Commentary

The many dimensions of cAMP signaling

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In the previous issue of PNAS, Rich *et al.* (1) provide dynamic evidence that cAMP is produced in a restricted microdomain near the surface membrane of human embryo kidney cells transformed with adenovirus containing cyclic nucleotide-gated (CNG) channels. When activated by cAMP, the channels conduct Ca^{2+} into the cell, thereby offering an on-line assay for the cyclic nucleotide through Ca^{2+} imaging. When the cells were stimulated with prostaglandin E1, the Ca^{2+} signal increased abruptly, then rapidly fell back to baseline. In contrast, total cAMP (assayed biochemically in the cells prelabeled with [^{3}H]adenine) rose rapidly to a plateau and then remained elevated for some time. The rapidly declining cAMP measured by Ca^{2+} influx through CNG channels was taken to be "membrane localized", whereas the radiolabeled cAMP represents the "total cellular" pool. These two pools are present within distinct compartments that are separated by diffusion barriers. Although subcellular compartmentalization of cAMP action was recognized at least 20 years ago (2), only recently has there been direct evidence for the idea that cAMP can act in special cellular domains rather than uniformly and everywhere (for recent general reviews, see refs. 3–5). An understanding of the molecular basis of compartmentalization should be achievable because of the extensive information now available concerning the molecular components of cAMP signaling pathways.

Techniques for Assaying cAMP

A major technical difficulty had been the lack of a suitable method for measuring cAMP at the subcellular level. Conventional immunological and radiochemical assays are not adequate for examining the regional flux and distribution of cAMP. In addition, only tentative results were obtained by using fluorescence–resonance energy transfer. This imaginative technique involves the microinjection of the cAMP-dependent protein kinase (PKA) subunits labeled with both fluorescein and rhodamine, and then observing, by confocal fluorescence microscopy, the loss of energy when cAMP causes the subunits to dissociate. Because relatively large amounts of fluorescently labeled PKA must be injected, the fluorescence–resonance energy transfer method has been used primarily with large invertebrate neurons [the marine mollusk *Aplysia* (6) and the spiny lobster, *Palinuris* (7)].

The use of CNG channels as sensors has the distinct advantage of detecting cAMP associated with the plasma membrane. CNG channels are nonselective cation channels made up of four subunits, each with a six-transmembrane segment related to voltage-gated K^+ channels (8, 9). Typically, they are sensitive to both cGMP and cAMP and are regulated by Ca^{2+}/calmodulin. The cyclic nucleotides bind to sites that are homologous to the two cAMP-binding sites present in each regulatory (R) subunit of PKA. Because this site is situated at the cytoplasmic surface of the cell's plasma membrane, this technique detects the cAMP produced in submembrane microdomains. The use of CNG channels as sensors was inspired by Richard H. Kramer, now at University of California, Berkeley (10, 11), who "crammed" patch pipettes containing inside-out membrane patches with CNG channels into neuroblastoma cells, thus bringing the exogenous cyclic nucleotide-binding sites into contact with the cytoplasm for microassay. There are only a few examples in which endogenous CNG channels have been used to monitor the production of cyclic nucleotides: neurons of the gastropod mollusk, *Pleurobranchia californica* (12), and rat olfactory receptors (13). The utility of the endogenous channels is obviously limited, however, to the particular cells that happen to be endowed with the CNG channels naturally.

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To extend the utility of the CNG-channel method, Jeffrey W. Karpen and his interdisciplinary collaborators (1, 14–17) at the University of Colorado Health Sciences Center (Denver) have engineered an adenovirus vector encoding the rat olfactory CNG channel mutated for enhanced sensitivity to cAMP (concentrations in the 100 nM range) and for greatly diminished sensitivity to cGMP and Ca^{2+}/calmodulin. The construct is a Ca^{2+} channel when activated by cAMP, and the influx of Ca^{2+}, followed fluorometrically, serves as a measure of the activation of adenylyl cyclase.

The Spatial Dimension: The Molecular Basis of Compartmentalization

To discuss the molecular basis of compartmentalization, we must first describe the biochemical characteristics of the components that make up the cAMP-signal transduction pathway: a receptor, adenylyl cyclase (AC), phosphodiesterase (PDE), and PKA. Signal transduction begins when an external first message (neurotransmitter, hormone, or drug) binds to a receptor (typically a protein with seven-transmembrane segments) to activate AC through a trimeric G protein. ACs contain 12-transmembrane segments, and the interactions between the receptor and the G protein, and between the G proteins and the cyclase, as well as the production of cAMP itself, all take place close to the cytoplasmic surface of the plasma membrane. Once formed, cAMP binds to the R subunits of PKA. Binding of the cyclic nucleotide releases active catalytic (C) subunits from inhibition by the R subunits releasing them to phosphorylate substrate proteins. Usually the action of the kinase ceases when the cAMP is removed by enzymatic degradation by phosphodiesterase. In the absence of cAMP, C subunits again become inhibited by reassociation with R subunits.

Fundamental to compartmentalization of the cAMP signaling pathway is the great variety of each molecular component. Each component is represented by several isoforms, and each isoform can be differentially regulated, for example, by phosphorylation or by Ca^{2+}, and be ticketed to a specific place within the cell, presumably because of special signal sequences or domain specificities. A particularly striking example is the differential

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distribution of β-adrenergic receptors, the first component in the signaling pathway. There are two β-adrenergic receptor subtypes in rat cardiomyocytes. The β2 receptor subtype is confined totally to caveolae, largely excluding the β1 receptor subtype that is distributed in other parts of the membrane of cardiomyocytes (2, 18). This differential distribution of receptor subtypes has physiological consequences: first, because the two subtypes have somewhat different properties; and second, because the two compartments (caveolae and the other portions of the membrane) not only contain different components of the cAMP pathway but also segregate receptors other than β-adrenergic ones. Thus, caveolae are largely depleted of the metabotropic acetylcholine receptor that is known to modulate the activity of β-adrenergic receptors (18).

The next component of the cAMP-signaling pathway is AC. There are more than nine closely related enzymes that can be regulated in several different ways (19–21). Some cyclases are activated by Ca^{2+}/calmodulin, others are inhibited by low concentrations of Ca^{2+}, and still others are inhibited by calcineurin, the Ca^{2+}-dependent protein phosphatase, or by phosphorylation with the Ca^{2+}/calmodulin dependent protein kinase II (CAMK II). Still others are activated by protein kinase C. As would be expected, there is evidence for colocalization of ACs with their specific regulators. Thus Ca^{2+}/calmodulin-dependent AC is a prominent constituent of dendritic spines in hippocampal neurons, where influx of Ca^{2+} through *N*-methyl-D-aspartate (NMDA) receptors leads to the synthesis of the cAMP needed for the induction of long-term synaptic plasticity (22). Ca^{2+}-regulated ACs also have been shown to be situated together with L-type Ca^{2+} channels (23) and channels mediating capacitative Ca^{2+} entry (24), and yet another with CAMK II in olfactory cilia (25).

Many enzymes degrade cyclic nucleotides. At present, there are at least eleven families of PDEs specific for cAMP (26, 27). These degradative enzymes differ in substrate and inhibitor specificities and in how they are regulated (principally by Ca^{2+} and by protein phosphorylation). Differential distribution again is fundamental to compartmentalization (28); for example, some PDEs are targeted specifically to the cortical cytoskeleton subjacent to the plasma membrane (29) or to the plasma membrane itself (30). In rat messengial kidney cells, PDE 3 and PDE 4 each have been shown to regulate distinct cAMP-signaling pathways (31).

There is less variety in the subunits of PKA. The holoenzyme is a heterotetramer consisting of two C and two R subunits (32). There are several (three mammalian) C isoforms, and two types of R, I and II, each with an α and β subtype. When the R subunit dimers bind four molecules of cAMP and dissociate from the two C subunits, the R subunits remain dimerized. The subunit types do not get scrambled because the R subunits remain dimerized when they reassociate with C subunits. Although there are some differences in the properties of RI and RII type PKAs, the major feature pertinent to compartmentalization is the interaction with PKA-binding proteins called A kinase anchor proteins (AKAPs). In many tissues, a large proportion of PKA is associated with cytoskeleton and membrane; for example, more than two-thirds of the PKA in hippocampal neurons is localized to the postsynaptic density and cytoskeletal elements of dendritic spines. Almost all of the anchored kinases are type II (5, 33, 34). The molecular details of how RII subunits bind to AKAPs are not understood fully. The N-terminal domains of the subunit dimer are critical. AKAPs do not have a consensus binding sequence, however. Therefore, it is likely that the binding occurs to regions of AKAPs with differing amino acid sequences but with a similar conformation.

The examples of cAMP-signal transduction described thus far are all short-term: signaling stops when the concentration of cAMP drops to baseline.

At least 20 AKAPs have been cloned, representing a family of functionally related proteins that serve to localize PKAs. Specific AKAPs anchor the kinase to particular subcellular components, for example, cytoskeleton, nucleus, endoplasmic reticulum, peroxisomes, microtubule associated protein 2-centrosome, and ion channels. Importantly, specific AKAPs can bring together several functionally related constituents; thus, for example, AKAP 79/150 anchors PKA II, PKC, and a phosphodiesterase, together with the β-adrenergic receptor (35).

The Time Dimension

The examples of cAMP-signal transduction described thus far are all short-term: signaling stops when the concentration of cAMP drops to baseline. The differences between the two pools of cAMP that Rich *et al.* (1) found in human embryo kidney cells treated with prostaglandin E1 is in the duration of the cAMP signal—brief in the membrane microdomain and longer-lived in the total cellular cAMP. Elegant experiments with inhibitors of PDE indicated that the differences observed were caused by differential activation of PDE in the membrane microdomain (1), but the signaling in both compartments ends rapidly when the cAMP is degraded.

But, cAMP signaling can be extended past the time when PDE has degraded the second messenger. An extra time dimension opens up with prolonged stimulation. Activated C subunits of PKA then are imported into the cell's nucleus, an outstanding cellular compartment! [see, for example, refs. 6 and 36; C subunits have putative nuclear localization signals (NLSs) in their amino acid sequence that facilitate passage through nuclear pores.] In the nucleus, the activated kinase subunits phosphorylate cAMP response element binding protein (CREB) and other transcription factors. CREB binds to the 5′ untranslated regions of genes that contain a cAMP-response element (CRE) and, upon phosphorylation, initiates a cascade of gene expression that persists long after the original stimulating cAMP has been degraded (37–39). Activation of CREB-mediated protein synthesis is a means of transforming short-term programs produced by cAMP into long-term durable changes of the cell. The formation of long-term synaptic plasticity thought to underlie learning and memory is an important example of this transformation (40–43).

Entry of C subunits into the nucleus is quite slow, and the phosphorylation of CREB may require periods of time longer than the life-time of cAMP in the cytoplasm. The action of the kinase can be prolonged, however, if some R subunits are degraded, the resulting imbalance rendering some C subunits autonomous and persistently active (44–46). During consolidation of long-term memory in *Aplysia* mechano-sensory neurons, one of the two immediate early genes activated by phosphorylated CREB encodes a ubiquitin C-terminal hydrolase (47) that stimulates the ubiquitin-proteasome-mediated degradation of R subunits. As a result, autonomous C subunits remain active for at least 24 h after the cAMP has been degraded (48).

With the CNG-channel technique for measuring cyclic nucleotides sensitively at the subcellular level, and with so much information about the many dimensions of cAMP signaling, we now can look forward to understanding in detail the physiological functions of each compartment.

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