Commentary

Transfected Cyclic Nucleotide–gated Channels as Biosensors

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The cyclic nucleotides, cyclic AMP and cyclic GMP, like intracellular calcium, are involved in a vast array of intracellular signaling processes. But knowledge of the cellular and subcellular concentrations of cAMP and cGMP, and of the kinetics and spatial distributions of the changes of their concentration in situ, have lagged far behind our knowledge of the corresponding features of calcium signaling, which were propelled by developments in calcium-sensing dyes and imaging technology (Tsien, 1989, 1998). In this issue, J.W. Karpen and colleagues advance the issue of cAMP measurement in living cells by developing and convincingly calibrating the use of transfected cyclic nucleotide–gated channels as cAMP sensors (Rich et al., 2000).

Cyclic AMP Signaling and Some Physiological Puzzles

Sutherland's seminal discovery of the role of cAMP as an intracellular "second messenger" (Sutherland, 1962), and the subsequent connection of adenylyl cyclase (AC) activation to chemo-sensing heterotrimeric G protein–coupled receptors (GPCRs; G Protein Receptor Database, http://www.gcrdb.uthscsa.edu/GCR_Fam.html; SwiftEMBL, http://swift.embl-heidelberg.de/7tm/), paved the way for understanding the molecules that generate, shape, and receive cAMP signals. In addition to the seven-helix GPCRs that activate AC, and the phosphodiesterases that hydrolyze cAMP (Butcher and Sutherland, 1962), several downstream effectors for cAMP, including cAMP-dependent protein kinases (PKA; Walsh et al., 1968), cyclic nucleotide–gated (CNG) channels (Fesenko et al., 1985; Kaupp, 1995), and cAMP-regulated guanine nucleotide exchange factors (Kawasaki et al., 1998) have been identified and characterized. To date, more than 200 cellular targets are known to be regulated by PKA alone (Francis and Corbin, 1999). Yet, compared with our understanding of calcium signaling, relatively little is known about the biophysical details of cAMP signaling in situ. An oversimplified, current conception of cAMP signaling is as follows: an extracellular signal such as norepinephrine binds to a GPCR in the cell plasma membrane, which then activates the membrane-associated G protein, G_S; the latter in turn activates AC, also located in the plasma membrane. Subsequently, the concentration of cAMP in the bulk cytoplasm of the cell rises to a concentration of several micromolar, activating PKA ($K_{1/2} \approx 80$ nM), which then phosphorylates its target molecule. Subsequently, cAMP phosphodiesterase hydrolyzes the cAMP to terminate the response.

This "one-compartment" conception of cAMP signaling raises a number of problems for the physiologist, among which are the following. First, there is the problem of signaling specificity: in any individual cell, PKAs have many different molecular targets, and it is difficult to understand how these different targets could be differentially regulated (for example, by different GPCR cascades) as a chemo-signal activating any one class of GPCR would lead to activation of all the PKAs. Second, there are the biophysical problems of energetics and kinetics: it seems energetically wasteful for the cytoplasmic concentration of cAMP to be raised throughout the cell to activate PKAs, which are known to be localized to different parts of cells by A-kinase anchoring proteins (AKAPs; Scott et al., 2000); it furthermore it seems a priori difficult to achieve precise kinetics and regulation of the cAMP concentration near PKA targets when signaling components are anisotropically distributed and physically separated one from another. To begin to address some of these issues, it would be useful to have a method of sensing cAMP that is intrinsically local.

Transfected cAMP-gated Channels as cAMP Sensors

Rich et al. (2000) have incorporated the coding sequence of the α subunit of the olfactory CNG channel into an adenovirus construct, and transfected it into rat C6-2B glioma cells and HEK-293 cells, including a line (HEK-AC8) expressing AC; this construct should allow efficient expression in many other cell types. Whole-cell recordings establish that the homomeric channels are expressed, inserted into the plasma membrane, and rapidly respond to cAMP or analogs delivered through the whole-cell pipette, via photolysis of NPE-cAMP ("caged cAMP") or by activation of AC by forskolin.

Rich et al. (2000) convincingly calibrate the transfected channels as cAMP sensors by analyzing the dose–response curves of channels in excised inside-out patches from the transfected cells. Critical features of these channels for their role as sensors are the very weak dependence of the dose–response curve on membrane potential, and the absence of any inactivation/desensitization; i.e., the currents

J. Gen. Physiol. © The Rockefeller University Press • 0022-1295/2000/08/143/3 $5.00
Volume 116 August 2000 143–145
http://www.jgp.org/cgi/content/full/116/2/143

recorded at constant cAMP have long-term stability. It follows that once the saturating cAMP-activated whole-cell current is known, the relative current will provide a measure of the intracellular cAMP concentration near the channels. To measure cAMP quantitatively, whole-cell currents through CNG channels are monitored with the perforated patch-clamp technique, allowing electrical access to the cell while preventing exchange of cAMP between the cell and the pipette. Cyclic AMP concentrations are calculated from the measured currents using the dose–response relation of the channel determined in excised membrane patches. The authors apply several additional tests to show that this calibration is valid in the whole-cell setting, and carefully determine the uncertainties in the measurement. In separate experiments, the authors demonstrate that changes in cAMP concentrations can be detected by measuring Ca^{2+} influx through the channel, which may allow for the study of cAMP signaling in electrically inaccessible compartments such as dendritic spines.

It is interesting to contrast this new approach with previous methods for measuring cAMP. For more than 30 years after the discovery of cAMP, the only way to measure it was in large populations of cells. R.Y. Tsien and colleagues developed fluorescent methods that report average cytosolic cAMP levels in single cells (Adams et al., 1991; Zaccolo et al., 2000). The method presented here by Rich et al. (2000) complements these global measures by allowing localized cAMP signals to be monitored in real-time.

Evidence for cAMP Signaling Compartments

Based on the readout of their transfected channel sensors, Rich et al. (2000) build a case that cAMP is produced in microdomains that have restricted diffusional access to the bulk cytosol. Three lines of evidence are presented for these domains. First, in response to forskolin stimulation of adenylyl cyclase, the cAMP concentrations detected by the channels are much higher than those found throughout the cytosol. Second, cAMP builds up to high concentrations near the channels even when the cytosol is being rapidly dialyzed in the whole-cell patch-clamp configuration. Third, the time for cAMP to diffuse from the whole-cell patch pipette to the channels appears considerably slower than the exchange of material between the whole-cell pipette and the bulk cytosol. From these lines of evidence, Rich et al. (2000) conclude that AC activation causes cAMP to build up in discrete cellular compartments before its rising in the cytosol. Rich et al. (2000) also show that their findings can be captured in a simple three-compartment model with permeability barriers (diffusional restrictions of finite thickness) between the microdomain, bulk cytosol, and whole-cell patch pipette. Thus, the authors concluded that "diffusional microdomains," cellular compartments between which cAMP cannot diffuse freely, underlie the apparent discrepancies between bulk cytoplasmic measurements of cAMP and the reports of their transfected sensors.

There is a large literature in recent years showing that many important signaling molecules colocalize within the membrane (e.g., in lipid rafts or organized by scaffolding proteins; reviewed in Brown and London, 1998; Fanning and Anderson, 1999). Thus, the movements of these molecules are restricted within that two-dimensional space. This, of course, facilitates encounters in the plane of the membrane, which are known to be of great importance in many bi-macromolecular interactions, such as occur in abundance phototransduction. The novelty of the claim of Rich et al. (2000) is that cAMP movements are argued to be restricted in three dimensions by an intracellular barrier, whose physical nature remains to be determined. The authors speculate that the barrier may be formed by ER (which has been shown in a number of studies to come in close proximity to the surface membrane) and caveolae (cholesterol-rich invaginations of the membrane).

Caveats

Despite the importance of the work by Rich et al. (2000), some words of caution are in order. Until the physical nature of the structures hypothesized to underlie the apparently hindered diffusion of cAMP are unequivocally identified with ultrastructural methods, alternative explanations remain viable. One alternative explanation of some of the findings is that PDE activity within cells may create cAMP gradients due to "diffusion with hydrolysis" (Jurevicius and Fischmeister, 1996), a phenomenon known to play a role in rod outer segments where cGMP serves as a diffusible intracellular messenger, acting over multi-micrometer distances (Olson and Pugh, 1993; Koutalos et al., 1995); Rich et al. (2000) have done experiments with PDE inhibitors that argue against this alternative, but parallel biochemical measurements of the kinetic parameters (and other details) of the specific PDEs involved need to be investigated. Another potential problem concerns experiments in which pCPT-cGMP and Na^+ are used to estimate the time for equilibration of the bulk cytosol with the whole-cell pipette (20–60 s), providing evidence that the latter time is 4–10× faster than the equilibration time (200 s) of cAMP with the channel sensors—and thus crucial evidence for the microdomains. The difficulty is that diffusion is a linear process, so that (absent a nonlinear degradation process) the time course for equilibration of a given substance is independent of the pipette concentration of the agent introduced, whereas the channel sensors have a variable and nonlinear sensitivity to each agent tested. Quantitative arguments were thus required to interpret the kinetic reports of the transfected channels from which equilibration times were extracted. And while

the 4–10-fold ratio seems robust against reasonable changes in parameter values, this kinetic issue should be definitively resolved in future experiments in which the diffusion of fluorescent cyclic nucleotide analogs is measured simultaneously with the induced currents.

Physiological Significance of cAMP Microdomains

What could be the physiological significance of cAMP microdomains? Again, a comparison with calcium signaling is informative. Even without diffusional barriers, a protein near a Ca^{2+} channel experiences a high concentration of Ca^{2+} due to the relatively enormous influx ($\sim 5 \times 10^5$ s^{-1}) at the channel mouth (Naraghi and Neher, 1997). In contrast, diffusional calculations by Rich et al. (2000) based on the turnover number of AC (60 s^{-1}) show that the cAMP concentrations that can be reached within molecular dimensions of adenylyl cyclase are insufficient to cause cAMP to rise to the level needed to activate PKA, or certainly CNG channels, which have a much lower apparent affinity. In essence, each cAMP molecule diffuses away faster than the next molecule is synthesized. Thus, unlike the situation with calcium channels, two-dimensional proximity to AC is not enough to achieve cAMP concentrations sufficient for activation of most cAMP-effector proteins.

Cyclic AMP microdomains would provide energetically efficient activation of PKA and other effector proteins, by removing the need for cAMP to accumulate throughout the cytosol. The concept of diffusionally restricted microdomains also provides a framework useful for understanding the differential regulation of cellular targets by cAMP. For example, two different hormones might cause cAMP to increase in distinct microdomains of the cell. As such, AKAPs may serve to target PKAs to specific subcellular compartments so that they can respond to appropriate cAMP signals. While this remains to be demonstrated, the current experiments prompt such investigations. Microdomains would also allow for rapid cross-talk between cAMP and Ca^{2+}, as appears to occur in dendritic spines (Finch and Augustine, 1998; Takechi et al., 1998). The signaling pathways for these two messengers are intimately coupled, and the coexistence of the signaling components in microdomains may facilitate their interactions. This makes it seem likely that cAMP will fluctuate dynamically, increasing the information content of cAMP signals (Cooper et al., 1995). In the future, the method described by Rich et al. (2000) should have the time resolution to decipher such covariant signals, and lead to a deeper understanding of the complexity of cellular regulation by cAMP.

Submitted: 16 June 2000
Revised: 21 June 2000
Accepted: 26 June 2000

REFERENCES

Adams, S.R., A.T. Harootunian, Y.J. Buechler, S.S. Taylor, and R.Y. Tsien. 1991. Fluorescence ratio imaging of cyclic AMP in single cells. *Nature*. 349:694–697.

Brown, D.A., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol*. 14:111–136.

Butcher, R.W., and E.W. Sutherland. 1962. Adenosine 3′, 5′, -phosphate in biological materials. *J. Biol. Chem*. 237:1244–1250.

Cooper, D.M.F., N. Mons, and J.W. Karpen. 1995. Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature*. 374:421–424.

Fesenko, E.E., S.S. Kolesnikov, and A.L. Lyubarsky. 1985. Induction by cyclic GMP of cationic conductance in plasma membrane of rod outer segment. *Nature*. 313:310–313.

Fanning, A.S., and J.M. Anderson. 1999. Protein modules as organizers of membrane structure. *Curr. Opin. Cell Biol*. 11:432–439.

Finch, E.A., and G.J. Augustine. 1998. Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. *Nature*. 396:753–756.

Francis, S., and J.D. Corbin. 1999. Cyclic nucleotide-dependent protein kinases: intracellular receptors for cAMP and cGMP action. *Crit. Rev. Clin. Lab. Sci*. 36:275–328.

Jurevicius, J., and R. Fischmeister. 1996. cAMP compartmentation is responsible for local activation of cardiac Ca^{2+} channels by β-adrenergic agonists. *Proc. Natl. Acad. Sci. USA*. 93:295–299.

Kaupp, U.B. 1995. Family of cyclic nucleotide gated ion channels. *Curr. Opin. Neurobiol*. 5:434–442.

Koutalos, Y., K. Nakatani, and K.W. Yau. 1995. Cyclic GMP diffusion coefficient in rod photoreceptor outer segments. *Biophys. J*. 68: 373–382.

Kawasaki, H., G.M. Springett, N. Mochizuki, S. Toki, M. Nakaya, M. Matsuda, D.E. Housman, and A.M. Graybiel. 1998. A family of cAMP-binding proteins that directly activate Rap1. *Science*. 282: 2275–2279.

Naraghi, M., and E. Neher. 1997. Linearized buffered Ca^{2+} diffusion in microdomains and its implications for calculation of [Ca^{2+}] at the mouth of a calcium channel. *Biophys. J*. 17:6961–6973.

Olson, A., and E.N. Pugh. 1993. Diffusion coefficient of cyclic GMP in salamander rod outer segments estimated with two fluorescent probes. *Biophys. J*. 65:1335–1352.

Rich, T.C., K.A. Fagan, H. Nakata, J. Schaack, D.M.F. Cooper, and J.W. Karpen. 2000. Cyclic nucleotide-gated channels colocalize with adenylyl cyclase in regions of restricted cAMP diffusion. *J. Gen. Physiol*. 116:147–161.

Scott, J.D., M.L. Dell'Acqua, I.D. Fraser, S.J. Tavalin, and L.B. Lester. 2000. Coordination of cAMP signaling events through PKA anchoring. *Adv. Pharmacol*. 47:175–207.

Sutherland, E.W. 1962. The biological role of adenosine-3′,5′-phosphate. *Harvey Lect*. 57:17–33.

Takechi, H., J. Eilers, and A. Konnerth. 1998. A new class of synaptic response involving calcium release in dendritic spines. *Nature*. 396:757–760.

Tsien, R.Y. 1989. Fluorescent indicators of ion concentrations. *Methods Cell Biol*. 30:127–156.

Tsien, R.Y. 1998. The green fluorescent protein. *Annu. Rev. Biochem*. 67:509–544.

Walsh, D.A., J.P. Perkins, and E.G. Krebs. 1968. An adenosine 3′, 5′, -monophosphate-dependent protein kinase from rabbit skeletal muscle. *J. Biol. Chem*. 243:3763–3765.

Zaccolo, M., F. De Giorgi, C.Y. Cho, L. Feng, T. Knapp, P.A. Negulescu, S.S. Taylor, R.Y. Tsien, and T. Pozzan. 2000. A genetically encoded indicator for cyclic AMP in living cells. *Nat. Cell Biol*. 2:25–29.