a, 61). The central role in the formation of the microdomains was attributed to different isoforms of PDEs present in cardiac tissue, mostly PDE3 and PDE4 (34, 61). Recently (56), the sensor was expressed in adult guinea pig ventricular myocytes using adenoviral gene transfer and demonstrated opposite cAMP responses to adrenergic and muscarinic stimulation. However, the issue of compartmentation was not addressed in this study. More recently (32), PKA adenoviruses were used to study cAMP dynamics in adult mouse cardiomyocytes from PDE4D-knockout mice, where the localized cAMP increases at the sites of ryanodine receptor localization (the so-called Z-
lines) after adrenergic stimulations were larger than in wild-type animals. A general point about the measurements of localized cAMP increases using the PKA probe is the fact that the sensor itself is localized by binding to AKAPs (S9a). This localization presumably enables the sensor to specifically measure cAMP in such AKAP-induced microdomains; it remains to be studied whether this affects ratiometric measurements and whether it may, for example, contribute to a striated pattern in cardiomyocytes. Even though the latest PKA-based indicators are genetically encoded, do not require microinjection, and can be expressed in different cell types by transfection with two DNA constructs encoding for R-CFP and C-YFP subunits, they still have some disadvantages. First, they are comprised of two large chimeric proteins that have to be expressed at equal levels to form a functional tetramer. Individual R and C subunits tagged with CFP or YFP can reassociate with unlabeled endogenous subunits, such that the kinetics of cAMP signals become difficult to ascertain with high accuracy. Second, the catalytic subunit is an active one, and its overexpression can have detrimental consequences for a cell, including apoptosis (30). Furthermore, activation of PDE4 by PKA-dependent phosphorylation leads to cAMP hydrolysis and makes accurate cAMP measurements difficult (27). Moreover, PKA dissociation occurs via a multistep, cooperative process through a series of conformational changes before C subunits are released (12, 29), which results in slower kinetics of the sensor compared with real cAMP kinetics. The FRET signal reported by the PKA sensors reflects, therefore, the complex dissociation of PKA rather than acute changes in cAMP concentrations per se (20).

Methods using CNGCs

Another technique for live-cell cAMP measurements has been developed based on CNGCs. These are non-selective cationic channels comprised of four subunits with an intracellular regulatory binding site for cAMP/cGMP (16). DNA encoding for olfactory CNGA2 α-subunits can be transfected into cells where they form functional homomultimeric channels integrated in the plasma membrane. Increases in cAMP lead to a fast gating of the

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**FIGURE 1. Principles of cAMP sensors constructed using PKA, CNGCs and Epac**

A: in absence of cAMP, PKA sensor is a complex of two regulatory (R) and two catalytic (C) subunits tagged with rhodamine (Rh)/CFP and fluorescein (Fl)/YFP, respectively, and high FRET is observed. After cAMP binding to the two sites in each regulatory subunit, catalytic subunits are released from the complex that results in the loss of FRET. B: olfactory CNGCs are gated by subsarcolemmal pools of cAMP. Increases in cAMP result in a current, which can be measured electrophysiologically for monovalent cations and calcium. Calcium influx can also be visualized in cells by imaging of a calcium-sensitive dye, e.g., Fura. C: Epac-based cAMP sensors (camps) are comprised of a single cyclic nucleotide binding domains (CNBD) fused between CFP and YFP. cAMP induces a conformational change leading to an increase in distance between the fluorophores, which is measured as a decrease in FRET.
channels, and this can be monitored by electrophysiological recording or calcium imaging (FIGURE 1B). Native CNGCs have a much lower sensitivity for cAMP (half-maximal response of the mostly used CNG A2 isoform at \(-36 \mu M\)) compared with PKA (40, 43), whereas their affinity for cGMP is much higher (\(-1.6 \mu M\)). Therefore, several mutants have been created to increase affinity and selectivity of these channels for cAMP (see Table 1; Ref. 43). Because CNGCs are membrane-localized sensors, they can measure cAMP concentrations exclusively at the plasma membrane. Compared with PKA, these sensors have no catalytic activity and do not undergo desensitization. They are not voltage sensitive and respond rapidly to cAMP. However, cAMP-mediated activation of the channels leads to calcium influx, which might result in some feedback effects. Measuring monovalent cation current in this case allows one to avoid potential problems caused by calcium (42, 43).

Use of CNGCs as cAMP sensors has further supported the concept of compartmentation. This method demonstrated the existence of local cAMP pools at the plasma membrane of C6-2B glioma cells and human embryonic kidney 293 (HEK293) cells. cAMP dynamics in these domains (measured with CNGCs) and in the "bulk cytosol" (assayed by a biochemical technique) have been reported to show distinct kinetics (41). Activation of cAMP synthesis in HEK293 cells by prostaglandin leads to a transient increase in cAMP at the plasma membrane, whereas a sustained signal was observed in the cytosol (41). Regulation of cAMP-levels in these highly dynamic subsarcolemmal domains was studied in rat adult ventricular cardiomyocytes expressing CNGCs after adenovirus-mediated transfection (15) and measured electrophysiologically (44). β-Adrenergic stimulation caused a compartmentalized cAMP signal that was measurable within some subsarcolemmal domains but not under the whole membrane. It was also demonstrated that the cAMP dynamics at the plasma membrane are regulated by a negative feedback via PKA and PDEs, confirming that cAMP hydrolysis by these enzymes "shapes" diffusing cAMP gradients and plays a central role in the formation of localized cAMP pools (44).

The use of CNGCs as cAMP sensors is restricted by some aspects that do not allow one to unequivocally demonstrate the presence of compartmentalized cAMP domains. First, the channels lack an ability to report cAMP in compartments other than the submembrane compartment, so that the visualization of cAMP in the cytosol needs to be based on another method, e.g., biochemical, with distinct sensitivity and resolution (41). Second, calcium influx through the channels might lead to inhibition of cAMP-sensitive ACs, affecting cAMP production (14). Finally, the visualization of low levels of cAMP pools by the PKA sensor or CNGCs might result solely from the fact that these sensors are themselves localized in cells at the site where the microdomains are observed. Thus it appears important to confirm the existence of local cAMP pools by using a sensor that is uniformly distributed in the cytosol and would report localized increases in cAMP. However, it remains unclear whether such signals could be "blurred out" due to the diffusion of such a uniformly distributed sensor.

**Sensors based on Epac**

To develop a new generation of cAMP sensors, different research groups used the third cAMP effector, Epac, as a backbone. Epac serves as an important intracellular effector for cAMP (7). It consists of a regulatory cAMP-binding site and a catalytic domain, which has the function of GTP loading of the small G protein Rap1 and maintaining its active state. Two groups independently created similar sensors comprised of Epac1 or its truncated (lacking the membrane interacting domain) and catalytically inactive mutants sandwiched between CFP and YFP (13, 38). Binding of cAMP to the sensors induces a conformational change that results in a decrease of FRET between the fluorophores. The affinities of such sensors for cAMP are, however, much lower than those of PKA and lie between 14 and 50 μM (Table 1). This allows one to visualize higher cAMP concentrations (a typical plateau of the PKA sensor, indicating that cAMP concentrations exceed the upper detection limit, is not observed using the Epac1 probe; Ref. 38) but might
result in an inability to detect lower concentrations of cAMP, which can already activate native PKA. When compared in one experimental system with the PKA sensor, the Epac1 probe demonstrated faster kinetics, suggesting that Epac activation occurs more rapidly than the PKA complex dissociation (38). The two sensors (PKA and Epac1 sensor lacking the membrane targeting domain) were also compared in one setup to study the effects of calcium oscillations on intracellular cAMP in HEK293 cells stably expressing extracellular Ca\textsuperscript{2+}-sensing receptor (18). In these cells, both sensors demonstrated similar results. Low-frequency, long-duration calcium oscillations generated a dynamic staircase pattern of cAMP-inhibition, whereas higher frequency spiking had no effect. In such cells, the localization of the sensor might not play an important role for calcium-cAMP cascade interaction, whereas in cardiac myocytes these sensors might behave differently.

A whole-length Epac1-based sensor ICUE has been used to compare cAMP dynamics in different subcellular compartments by targeting it with plasma membrane (pmICUE), mitochondrial, and nuclear localization sequences (13). The cAMP dynamics at the plasma membrane after adrenergic stimulation were found to be slightly faster than in cytosol and mitochondria. Interestingly, application of prostaglandin in HEK293 cells produced a sustained response of pmICUE at the plasma membrane (even though the sensitivity of ICUE might be lower than that of CNGCs; see Table 1) in contrast to a transient signal measured under the same conditions with CNGCs (13, 41). Since pmICUE is supposed to localize uniformly to the plasma membrane, we believe that a transient cAMP signal measured by CNGCs results from their localization in distinct compartments within the plasma membrane, which are regulated by PDE activity. This hypothesis is confirmed by studies in cardiac myocytes (44) describing the dynamic regulation of these domains by PDE isoforms. Further experiments are needed to more precisely compare cAMP compartmentation effects using different sensors under similar experimental conditions.

Crystallographic studies of cyclic nucleotide binding domains (CNBDs) of Epac and PKA revealed that cAMP-induced conformational changes occur at the level of a single binding domain and do not necessarily require other parts of the protein (12, 39). We took advantage of this conformational change to create probes based on a single CNBDs of Epac2, Epac1, and PKA (36). Such cAMP sensors (camps) are depleted of all catalytic and interaction domains, containing only a single CNBD fused between YFP and CFP. They demonstrate a cAMP-dependent decrease in FRET and affinities between 0.9 and 2.4 μM (FIGURE 1C, Table 1), allowing one to measure cAMP within the physiological range of concentrations. These sensors are uniformly distributed in the cytosol, and their lack of catalytic and protein-binding activities appears to make them inert for the cells. They demonstrate rapid changes of cAMP after stimulating the cells with agonists of G\textsubscript{s}-protein-coupled receptors. Importantly, the activation of Epac-camps in vitro and in intact cells is much faster than that of the tetrameric PKA sensors, allowing one to achieve higher temporal resolution (36).

**Imaging cAMP Synthesis and Hydrolysis Using Epac-camps**

As mentioned above, cAMP compartmentation appears to be regulated by certain isoforms of PDE through cAMP hydrolysis. Little is known, however, about the kinetic properties of PDEs themselves and the mechanisms responsible for compartmentation. PDE activity in intact living cells has been assessed only indirectly by the use of PDE inhibitors (43). To study kinetic properties of one of the cAMP-hydrolyzing isoforms, PDE2, we used Epac2-camps transferred into bovine adrenal zona glomerulosa cells via adenovirus (37). These primary cells exclusively express the PDE2 isoform, which is directly activated by cGMP (33), whereas other PDEs and cGMP-binding proteins (kinases and CNGCs) are not present. Epac2-camps was able to accurately visualize cAMP degradation by PDE2, because the dissociation of cAMP from the sensor was much faster than the speed of hydrolysis (37). It was demonstrated that cAMP hydrolysis itself is much faster than its synthesis: PDE2 was virtually instantaneously activated by cGMP and hydrolyzed extensive amounts of cAMP, even in the continuous presence of AC stimulation (FIGURE 2). The rapidness of PDE2 action, which kinetically overcomes cAMP production, might be an important mechanism for PDE-mediated compartmentation, i.e., it might "shape" the local pools of cAMP by hydrolysis. In the future, it would be necessary to study the direct kinetics of other PDE isoforms, such as PDE3 and PDE4, that appear to provide for cAMP compartmentation in cardiac myocytes.

PDE2 is an example of a PDE, whose activity is induced by atrial natriuretic peptide-stimulated cGMP production. Other PDE isoforms have been described to be activated by calcium (21). Calcium plays a dual role in regulation of cAMP balance in cells. It can increase cAMP via activation of several AC isoforms (ACI, ACVIII) and decrease cAMP by inhibition of other isoforms (ACIII, ACVI) or via stimulation of cAMP degradation by PDE1 (9, 21). In several types of cells, calcium oscillations have been described in response to physiological stimuli (10, 23, 57). Such oscillations and cAMP play an important role in insulin secretion (28). Is there any relationship between calcium oscillations and cAMP in insulin-producing cells? To answer this question, Epac1-camps and Fura2 were used for simultaneous imaging of cAMP and calcium in insulin-secreting MIN6 cell line (31). This double-imaging technique demonstrated that calcium oscillations and changes in cAMP were synchronized. cAMP levels also oscillated, and, interestingly, increases in calcium were accompanied by decreases in cAMP, suggesting the activation of...
PDE1 (31). An earlier study in glioma C6-2B cells, where cAMP and calcium were simultaneously imaged by FlCrRh and Fura2, demonstrated a sustained calcium-dependent decrease in cAMP via the inhibition of ACV (11). Concurrent measurements of calcium oscillations and cAMP in intact cells expressing Ca²⁺-sensing receptor showed a lack of cAMP-oscillations but a staircase pattern of cAMP inhibition (18). Different modes of calcium-cAMP interaction appear to be clearly cell specific and can now be more precisely investigated by combining both calcium and cAMP imaging techniques for simultaneous measurements. Further studies should demonstrate the correlation of calcium and cAMP oscillations with insulin secretion and investigate how calcium and cAMP interact in other cells with distinct physiological functions.

In summary, Epac-camps sensors can be successfully applied to dynamic monitoring of cAMP synthesis and hydrolysis. This makes them good candidates to study molecular mechanisms of cAMP compartmentation as sensors uniformly distributed in the cytosol. Since several cAMP pools might coexist in cells (some fractions localized to sites of cAMP production and uniformly distributed cytosolic pools), the different sensors described here are important and complementary to analyze cAMP signaling at distinct cellular locations. It becomes evident that only the combination of several approaches is likely to give a more precise idea of cAMP intracellular dynamics.

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