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REVIEW ARTICLE

Illuminating Cyclic Nucleotides: Sensors for cAMP and cGMP and Their Application in Live Cell Imaging

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Abstract | Cyclic nucleotide monophosphates (cNMPs) act as second messengers in eukaryotic and prokaryotic signaling pathways. Even though cNMPs regulate a wide variety of processes, the effects they bring about can be highly specific, and are governed not just by the set of effectors present in a cell type, but also by upstream extracellular factors that modulate cNMP levels in the cell. Here, we describe various eukaryotic effectors of cAMP and cGMP and their use as tools to understand cNMP compartmentalization and temporal fluctuations inside cells.

Keywords: *cAMP, cGMP, Sensor, FRET, BRET*

1 A Brief History of Cyclic Nucleotides in Cellular Signaling Processes

Cyclic AMP was the first molecule to be identified as an intracellular mediator for hormonal activity. Adrenaline was known to activate liver phosphorylase in liver homogenates, resulting in conversion of glycogen to glucose-1-phosphate. Seminal work by Earl Sutherland and co-workers showed that adrenaline stimulation in liver homogenates produced a soluble and heat-stable factor that could activate phosphorylase. This factor could activate phosphorylase even in the supernatant fraction of liver homogenates where adrenaline administration had no effect.[1] The factor was purified and identified as 3′,5′-cyclic adenosine monophosphate (cAMP).[2–4] Later, in 1963, the presence of cAMP was also reported in prokaryotes, and shown to be involved in catabolite repression .[5, 6] Subsequently, 3′,5′-cyclic guanosine monophosphate (cGMP) was isolated from rat urine [7] and was identified as a second messenger in phototransduction in retina.[8] Since then, a number of cyclic nucleotides have been identified, including cyclic CMP,[9] cyclic UMP,[9] cyclic di-GMP,[10] cyclic di-AMP[11] and cyclic GAMP.[12]

2 Cyclic AMP and cGMP Synthesis and Degradation Enzymes in Eukaryotes

After cAMP and cGMP were identified as second messengers, a large number of studies led to an understanding of cNMP synthesis, degradation, and their mode of action inside cells.[13] Cyclic nucleotide synthesis and degradation occurs in almost all tissues.[14–17]

The enzymes that synthesize cAMP and cGMP are referred to as adenylyl cyclases and guanylyl cyclases, respectively (Fig. 1). These enzymes are sensitive to various ligands and factors, thus making cAMP and cGMP play the roles of second messengers in signaling events.[18, 19] Cloning and sequencing of adenylyl cyclases revealed that enzymes from bacteria, eukaryotes and secreted adenylyl cyclases from pathogenic bacteria, can be classified into six classes, based on amino acid sequence similarity.[20–22] Mammals encode ten Class III adenylyl cyclases (AC1-AC10). AC1 to AC9 encode membrane-bound cyclases, while AC10 encodes for a soluble cyclase. All adenylyl cyclases are active as homodimers.[23]

Mammals express four soluble guanylyl cyclase subunits (α1, α2, β1 and β2) and seven single membrane-spanning forms named as GC-A to GC-G.[24] Soluble cyclases function as heterodimers and membrane guanylyl cyclases as homodimers.

Cyclic nucleotide phosphodiesterases (PDEs) degrade cNMPs by converting them to 5′NMPs following cleavage of the cyclic phosphodiester bond. There are three different classes of PDE, out of which, class I contains all mammalian PDEs. Mammalian PDEs have been grouped into 11 families based on sequence homology, substrate-specificity and mode of regulation.[25]

Catabolite repression: System of gene control in some bacterial operons in which glucose is used preferentially and the metabolism of other sugars is repressed in the presence of glucose.

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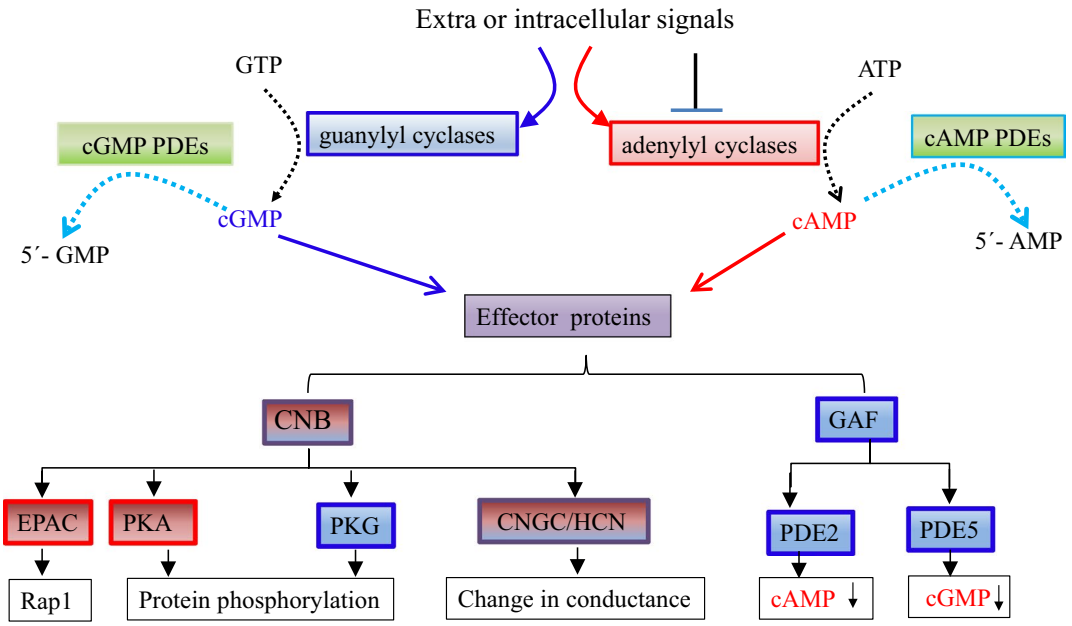

Figure 1: A schematic summarizing the cNMP synthesizing, degrading and effector proteins. ***Boxes*** outlined in ***red*** and ***blue*** indicate cAMP and cGMP effector proteins, respectively, and in ***purple*** domains that bind either cAMP or cGMP.

3 Cyclic NMP-Dependent Kinases: cAMP and cGMP Effectors in Eukaryotes

Patch clamping: A technique used to measure the electrical activity across a small patch of membrane containing one or more channels. It comprises a glass micropipette containing electrode in tight contact with a small patch of membrane and a reference electrode inserted in extracellular media.

Although cAMP stimulates ATP-dependent phosphorylation of phosphorylase kinase in vitro, no direct interaction between cAMP and phosphorylase kinase was observed.[26, 27] This led to the purification of a novel protein kinase activity from rabbit skeletal muscles that was dependent on cAMP.[28] Analogous to cAMP, it was speculated that cGMP may act via protein kinases, and indeed, a cGMP-dependent protein kinase was subsequently purified from lobster muscles.[29] Cyclic AMP- and cGMP-dependent protein kinases are referred to as PKA and PKG, respectively.

4 Non-Kinase Effectors of cAMP and cGMP

Nucleotide exchange factor: These are proteins that activate G proteins by enhancing the exchange of GDP for GTP.

Cyclic AMP-dependent protein kinase activity was purified from nine phyla of the Animal Kingdom.[30] This strengthened the hypothesis that all actions of cAMP were brought about by the set of protein kinases and their specific targets present in a cell. However, the discovery of non-kinase targets of cAMP, such as cation channels and guanine nucleotide exchange proteins added an increased level of complexity to cyclic nucleotide signaling.

In 1987, ion channels were identified as new effectors. The presence of odorant-stimulated adenylyl cyclase in olfactory receptor cells suggested a role for cAMP in olfactory transduction.[31] Patch clamping of olfactory receptor cells demonstrated the presence of cAMP-inducible cation channels.[32] In parallel, rod cells were found to contain a cGMP-specific PDE and guanylyl cyclases.[33, 34] PDE was found to be activated by light[35] and led to the identification of cGMP-stimulated, cation conductance in rod cells.[36] These cGMP-sensitive channels were purified from bovine retinae, and were shown to transport both monovalent and divalent cations, in a cGMP-dependent manner.[37]

An additional cyclic nucleotide gated channel, or CNGC, is the hyperpolarization-activated cyclic nucleotide-gated channel (HCN). HCN was identified in a study aimed at understanding the mechanism of cAMP-dependent, but phosphorylation-independent, increase of polarizing currents in neurons and heart cells.[38]

At the time of writing, the most recently identified target protein for cAMP in eukaryotes is an exchange protein directly activated by cAMP (Epac). Epac proteins were identified independently by two groups, and share sequence homology with a guanine nucleotide exchange factor (GEF).[39, 40]

5 Cyclic NMP-Binding Domains

The general understanding is that cyclic nucleotides bind to specific domains in proteins, thereby allosterically regulating the effector activity of the

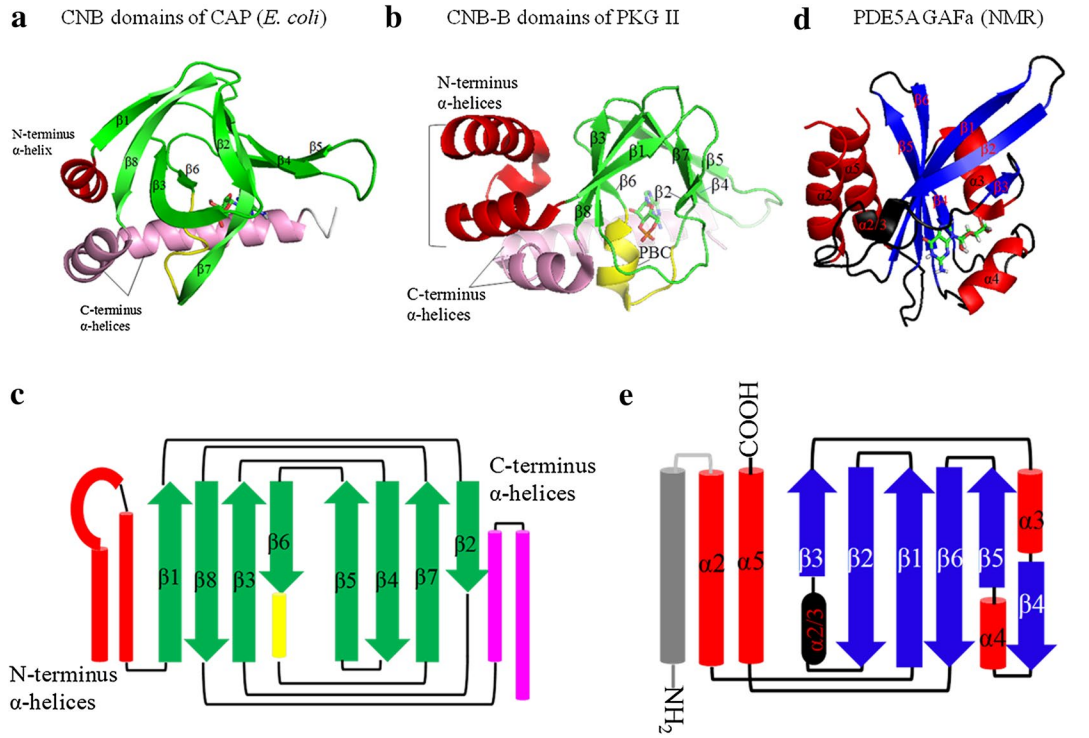

Figure 2: Structure and topology of the CNB and GAF domains. **a** CNB domain of catabolite activator protein from ***E. coli*** bound to cAMP. **b** C terminus GAF domain of human PKG II bound to cGMP. **c** The topology of C terminus PKG II domain, with ***color*** scheme similar to that in **b**. Note that the topology of the β-sandwich is conserved in all CNB domains. **d** GAFa domain of PDE5A bound to cGMP. **d** Topology of cNMP-binding GAF domains adopted from Heikaus et al.[85] The region shown in back and gray is not present in all the GAF domains. The structures in **a**, **b** and **d** were generated from the Protein Data Bank accession codes 1CGP,[184] 5BV6[83] and 2K31[89] using PyMOL Win[185].

full-length protein. The cyclic nucleotide binding (CNB) domain present in PKA, PKG, CNGC, HCN and Epac share sequence and structural similarity (Fig. 2). In addition to these eukaryotic cNMP-binding proteins, the CNB domain is also present in bacterial cAMP effector proteins. The most well-understood cAMP responsive protein (CRP) in prokaryotes is CAP. CAPs are transcription factors, and possess a CNB and a DNA-binding domain. They regulate the transcription of various genes in a cAMP-dependent manner. A unique cAMP-binding protein that has been characterized in mycobacteria possesses cAMP-induced protein lysine acetyltransferase activity.[41] *Pseudomonas aeruginosa* expresses a protein referred to as cAMP-binding protein A (CbpA) that has cAMP-binding activity and two CNB domains.[42]

A second domain has been found to bind cyclic nucleotides and is called the GAF (c**G**MP-specific and stimulated PDEs, ***A****nabaena* adenylyl cyclases and *Escherichia coli* **F**hla) domain (Fig. 2d, e). Sequence alignment of the photoreceptor PDE from rods and cone cells, and the cGMP-stimulated specific PDEs showed the presence of a conserved domain, distinct from the cGMP-hydrolytic domain in these PDEs. Limited proteolysis experiments indicated that the conserved domain contains non-catalytic cGMP-binding activity.[43] Subsequently, this cGMP-binding domain was found to be present in a large family of proteins present in all kingdoms of life, and was named the GAF domain.[44] The role of the GAF domain in PDEs is to bind cGMP and stimulate or inhibit phosphodiesterase activity.[45, 46] Analogous to the GAF domains of PDEs, the GAF domain in the Anabaena adenylyl cyclase was shown to bind cAMP and increase cAMP synthetic activity. However, not all GAF domains bind cyclic nucleotides. The *E. coli* FhlaA is activated by binding of formate to tandem GAF domains,[47] and we now know that proteins containing the GAF domain represent one of the largest families of proteins regulated by binding to a variety of small molecules.[48–50]

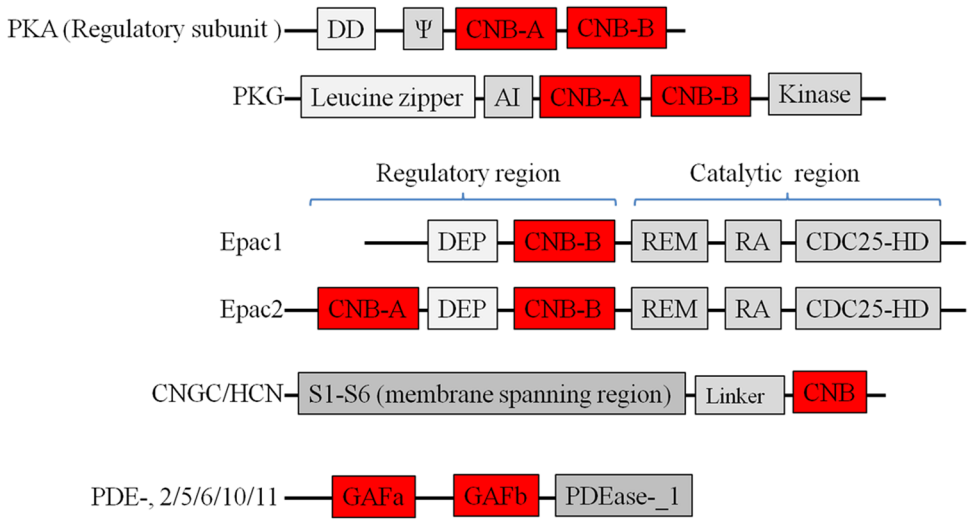

Figure 3: Domain organization of CNB and GAF domain-containing proteins in eukaryotes. Ψ is the pseudosubstrate sequence, and AI is the autoinhibitory sequence. Other domains have been described in the text.

6 Structural Features of the CNB Domain

The first reported crystal structure of a CNB domain bound to cAMP was of CAP from *E. coli*.[51, 52] Interestingly, when sequences of PKA type II,[53] PKA type I [54] and PKG [55] became available, it was seen that they share a significant sequence similarity amongst themselves and with CAP. Hence, it was predicted, and is now known, that all these cyclic nucleotide-binding proteins from eukaryotes and prokaryotes have structurally similar CNB domains.

The CNB domain is ~120 amino acids in length. The basic topology of a CNB domain is a relatively rigid, eight-stranded antiparallel β-sandwich, flanked on both termini by flexible α-helical regions with variable number of helices.[56, 57] The most conserved region of the β-barrel is involved in binding to the phosphate group attached to the ribose sugar of the cNMP, and is hence referred to as the phosphate-binding cassette (PBC, shown in yellow in Fig. 2). PBC lies between two strands of a β-sandwich, and is 12–14 amino acids in length. The two most conserved residues in PBC are arginine and glutamate.[58, 59] Arginine interacts with the equatorial oxygen of the phosphate of ribose moiety (O1P)[52] and glutamate makes hydrogen bond with 2′ OH of cAMP.[60]

The helical region undergoes large conformational changes upon cNMP binding and hence is important for cNMP-mediated allosteric regulation. The helical region which is N-terminal to the β-sandwich is often referred to as N-terminal helical bundle. The helical region present towards the C terminus can be divided into hinge and lid regions. The lid is the most variable part of the CNB domain. The variability of the C terminus helical region can be appreciated by the fact that the hinge of the second CNB domain of PKA type II acts at lid for first CNB domain.[57, 61, 62] The amino acids in the lid make hydrophobic interactions with the base of the bound cyclic nucleotide and protect it from the surrounding environment. In the absence of cyclic nucleotides, the lid helix is relieved of these interactions, and initiates structural changes in the CNB and other domains present in the protein.[62] The conformational changes induced by binding of cyclic nucleotides to CNB domains have been utilized to develop tools for detection of these second messengers inside live cells in real time, as described in subsequent sections.

α-helical: A protein structure composed of α-helices. An α-helix refers to a conformation adopted by a stretch of polypeptide in which backbone N–H of an amino acid residue forms a hydrogen bond with C=O of every third or fourth residues.

β-sandwich: A domain structure in a protein that is characterized by the presence of two antiparallel β-sheets facing each other.

G protein: G proteins are proteins that bind the guanine nucleotide. They are activated when bound to GTP and inactivated in a GDP-bound state.

7 Eukaryotic Proteins with CNB Domains

7.1 Epac

Epac contains CNB domains fused to a GEF domain and is involved in activation of the G protein RapI.[39, 40] There are two genes in humans referred to as EpacI and Epac2. Both these Epacs are active as monomers. A single polypeptide chain harbors an N-terminal catalytic and a C-terminal regulatory region. The regulatory region contains an N terminus DEP domain (Disheveled, Egl-[10,] and Pleckstrin) and the CNB domain (Fig. 3).[39, 40] DEP domains are involved in anchoring the protein to the cell membrane. The catalytic region contains the Ras-Exchanger

Motif (REM), Ras-association (RA) and CDC25-HD domain.[39, 40] CDC25-HD is the catalytic domain and is sufficient for acting as a nucleotide exchange factor for members of the Ras family of G proteins.[63]

Epac1 is localized in the cytoplasm in the apo state. After binding cAMP, conformational changes expose the DEP domain, and this is followed by translocation to the plasma membrane. Epac2 is always localized on the plasma membrane due to its interaction with the Ras protein. Thus, Epacs are involved in the regulation of multiple processes such as cardiovascular contraction, insulin secretion and neuronal transmission, all of which occur in a cAMP signaling-dependent manner.[64]

7.2 PKA/PKG

Protein kinase A (PKA) is composed of two catalytic (C) and two regulatory subunits (R) and forms a heterotetrameric complex (R2C2).[65] Two regulatory subunits, RI and RII, define the enzyme as either PKA Type I or PKA Type II. The R subunit contains a dimerization domain (DD), a pseudosubstrate sequence and two tandem CNB domains. The catalytic activity of the C subunit is inhibited by the pseudosubstrate sequence (Ψ) present in the R subunit in the tetrameric holoenzyme. Binding of four cAMP molecules to the heterotetramer releases the C subunits, which now become catalytically active (Fig. 3).[66]

Mammals encode two genes that encode cGMP-dependent protein kinases PKGI and PKGII. PKGI has two isoforms PKGIα and PKG1β.[67, 68] Both PKGI and PKGII have similar domain architecture and catalytic and regulatory domains are present in a single polypeptide chain, and contain an N-terminal leucine/isoleucine zipper for dimerization, followed by an autoinhibitory sequence and two tandem CNB domains, CNB-A and CNB-B. The C terminus harbors the catalytic kinase domain. CNB-A has a higher affinity for cGMP [69, 70] and cyclic GMP binding allosterically activates the catalytic domain.

7.3 CNGC/HCN

CNGC channels open and close depending on the binding of cyclic nucleotides. HCN open and close in response to membrane potential, and cNMP binding shifts the threshold of the potential needed for opening or closing of the channel. [71, 72] Both CNGC and HCN are tetrameric in nature and made of various combinations of two different subunits. Each subunit has a six-pass membrane-spanning region, and a CNB domain at the C terminus (Fig. 3). Full-length crystal structures for CNGC or HCN have not been reported. However, a reasonable model of the structure based on the crystal structure of isolated CNB domain of such channels,[73, 74] and the membrane spanning domain of other related channels, is available, that aids in understanding cNMP-induced conformational changes that results in opening and closing of the channel.[62, 75]

The structural basis of cAMP and cGMP-induced conformational changes and the regulation of activity in all these CNB domain-containing proteins has been excellently summarized in a review by Rehmann et al.[62]

8 Selectivity in CNB Domain-Containing Effector Proteins

A large number of signaling pathways involve cAMP and cGMP as second messengers in eukaryotes. The selectivity of CNBs for cAMP and cGMP is very crucial for keeping these pathways segregated. Mammalian cAMP and cGMP-specific protein kinases show considerable similarity in sequence but also can be selective for cAMP or cGMP. One residue responsible for cGMP selectivity is an amino acid adjacent to the conserved arginine in PBC. An alanine residue at this position in PKA was seen to be replaced by a threonine in PKG. It was proposed that the OH group of the threonine side chain makes a hydrogen bond with 2-amino group of cGMP.[76] When the conserved alanine in PKA was replaced with threonine, the affinity for cGMP increased tenfold and that for cAMP remained unaltered.[77] Further, replacing the threonine in PKG and CNG with alanine resulted in a significant decrease in the affinity for cGMP, whereas that of cAMP remained unchanged.[69, 78]

The specificity for cyclic nucleotide-mediated regulation of effector protein activity can also be brought about by the regions that are involved in allosteric transition, as opposed to residues those are involved in interaction at the binding pocket. Bovine rod cyclic nucleotide-gated channel is highly selective for cGMP. Replacement of an aspartic acid residue located at the C-terminal α-helical region of the CNB domain makes it selective for cAMP.[79] The ability of the carboxylic acid group of the aspartate residue allows favorable interactions with N1 and N2 hydrogen atoms of cGMP, and unfavorable electrostatic effects with unshared electrons at N1 of cAMP. Analyses based on a two-step model involving initial binding of cNMP to the channel, and a second step of structural transition resulting in opening of the

channel, suggested that the change in selectivity was the outcome of the ability of cNMP to trigger structural transitions, rather than affecting its initial binding affinity.[79]

Selectivity in PKGI is known to be imparted by the lower affinity CNB-B domain. Despite the presence of aforementioned threonine residue at the PBC, the affinity of the CNB-A domain of PKGI-β for cAMP is similar to cGMP.[80] However, binding of cyclic nucleotides to the CNB-B domain is required for complete activation of PKG I.[81] This makes binding at CNB-B a limiting step for the activation of PKG I by cAMP. The need of very high cAMP levels (~50 μM) for binding at CNB-B thus limits the cross-talk between cAMP and cGMP signaling. The crystal structure of this domain bound to cGMP showed an arginine present at β-5 of CNB that makes a hydrogen bond contact with the guanine moiety. Mutational analysis showed that this interaction provides the basis for selectivity for cGMP over cAMP.[82] Similarly, crystal structures' cGMP selective CNB-B domain of PKG II bound to cGMP showed the presence of two specific aspartate and arginine residues that make contact with the guanine moiety and provide a basis for selectivity.[83]

Overall, this indicates that mechanisms by which selectivity is achieved are directed by the proteins in which the CNB domain is found.

9 Cyclic Nucleotide-Binding GAF Domain

The amino acid sequences of GAF domains have diverged significantly due to their long evolutionary history. However, all the characterized cNMP-binding GAF domains contain a conserved NKFDE motif [44, 49] and mutational analyses have shown that these residues are essential for cNMP-binding.[43, 84] However, none of these residues are located near the binding pocket as seen in crystal structures. It has been suggested that they, therefore, may play a role in providing structural stability to GAF domains.[85]

Nucleotide-binding GAF domains have a core structure composed of six stranded antiparallel β-sheets (3-2-1-6-5-4) and four α-helices referred to as α-2, α-3, α-4 and α-5 (Fig. 2d, e). The central β-sheet faces α-2 and α-5 on one side and α-3 and α-4 on another. The cNMP-binding pocket is present in between the β-sheet and α-3 and α-4 helix. Based on biochemical and structural data it is believed that all tandem GAF domain-containing PDEs form parallel dimers.[85] The α-2 and α-5 helices are involved in making dimerization contacts in GAFa of PDE2A [86, 87] and GAFb domain of PDE10 A.[88] Other than the four α-helices mentioned earlier, additional α-helices are seen in the GAF domains of some PDEs. For example, a fifth α helix α-1 is present in GAF domain of PDE5, and is needed for making dimerization contacts.[89, 90]

β-sheet: A secondary structure motif in proteins composed of two or more β-strands interconnected by hydrogen bonds, forming a planar structure. β-Strands are polypeptides of 3–10 amino acids in length whose backbones are present in an extended conformation.

10 Eukaryotic Proteins with cNMP-Binding GAF Domains

PDE2, PDE5, PDE6, PDE10 and PDE11 contain tandem cNMP-binding GAF domains in an N-terminal regulatory region, referred to as GAFa and GAFb. High affinity binding of cGMP to the GAFb domain of PDE2 activates the enzyme and results in enhanced cleavage of both cAMP and cGMP.[91] PDE3 has higher catalytic activity for cAMP as compared to cGMP, but the K_m for the two substrates is similar. This leads to competitive inhibition of cAMP hydrolysis at high cGMP concentration.[92] Therefore, PDE2 and PDE3 play an important role in cross-talk between cAMP and cGMP signaling.[93] Cyclic GMP binding to the GAFa domain of PDE5 increases the V_{max} and K_m of the enzyme for cGMP [94] and also increases the affinity of PDE5 for its inhibitor, sildenafil citrate.[95, 96]

11 Selectivity for cAMP and cGMP Binding in GAF Domains

The cGMP-binding GAF domains have very similar nanomolar affinities for cGMP. However, they show different extent of selectivity for cAMP. For example, PDE6 GAFa, PDE5A GAFa and PDE2GAFb have ~10000-, 1000- and 20–30-fold higher affinity for cGMP as compared to cAMP.[89, 91, 97, 98] The fact that the affinities of cGMP-binding GAF domains are very high and are very similar for all GAF domains suggests the selectivity in these domains is brought about by negative selection against cAMP. Mutational analysis of the GAFb domain of PDE2 showed that residues that interact with the phosphate-ribose moiety or the imidazole ring of cNMP confer high affinity binding, while residues that interact with the pyrimidine ring provide cyclic nucleotide specificity.[91] We have shown that a single cAMP-specific GAFb domain from an Anabaena adenylyl cyclase showed lower selectivity for cAMP in comparison with the tandem GAFab domain.[99] This indicates that the presence of a second adjacent GAF domain in PDEs could also be a mechanism to achieve cyclic nucleotide selectivity in PDEs.

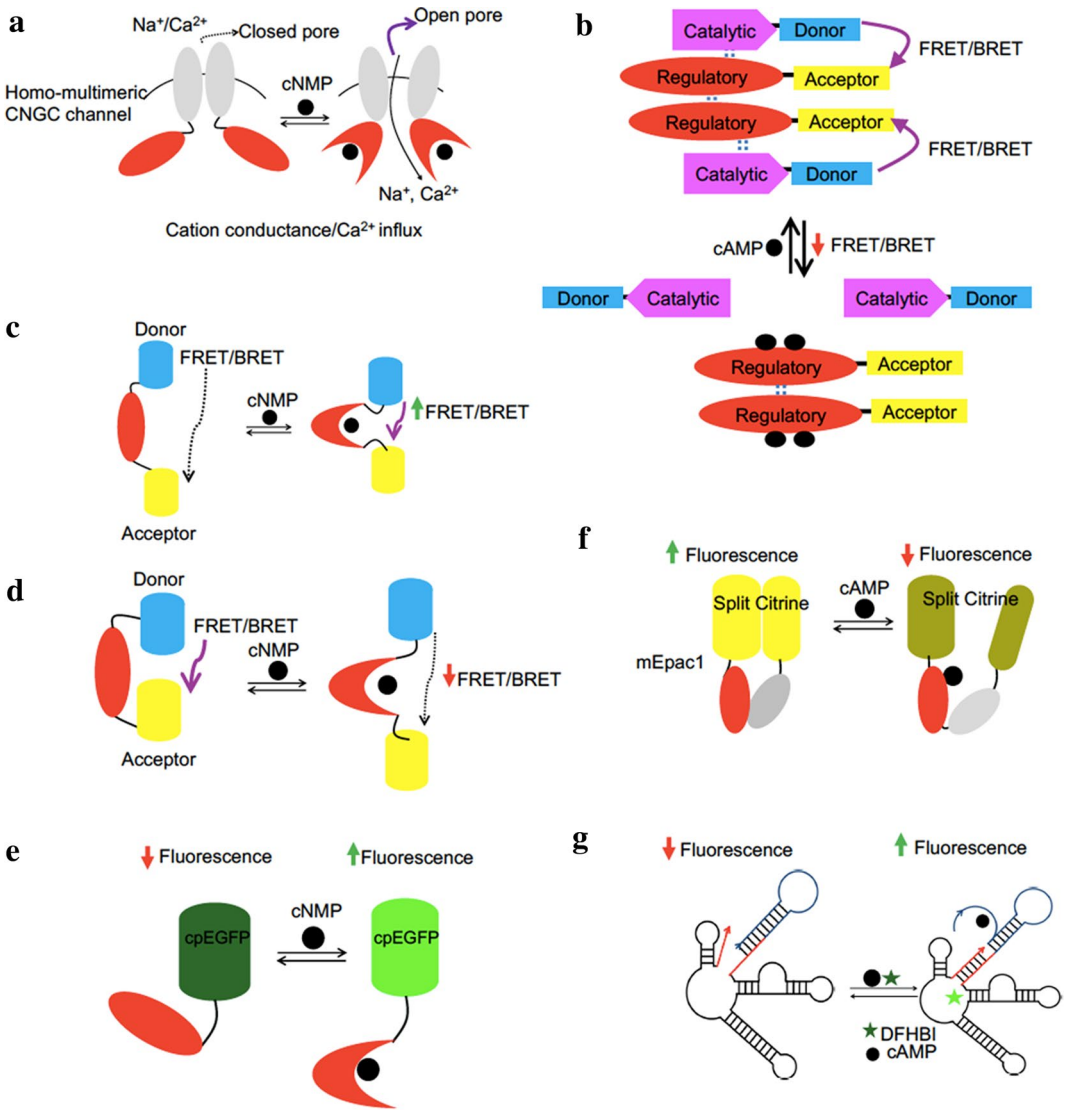

Figure 4: Schematic representation of the design strategy of various cAMP sensors. **a**–**f** Protein-based sensors for cyclic nucleotides. ***Red ovals*** represent the domain that binds the cyclic nucleotide, and the ***filled arcs*** represent the conformational change that occurs on cAMP binding. **g** Nucleic acid-based cAMP sensor. ***Blue lines*** correspond to cAMP binding sequence and ***black lines*** indicate the DFHBI binding sequence.

12 Cyclic Nucleotide-Binding Proteins as Cellular Sensors that Monitor cAMP and cGMP Levels in Time and Space

Traditionally, measurements of cAMP and cGMP are performed using cell population assays, such as radioimmunoassay or ELISA methodologies.[100, 101] However, these methods cannot provide information about the localization of cNMPs within single living cells, heterogeneity in concentration in a population of cells, and/or fluctuations that occur very rapidly. It is reasonable to propose that methods of detecting these analytes at the level of the single cell could provide new information about cNMP-mediated signaling. Since cNMP-binding target proteins undergo conformational changes upon binding to cNMPs, one could monitor these conformational changes for visualization and estimation of cAMP and cGMP nucleotides inside single cells. Figure 4 depicts the design strategies employed for making cNMP sensors, which are discussed further below. Some of these sensors utilize the CNB domain alone, opposed to full-length effector proteins. The region of the CNB domain that is sufficient for binding and undergoing cNMP-induced conformational changes are selected based on the

Table 1: A summary of the cAMP and cGMP sensors described in this article

Sensor design	Recognition module	EC_{50} (μM)	References
cAMP sensors			
Conductance of CNGC	Rat olfactory CNGC α subunit (roCNGCα)	40	108
	roCNGCα (C460W, E583M); C460W and E583M make it more sensitive and selective for cAMP over cGMP	1	105
	roCNGCα (C460W, E583M and Δ61–90); Δ61–90 to impart Ca^{2+} insensitivity.	14	
Inter-molecular FRET	R1 subunit of human PKA	0.080	112
	RII subunit of PKA	NA	113
Intra-molecular FRET	*Epac1-camps*: CNB domain of human Epac1 (E157–E316)	2.3	115
	Epac2-camps: C terminus CNB domain of murine Epac2 (E285–E443)	0.9	
	PKA-camps: CNB domains of and RII β isoform of murine PKA (M264–A403)	1.8	
	Full-length human Epac1 (hEpac1)	50	114
	(Δ1–148) hEpacI; deletion to get rid of membrane localization	35	
	(Δ1–148, T781A, F782A) h Epac1; T781A and F782A remove catalytic activity	14	
	CNB domain of murine HCN2 (A467–K638)	6	118
	(Δ1–148, T781A, F782A) hEpac1	10	117
	(Δ1–148, T781A, F782A, Q273E) hEpac1; Q273E is to increase cAMP affinity.	4	
Inter-molecular BRET	RI α of human PKA	~1	125
	hPKA RI α (R210K); R120K lowers cAMP affinity	~0.1	
	hPKA RII α	NA	
Intra-molecular BRET	Human EpacI (149–881, T781A, F782A)	8	128, 129
	Human EpacI (149–881, T781A, F782A). In addition an N terminus tag (1–196 aa of PDE2A3) for plasma membrane localization	10	
	hEpacI (149–881)	NA	130
	Full-length KATms (1–333)	0.15	41
	CNB domain of KATms (1–215)	0.07	
Fluorescence (not FRET)	*Flamindo*: Mouse Epac1 CNB domain (157–316)	3.6	123, 124
	PM-Flamindo: mEpacI (157–316) and N terminus tag (20 amino acid of GAP43) for plasma membrane targeting	NA	
	Flamindo2: mEpacI (157–316), longer linker between Citrine and N terminus of mEpac	2.1	
	NLS-Flamnido2: NLS at N terminus for nucleus targeting.	NA	
	Class II RNA aptamer [182]	985	142
cGMP sensors			
CNGC conductance	Chimeric CNGC known as "RONS2" [183]	3–4	107
FRET	*CGY-A12*: h PKG 1α (L, I, C1–47A); leucines/isoleucine zipper mutated to prevent dimerization.	NA	119
	CGY-Del1: PKG Iα (Δ1–47); dimerization domain deleted	0.02	
	Cygnet 1: PKG Iα (Δ1–77); dimerization domain deleted.	1.5	120
	Cygnet 2.1: PKG Iα (Δ1–77, T516A); T516A removes catalytic activity	1.8	
	cGES-DE2: PDE2A (Q392–A525); GAFb domain	0.9	121
	cGES-DE5: PD5A (Q154–A308); GAFa domain	1.5	
	cGES-GKIB: PKGI (L231–A350)	5	

(Continued)

Table 1: (Continued).

Sensor design	cAMP sensors		
	Recognition module	EC_{50} (μM)	References
BRET	Human PDE5A2 (S118–H273); GAFa domain	0.030	96
Fluorescence (not FRET)	Regulatory domain of PKG1 α (1–356)	0.035	122
	Regulatory domain of PKG1 β (1–372)	1.1	
	Regulatory domain of PKG1 α (77–356)	0.17	

NA means EC_{50} values were not reported

information available from the crystal structures (see Table 1).

13 CNGC and HCN Channels for Sensing Using Electrophysiology

To monitor rapid changes in cGMP and cAMP concentrations near the plasma membrane, CNGCs are very useful. Since homotetrameric CNGCs are functional, expression of a signal subunit in the cell of interest is sufficient for making use of them as a sensor. CNGC sensors are either expressed in cells using adenovirus transduction method or patch-crammed into cells of interest.[102, 103] The amount of cNMP generated in the cell is estimated from the extent of cation conductance through these channels, which can be measured using standard electrophysiological methods. Alternatively, cation conductance can also be measured by detecting Ca^{2+} entry into the cytoplasm using a Ca^{2+}-sensitive dye.[104–106]

The first real-time measurement of cGMP in living cells was performed in differentiated neuroblastoma cells using the patch-cram method.[107] A patch from a Xenopus oocyte containing a high density of CNGC was excised and inserted (crammed) into recipient cells. Prior to insertion, the patch was calibrated for cGMP-stimulated conductance. In a second study, a rat wild-type olfactory CNGC (oCNGC), mutant, which is more selective for cAMP, was used for cAMP measurement in cultured cells using adenovirus-based expression methods.[105, 108]

The advantages of using CNGC for live cell measurements of cAMP or cGMP are that measurements can be performed rapidly, result in lower swamping of cNMPs, and provide high resolution of cNMP concentrations following calibration using excised patches from membranes of cells expressing the channels. However, electrophysiological measurements are technically challenging and time consuming; therefore, the use of

Adenovirus transduction: It is a method of delivery of foreign DNA into the cells, achieved by packing the DNA into adenoviral-like particles. These infect cells and release their contents, but cannot replicate and cause pathogenesis.

Patch-crammed: This is a method where a patch of membrane from a donor cell, expressing the membrane protein of interest, is excised and inserted into another cell of interest. This method is mainly employed to understand the properties of a channel inside the membrane patch.

Electrophysiological methods: The methods for recording electrical activity of a cell.

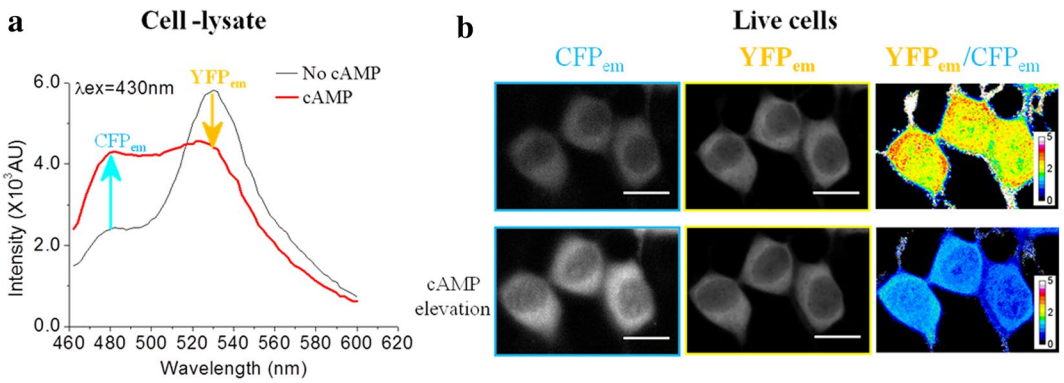

Figure 5: Output from a typical FRET-based cAMP sensor. **a** The emission spectrum of cell lysates prepared from cells expressing an Epac-based cAMP sensor. The sensor has CFP and YFP variant proteins as FRET donor and acceptor, respectively. In the presence of cAMP, the emission of CFP increases, whereas emission of YFP decreases, indicating loss of FRET in the presence of cAMP. **b** Images of cells expressing the sensor, acquired using widefield epifluorescence microscopy, with CFP excitation (408 nm) and collection of emission using CFP (CFPem) and YFP (YFPem)-specific filters. Upon cAMP elevation, an increase in intensity in CFPem can be seen. The changes in FRET can be visualized in pseudocolored images obtained by dividing the intensity acquired in the YFPem channels by the corresponding intensity in the CFPem channel. ***Scale bar*** 10 μm. The data shown was generated in our laboratory and the sensor was generously provided by Prof. K. Jalink (Netherland Cancer Institute)[117]

these sensors has been restricted to a few laboratories.[109, 110]

14 FRET Sensors

Förster resonance energy transfer (FRET) is the phenomenon of non-radiative transfer of energy from one fluorophore to another, when present in near proximity. Efficiency of FRET between a given pair of fluorophore depends on the distance between them, as well their orientation. In general, FRET sensors have an analyte-binding domain fused to a pair of fluorophores suitable for FRET. Analyte binding brings about conformational changes in the biosensor, resulting in an alteration in distance or relative orientation of the fluorophores, and a consequent change in the FRET signal.[111] An example that is commonly obtained from FRET-based imaging of cAMP has been illustrated in Fig. 5.

The first attempt to visualize cAMP in single living cells was made in 1991.[112] The C subunit and R subunits of PKA were labeled with fluorescein and tetramethylrhodamine, respectively, and the labeled subunits injected into the cells. The inactive holoenzyme complex, R2C2, showed high FRET because of proximity of fluorescein and tetramethylrhodamine-labeled C and R subunits. Upon addition of cAMP-elevating reagent such as forskolin or isoproterenol, the FRET signal was lost because of dissociation of the R2C2 complex.

The development of mutant GFPs with different excitation and emission wavelengths allowed replacement of chemical fluorescent dyes with a pair of fluorescent proteins in the PKA sensor, and made them genetically encodable.[113] Such a sensor was expressed in CHO, COS-7 and HEK293 cells and responses to forskolin and di-Br-cAMP were detectable. These PKA-based sensors were not monomeric. Therefore, this resulted in slower kinetics of the sensor. Moreover, fluctuations in the relative expression of C and R subunits resulted in variations in the FRET ratios, independent of cAMP. The second major improvement to cAMP sensors was to encode them in a single polypeptide chain, by attaching the FRET protein pair to the termini of either full-length Epac[114] or the CNB domains of PKA or Epac[115]. Ponsioen et al. used isoproterenol to elevate cAMP levels in GE11 cells and detected the increase using earlier PKA sensors as well as single-chain Epac sensors. Lysophosphatidic acid (LPA) treatment followed by isoproterenol lowered cAMP levels, and this rapid lowering could be detected by the Epac single-chain sensors, but not by the PKA sensor. In fact, the PKA sensors showed a lag period of several minutes before showing a recovery of the FRET signal after LPA addition.[114]

The single-chain sensors based on the CNB domains of Epac have a higher affinity for cAMP in comparison to the full-length Epac sensor [114, 115]. Single-chain Epac sensors were used to monitor the speed of cAMP diffusion in the cell body of hippocampal neurons, and cAMP levels in peritoneal macrophages upon β-AR stimulation.[115] Cyclic AMP-induced FRET changes are more dramatic in the PKA sensors, due to complete separation of the fluorophores present on different polypeptides following cAMP binding. In contrast, single-chain sensors show smaller cAMP-induced changes in FRET, because cAMP binding to the central CNB domain induces conformational changes that result in relatively smaller alterations in distance and orientation of the fluorophores. Currently, a pallet of cAMP sensors is available, based on both Epac and PKA, encoding a variety of fluorescent proteins.[109, 116, 117] The CNB domain of HCN2 has also been used to make a FRET-based sensor.[118]

A series of FRET-based sensors based on full-length and N-terminal truncated PKG 1α have been reported.[119] ECFP and EYFP were fused at the N and C termini of PKG I, using various linker combinations. These sensors are known as CGY (for ECFP, PKG Iα, and EYFP).[119] Other FRET-based sensors for cGMP, named Cygnet-1 and Cygnet-2 (cyclic GMP indicator using energy transfer) were developed by the Dostmann group.[120] These sensors are also based on N-terminally truncated PKG 1α. In Cygnet-1, PKG 1α lacks dimerization and autoinhibitory domains while Cygnet-2 lacks these two domains and is also catalytically inactive. Cygnet-2 was used to image cGMP elevation induced by NO and C-type natriuretic peptide (CNP) in rat fetal lung fibroblasts.[120] Nikolaev and co-workers developed a series of cGMP-energy transfer sensor (cGES) based on the C-terminal CNB domains of PKG1 (cGES-GKIB), GAF domains from PDE2 (cGES-DE2) and PDE5 (cGES-DE-5).[121] Comparison of cAMP selectivity of these sensors indicated that cGES-DE5 and Cygnet are selective for intracellular cGMP. Further, it was observed that the response of cGES-DE5 to increasing concentrations of cGMP is faster than Cygnet, and also showed a higher change in signal. This places cGES-DE5 as the most suitable FRET sensor for cGMP imaging.

15 Non-FRET Fluorescence Sensors for cAMP and cGMP

Nausch and co-workers developed non-FRET fluorescent indicators for cGMP (FlincGs). They generated three FlincGs by attaching at the N terminus of a circularly permuted eGFP (1) the regulatory domain of PKG1-α (α-FlincGs), (2) PKGI-β (β-FlincGs) (3) and the N-terminal deletion mutant of PKG1-α (δ-FlincGs).[122] Binding of cGMP to all these constructs resulted in an increase in fluorescence of GFP. These sensors had an additional excitation peak at 410 nm, and intensity corresponding to that excitation did not change significantly upon cGMP addition. Hence, these proteins could be used as ratiometric sensors. These sensors show the highest dynamic range (~3.7-fold change in signal) amongst all the sensors available for live cell imaging of cGMP. δ-FlincGs was used to monitor cGMP elevation mediated by NO and atrial natriuretic peptides (ANP) in vascular smooth muscle cells. While NO induced sustained and global elevation in cGMP, ANP-induced cGMP elevation was localized and transient.[122]

A non-FRET protein-based sensor for cAMP is Flamindo (fluorescent cAMP indicator).[123, 124] This sensor is made by inserting a fragment of Epac at the 145th amino acid residue of Citrine, which is a modified YFP. Flamindo showed a decrease in fluorescence intensity upon cAMP binding. Kitaguhci et al. used Flamido1 to study cAMP elevation in pancreatic MIN6 cells, and showed that calcium influx from the extracellular milieu, and not the immobilization of Ca^{2+} from intracellular stores, elevates cAMP and potentiates insulin secretion. Flamido2 is a modified version of Falmido and has a higher dynamic range (~fourfold change in signal) and brightness.[124]

Flamindos are single wavelength sensors. This makes them valuable for multicolor imaging allowing one to study the dynamics of a second analyte along with cAMP in the same cell at the same time. Simultaneous imaging of Ca^{2+} and cAMP using Flamindo2 in HeLa cells showed that both cAMP and Ca^{2+} were elevated by noradrenaline in these cells.[124] FlincGs and Flamido are smaller in size in comparison with FRET sensors, which allows the attachment of additional sub-cellular-targeting sequences to them, without resulting in very large proteins being expressed in the cell.

16 BRET Sensors

Cyclic nucleotide sensors based on bioluminescence resonance energy transfer (BRET) have also been described. In BRET the donor fluorophore for FRET is replaced with a luciferase enzyme that acts as donor of chemiluminescence. The advantage of BRET sensors over FRET sensors is that they do not need any excitation light and, therefore, can be used in light-sensitive systems. Two types of BRET sensors for cAMP, based on PKA type I and type II, have been reported. These sensors contain GFP as the acceptor fluorescent protein attached to the catalytic subunit, and Renilla luciferase (Rluc) as the donor attached to either RI or RII.[125] Interestingly, it was observed in COS-7 cells that PKA type II was responsive to β-adrenergic stimulation whereas the type I sensor showed no change in BRET. This again supported the emerging concept of compartmentalized cAMP elevation by β-adrenergic receptor signaling.[126, 127]

A single-chain BRET-based cAMP sensor using Epac, YFP and Rluc named CAMYEL (cAMP sensor using YFP-Epac-RLuc) has been generated.[128] The N-terminal 196 amino acids of PDE2 splice-variant-3 were fused to the N terminus of CAMYEL, to get PDE2-CAMYEL. This construct localizes the plasma membrane and was used to study the localization of PDE activity in HEK 293 cells.[129]

Another BRET-based sensor using N terminus truncated Epac, Citrine and Rluc has been described.[130] BRET sensors based on a mycobacterial cAMP-binding protein lysine acyltransferase (KATms[41]) utilizes the CNB domain and Rluc and GFP as donor and acceptor for BRET.[131] The sensor was used to demonstrate the cAMP-induced allostery seen in full-length KATms.[131] Interestingly, in the sensors based on the KAT proteins, the BRET signal increases upon cAMP binding as opposed to PKA and Epac-based sensors.

A BRET-based sensor for cGMP has also been reported using the GAFa domain of PDE5.[96] It was seen that cGMP levels in HEK293 cells transfected with this sensor was higher than untransfected cells, indicating that these sensors can act as sinks for cGMP. This observation has two important implications. First, intracellular cNMP sensors could affect the physiology of cells by sequestering cyclic nucleotides. Second, the GAF domain of PDE5 may act as sink for cGMP, and hence play a dual role, one in lowering

Circularly permuted eGFP: is a modified eGFP having the same amino acids as eGFP; however, the order of the sequence of amino acid is changed. The new order is obtained by connecting the N and C termini to get a circular polypeptide and by inserting a break at the place of interest to make new C and N termini.

Chemiluminescence: It is the phenomenon of emission of light as a result of a chemical reaction. In living systems, the principal source of chemiluminescence is oxidation of members of a set of small molecules named luciferin, by the class of enzyme called luciferase.

cGMP levels by increasing PDE5 activity, and the other in acting as a store for cGMP, preventing its hydrolysis. Importantly, all BRET sensors have been used only for population-level live cell measurements and none were used for detection of cAMP or cGMP at the single cell level using imaging techniques.

17 Nucleic Acid-Based Sensors for cAMP and cGMP

Myocytes: The contractile cells present in muscles.

In the last few years, nucleic acids have been shown to be excellent biomaterials for making desired architectures and assemblies.[132] One of the applications of these nucleic acid-based nanodevices is in bioimaging.[133] Nucleic acid sensors have been used for quantitative imaging of H^+ and Cl^- in isolated cell culture and in whole organisms.[134–136] Furthermore, nucleic acid sequences called aptamers that specifically bind to an analyte of interest can be isolated using in vitro selection methods from synthetic libraries.[137, 138] These aptamers can be used to design nucleic acid nanodevices that can be used for detecting specific analytes.[139]

Receptor desensitization: Inactivation of the receptor after activation by a ligand, which can include mechanisms of internalization and recycling.

Recently, an RNA aptamer for 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) called Spinach [140] has been described. DFHBI is cell permeable and is non-toxic to the cell and has very low fluorescence. When DFHBI binds to Spinach, its fluorescence increases significantly. Therefore, Spinach attached to the terminus of an RNA of interest can be used to visualize that RNA inside the cells, upon bathing cells in a solution containing DFHBI. Spinach-DFHBI system has been referred to as RNA mimics of GFP for this reason. A series of genetically encodable sensors for various analytes were then prepared by attaching Spinach to aptamers specific for these analytes, in such a way that DFHBI could bind to Spinach only when the analyte is bound to the aptamer (Fig. 4g).[141]

An RNA aptamer for cAMP and a modified version of Spinach has been used to design a sensor for cAMP (Figure a).[142] This sensor needs a free 5′ end for sensing cAMP, which makes it challenging to stably express this construct inside the cell. Moreover, the sensor has a very low affinity for cAMP and hence is useful for monitoring cAMP fluctuations at very high cAMP levels (above 100 μM). Nevertheless, this study demonstrates the possibility of generating nucleotide-based sensors for cyclic nucleotides, which ideally in future, should be genetically encodable.

18 Applications of cNMP Sensors

Cyclic AMP is an important mediator of cardiac contractility regulated by catecholaminergic receptors, and also mediates responses to a variety of other hormone and neurotransmitters in heart.[143] Deregulated GMP signaling has been associated with specific cardiovascular diseases.[144] Similarly, cAMP and cGMP play an important role in neurotransmission.[145, 146] Hence, sensors for cAMP and cGMP have been extensively used to study the compartmentalization of cyclic nucleotides in cardiac myocytes and neuronal cells.[145, 147, 148] A brief summary of interesting studies about cNMP compartmentalization in these two cell types has been provided below.

The first real-time measurements of cGMP in neuroblastoma cells was performed by Kramer and co-workers using patch-cram method.[107] These measurements introduced the previously unknown concept of long-term suppression (LTS) of cGMP responses elicited by NO. Post-application of muscarinic agonists to mammalian neuronal cells, no response to NO donors can be seen for ~2 h (referred to as LTS). LTS is not a result of receptor desensitization, because cGMP elevation induced by application of NO donor agents is not mediated by receptors. The mechanism of LTS was later explained to be activation of unidentified non-specific PDEs, by CaM-Kinase II-mediated covalent modifications.[107, 149]

Shelly et al. showed that local application of cAMP and cGMP to a neurite in neuronal cultures results in their differentiation into axons and dendrites, respectively.[150] With the help of fluorescent biosensors for imaging cAMP and cGMP they were able to show that local application of cAMP to a neurite resulted in a decrease of cAMP in other neurites. The level of cGMP decreased in the neurite where cAMP was applied, and increased at distantly localized neurites.[150] In contrast, local elevation of cGMP resulted in a decrease in cAMP levels in the stimulated neurite, but had no long-range effects. This local and long-range reciprocal regulation of cAMP and cGMP ensures that a neuron has a single axon and multiple dendrites.[150]

Del Puerto et al. used cAMP-imaging in hippocampal neurons to understand the mechanism of coordination of three different purinergic receptors (P2X7, P2Y1 or P2Y1) in modulating the growth of the axon. They observed that these receptors alter the levels of cAMP at distal regions of axons, and the effect is brought about by the regulation of AC5.[151]

Nicol and co-workers reported the dynamics of interaction between Ca^{2+} and cAMP at the tip of growing axon, referred to as the growth cone, using Ca^{2+} and cAMP imaging.[152] They showed that at the filopodia of the growth cone, a transient elevation in cAMP leads to transient elevation of Ca^{2+}, whereas at the growth cone center, elevation in Ca^{2+} leads to elevation in cAMP.[152] Castro et al. used a cAMP sensor to show that dendrites of cortical neurons exhibit higher cAMP levels in response to isoproterenol as compared to rest of the cytosol, and PDE4 acts as barrier in propagation of cAMP signal from membrane to rest of the cytosol.[153] Imaging of cAMP in brain slices showed that cAMP signal elevation is faster, higher and long lasting in striatal neurons as compared to cortical neurons.[154] In medium spiny neurons, NO-cGMP signaling modulates dopaminergic transmission. Elevated cGMP levels activate PDE2 which in turn inhibits transient cAMP elevation, mediated by stimulation of dopaminergic receptors.[155]

Imaging of cAMP levels using sensors in rat neonatal cardiomyocytes showed that upon β-adrenergic stimulation, cAMP was enriched in specific microdomains. No such effect was seen when cAMP was elevated by the application of a phosphodiesterase inhibitor or forskolin to these cells. This suggested that cAMP stimulation via the β-adrenergic receptor may activate PKA localized to these microdomains.[126] Subsequently, cAMP was imaged in PKA type I or PKA type II-enriched domains in cardiac myocytes, using cAMP sensors targeted to these domains. It was seen that cAMP elevation in these domains was specific to the GPCR agonist used, indicating that these domains are coupled to different types of GPCRs, by a yet-to-be identified mechanism. Elevation of cAMP using specific PDE inhibitors showed that PDE2 regulated cAMP levels at RI domains whereas PDE4 decreased cAMP levels at RII domain.[156] Furthermore, cAMP and cGMP imaging in cardiomyocytes demonstrated that cGMP modulated the levels of cAMP in both these domains[93] is mediated by cGMP-activated PDEs. RII domain is enriched in PDE2. Hence, cGMP elevation negatively regulates cAMP level in this domain. RI domain in enriched in PDE3 which is a cGMP-inhibited PDE. Hence, cGMP elevation elevates cAMP levels in this domain.[93] Soluble GC stimulation results in similar levels of cGMP elevation in both the domains, while plasma membrane-associated receptor guanylyl cyclase stimulation elevated cGMP only in the RII domain.[93]

Fischmeister group measured cGMP-induced currents at subsarcolemmal compartments upon local application of NO donors in frog ventricular myocytes. They observed that the spread of cGMP from the site of NO donor application to a remote part of the cell was restricted.[157] Further, they monitored subsarcolemmal cGMP in rat cardiomyocytes using rat olfactory CNGC. They observed that the particulate GC (pGCs) have more pronounced localized effect in elevating cGMP in comparison to soluble GCs (sGC) activation.[158] The intracellular compartmentalization of cGMP is regulated by PKG activation in adult cardiomyocytes.[159]

In addition to cardiac myocytes and neurons, sensors for cyclic nucleotides have also been used in other contexts. Bagorda and co-workers studied changes in cAMP levels in response to stimulation of chemoattractant receptors in *Dictyostelium discoideum*. They showed that adenylyl cyclase A is specifically responsible for chemoattractant receptor-mediated elevation in cAMP.[160] Gomes et al. showed that under starvation, cAMP levels were elevated which in turn activated PKA.[161]

GPCRs, post-activation by their respective ligands, are endocytosed during receptor desensitization and recycling. Recently, cAMP-imaging techniques have shown that certain receptor–ligand pairs co-localize in internalized vesicles and actively produce cAMP. This phenomenon has been referred to as "sustained cAMP production",[162, 163] and could allow the spread of the signal to locations away from the plasma membrane. CAMYEL and plasma membrane-targeted CAMYEL were used to estimate the localization of the activity of various PDEs in HEK293 by treatment with specific PDE inhibitors, and measuring cAMP in cells transfected with these sensors.[129] Evidence for localized cAMP elevation near phagosomes in the activated macrophage[164] has also been described.

19 Perspective and Conclusions

Cyclic pyrimidine nucleotides (cUMP and cCMP), and PDE activity specific to them, were detected in animal tissues extracts.[165–169] However, due to irreproducibility of the experiments and methodological problems, the field of cyclic pyrimidine cyclic nucleotides had remained unexplored. It will be worthwhile to revisit the field of cyclic pyrimidine nucleotides, with newer methodologies to gain insight into their biological functions. To develop sensors to these,

Ventricular myocytes: Myocytes present in ventricle of the heart.

Cortical neurons: the neurons present in the cerebral cortex of the brain.

Striatal neurons: The neurons present in striatum of brain. Striatum is sub-cortical part of the fore-brain.

however, one would need to identify binding proteins for cUMP and cCMP which are structurally modulated on cNMP binding.

In addition to cyclic pyrimidine nucleotides, cyclic di-nucleotides have also been identified in bacteria and eukaryotes. Three well-known c-di-nucleotides are c-di-GMP,[170] c-di-AMP[171] and cGMP-AMP (cGAMP).[12, 172] C-di-GMP and c-di-AMP are mostly bacterial second messengers and cGAMP is present in eukaryotes. Cyclic di-GMP seems to be the most important second messenger in bacteria having surpassed the role of cAMP as a second messenger.[173] Cyclic di-GMP signaling has also been reported in *Dictyostelium discoideum*.[174] In contrast to cAMP and cGMP, where the target proteins either bind to CNB or GAF domain-containing proteins, the target molecules for c-di-GMP have a larger repertoire of proteins and binding domains.[170] The targets for c-di-AMP are also unique.[171]

Novel domains that can bind cAMP and/or cGMP may be discovered. In order to find such new domains, unbiased biochemical approaches should be utilized, instead of predictive bioinformatics approaches. For example, we have identified a mycobacterial protein that binds cAMP, which does not contain either a CNB or a GAF domain.[175] This protein is a universal stress protein (USP), and was identified following interaction of mycobacterial cytosolic extracts with cAMP-agarose beads. USP has a tenfold higher affinity for cAMP as compared to ATP. The crystal structure of USP bound to cAMP, as well as mutational analyses, showed that cAMP binds to USP at the same binding site as ATP.[175]

Single cell imaging methods have provided significant information about compartmentalization and regulation of cAMP and cGMP signaling in neuronal cells and cardiomyocytes. However, cAMP also plays an important role in innate immunity.[176] A plethora of bacterial pathogens have been known to exploit cAMP signaling to evade host immune response.[177] Surprisingly, cAMP sensors have not been used to study cAMP localization in immune cells, or in cells infected with cAMP-elevating pathogens. Similarly, cGMP has a critical role in regulating gastrointestinal homeostasis,[178] and the compartmentalization and temporal dynamics of cGMP in intestinal cells is not known. Interestingly, current attempts are directed towards imaging cNMP signaling at more relevant physiological levels, as opposed to isolated cells in culture, as in imaging in brain slices, and generation of transgenic animals expressing these sensors.[154, 179–181] We, therefore, anticipate an increase in the use of biosensors to get new insights into spatial and temporal properties of cAMP and cGMP signaling.

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