sured for skeletal muscle, which gave the lowest signal. In addition, differences in the relative amounts of individual SR proteins correlated with tissue type. For example, SRp30a/b was most abundant in thymus extracts, whereas this band was nearly undetectable in extracts of either skeletal or cardiac muscle. In brain as well as in both muscle types, SRp75 accounted for about 50% of the total mAb104 signal per lane, as compared with 20% of the total in the thymus. SRp55 is only faintly detectable in brain extract in which SRp40 and SRp30a/b are also only minor components. In view of the heterogeneity of cell types in each tissue, it is possible that variations in the relative abundance of distinct SR proteins may be much greater in single cells. These results show that the differentiated state of cells is associated with the expression of a particular set of SR proteins.

Other results have shown that many different SR proteins isolated from calf thymus, HeLa cells, and cultured *Drosophila* cells have the same effect on the splicing of introns from pre-mRNAs in vitro (*1, 4, 5, 8*). The high degree of size and sequence conservation of the different SR proteins among species, however, suggests that individual SR proteins have distinct and essential functions in vivo. The findings described here show that particular members of the SR family have distinct functions in vitro. The observation that particular SR proteins are associated with the preferential use of different 5′ splice sites suggests that SR proteins may function in alternative splicing in vivo. The absolute and relative amounts of SR protein family members expressed by a given cell may determine the preferred splice site usage and therefore contribute to the regulation of gene expression.

REFERENCES AND NOTES

1. A. M. Zahler, W. S. Lane, J. A. Stolk, M. B. Roth, *Genes Dev.* **6**, 837 (1992).
2. R. J. Bandziulis, M. S. Swanson, G. Dreyfuss, *ibid.* **3**, 431 (1989).
3. M. B. Roth, A. M. Zahler, J. A. Stolk, *J. Cell Biol.* **115**, 587 (1991).
4. A. R. Krainer, A. Mayeda, D. Kozak, G. Binns, *Cell* **66**, 383 (1991).
5. H. Ge, P. Zuo, J. L. Manley, *ibid.*, p. 373.
6. M. Vellard, A. Sureau, J. Soret, C. Martinerie, B. Perbal, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2511 (1992); X.-D. Fu and T. Maniatis, *Science* **256**, 535 (1992).
7. Result determined from the sequence of clone cm5d3 from the cDNA sequencing and mapping project of the nematode genome project of the Sequencing Consortium, Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom.
8. A. Mayeda, A. M. Zahler, A. R. Krainer, M. B. Roth, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1301 (1992).
9. A. R. Krainer and T. Maniatis, *Cell* **42**, 725 (1985).
10. J. D. Dignam, R. M. Lebovitz, R. G. Roeder, *Nucleic Acids Res.* **11**, 1475 (1983).
11. A. M. Zahler, K. M. Neugebauer, J. A. Stolk, M. B. Roth, in preparation.
12. B. Ruskin, A. R. Krainer, T. Maniatis, M. R. Green, *Cell* **38**, 317 (1984).
13. R. F. Johnston, S. C. Pickett, D. L. Parker, *Electrophoresis* **11**, 355 (1990).
14. J. E. Harper and J. L. Manley, *Mol. Cell. Biol.* **11**, 5945 (1991).
15. H. Ge and J. L. Manley, *Cell* **62**, 25 (1990).
16. SR proteins were purified by the use of denaturing SDS–polyacrylamide gel electrophoresis (PAGE). To test the effect of the denaturation and renaturation of SR proteins on splice site specificity, we compared alternative splicing activity of calf thymus SR protein Mg^{2+} precipitates (essentially a pure SR protein mixture containing all the family members) that were either resuspended in buffer D (*10*) or denatured in 6 M guanidine, renatured by dialysis in buffer D, precipitated with Mg^{2+}, and then resuspended in buffer D. When normalized for activity on β-globin pre-mRNA, both SR protein mixtures showed the same specific activity (essentially 100% recovery of activity after renaturation from guanidine) and the same kinetics of splice site selection on the SV40 t antigen pre-mRNA (A. M. Zahler, K. M. Neugebauer, W. S. Lane, M. B. Roth, unpublished data). From this we conclude that individual SR protein splice site selection activity is not affected by denaturation and renaturation of the SR proteins.
17. C. W. J. Smith, J. G. Patton, B. Nadal-Ginard, *Annu. Rev. Genet.* **23**, 527 (1989).
18. M. B. Roth, C. Murphy, J. G. Gall, *J. Cell Biol.* **111**, 2217 (1990).
19. C. T. Hardy, T. A. Darow, G. E. Kenny, *J. Clin. Microbiol.* **28**, 2534 (1990).
20. D. A. Hager and R. R. Burgess, *Anal. Biochem.* **109**, 76 (1980).
21. W. S. Lane, A. Galat, M. W. Harding, S. L. Schreiber, *J. Protein Chem.* **10**, 151 (1991).
22. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

23. We thank J. Manley for providing splicing construct pSVi66; J. Wallace for sequence analysis; R. Robinson for protein digestion and separation; M. Gordy for peptide sequencing; L. Bohor-Roth, J. Priess, M. Groudine, and S. Hahn for careful reading of the manuscript; and R. Tuma for comments. Supported by grant 42786-02 (M.B.R.) from the National Institute for General Medical Sciences. A.M.Z. is a Burroughs-Wellcome Fund Fellow of the Life Sciences Research Foundation. K.M.N. is a Postdoctoral Fellow of the American Cancer Society.

30 June 1992; accepted 28 January 1993

Spatially Resolved Dynamics of cAMP and Protein Kinase A Subunits in *Aplysia* Sensory Neurons

Brian J. Bacskai, Binyamin Hochner, Martyn Mahaut-Smith, Stephen R. Adams, Bong-Kiun Kaang, Eric R. Kandel, Roger Y. Tsien*

Cyclic adenosine monophosphate (cAMP)–dependent protein kinase, labeled with fluorescein and rhodamine on the catalytic and regulatory subunits, respectively, was injected into *Aplysia* sensory neurons either in culture or in intact cell clusters. Energy transfer between the subunits, a measure of cytosolic cAMP concentration ([cAMP]), and compartmentation of the dissociated subunits were monitored by confocal fluorescence microscopy. Bath application of serotonin produced a much greater elevation of [cAMP] in the processes than in the central bodies of the neurons. The resulting gradients must drive a sizable centripetal flux of cAMP because direct microinjection of cAMP showed that it diffused readily. Perinuclear increases in [cAMP] slowly caused the translocation of the freed catalytic subunit into the nucleus to an extent proportional to the percentage of its dissociation from the regulatory subunit.

Serotonin leads to an increase in [cAMP] and can elicit both short- and long-term synaptic changes in the monosynaptic connection between sensory and motor neurons of the gill-withdrawal reflex in the marine snail *Aplysia* (*1, 2*). The distinction between short- and long-term processes in sensory neurons seems to derive in part from the ability of the modulatory transmitter to select between a program involving only a cytoplasmic mechanism of regulation by cAMP or the induction of additional mechanisms that alter cell state through the activation of transcriptional regulatory proteins in the nucleus, such as CREB (*3*). To determine where transmitter generates cAMP, what concentrations are attained, and how the cAMP signal reaches the nucleus, we injected and imaged cAMP-dependent protein kinase labeled with both fluorescein and rhodamine (FlCRhR) (*4*). This labeled enzyme allows both the dynamic measurement of free cAMP and the tracking of the regulatory (R) and the catalytic (C) subunits in single cells.

The FlCRhR was introduced by pressure microinjection into the cell bodies of sensory neurons that were either isolated in culture (*5*) or in excised pleural ganglia. Fluorescence imaging was performed with a laser-scanning confocal microscope specifically designed for high-speed emission ra-

B. J. Bacskai, M. Mahaut-Smith, S. R. Adams, R. Y. Tsien, Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA 92093–0647.
B. Hochner, B.-K. Kaang, E. R. Kandel, College of Physicians and Surgeons of Columbia University, Center for Neurobiology and Behavior, New York, NY 10032.

*To whom correspondence should be addressed.

tioing (6), which permitted the simultaneous acquisition and ratioing of pairs of accurately registered images. We acquired the images at fluorescein and rhodamine emission wavelengths while exciting fluorescein with an Ar laser at 488 nm. The optical sectioning capability of the confocal microscope was used to image either the cell body and processes at a plane just above the glass substratum or the nucleus and perinuclear cytoplasm several tens of micrometers higher.

The concentration of free cAMP in unstimulated neurons was <50 nM (*7*) because coinjection of FlCRhR with R_p–cyclic adenosine monophosphorothioate (R_p-cAMPS), a nonhydrolyzable cAMP antagonist (*8*), did not reduce the apparent resting levels of cAMP. The R_p-cAMPS did inhibit increases in the kinase activity induced by serotonin, isobutylmethylxanthine (IBMX), and forskolin. The low concentrations of free cAMP in unstimulated sensory neurons contrasts with radioimmunoassays that indicate several micromolar or more total cAMP in sensory neuron clusters, which could be elevated two- to threefold after stimulation (*9*, *10*). A large difference in concentration between free and total cAMP is typical for second messengers and may reflect static binding or sequestration. Bath application of serotonin produced rapid increases and large spatial gradients in the concentration of free cAMP (Fig. 1). The [cAMP] increased monotonically as a function of distance along the processes away from the cell body. In the finer parts of the processes, elevations in [cAMP] were detectable at serotonin doses of 0.5 to 1 μM, although these responses required several minutes to reach their peaks. Higher transmitter concentrations produced faster and larger increases in [cAMP], which reached maximal values at doses of 50 to 100 μM serotonin and showed some depression despite the continuous presence of transmitter. These latter doses produced emission ratio changes that correspond to over 100-fold increases in the concentration of free cAMP and complete dissociation of the kinase in the more distal portions of the processes but only slight activation in the main body of the neuron. Spatial gradients in [cAMP] produced by serotonin were observed in 13 of 15 cells.

The gradients could be caused by (i) localization of serotonin receptors or adenylyl cyclase to the distal processes, (ii) restricted diffusion of cAMP, (iii) differential effectiveness of phosphodiesterases, (iv) perturbation of the injected kinase in one part of the cell, (v) differences in surface-to-volume ratio, or various combinations of these factors. Localization of serotonin receptors to the distal processes in *Aplysia* sensory neurons might be expected because synaptic connections in vivo occur at the presynaptic terminals, but no direct confirmation is available. However, receptor localization cannot be the only explanation because forskolin, a direct activator of the cyclase, produced roughly similar gradients in seven of seven cells (data not shown).

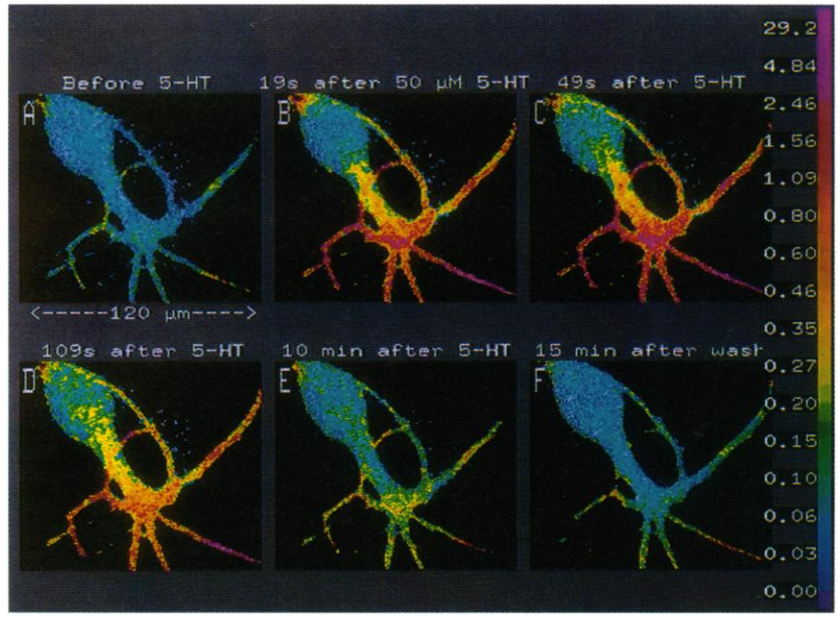


Fig. 1. Gradient in [cAMP] in *Aplysia* sensory neurons after uniform application of serotonin (5-HT). A single, cultured *Aplysia* sensory neuron was microinjected with FlCRhR (*23*). Digital fluorescence images are the result of simultaneous acquisition of two confocal single-wavelength emission images (500 to 530 nm and >560 nm) at a plane just above the glass substratum. After subtraction of background, a ratio was calculated (short over long wavelength), and the image was pseudocolored from blues to reds, which correspond to low to high ratios and low to high concentrations of free cAMP (scale on right in μM cAMP). After uniform bath application of 50 μM 5-HT (**B**), a striking gradient of [cAMP] develops between the cell body and the distal processes. The increase in [cAMP] persisted with some habituation while 5-HT remained in the bath (**C** through **E**), but after 5-HT was washed away (**F**), [cAMP] returned to close to control levels (**A**).

We tested the diffusibility of cAMP by directly microinjecting it into the cell body. Within 1 to 2 s after injection, the ratio change was obvious within a radius of ~50 μm of the site of injection, and, by 7 to 8 s, even the most distal processes (up to 100 μm away) showed saturation of the kinase (Fig. 2). The spatial spread of cAMP as a function of time conformed quantitatively to simple diffusion with an apparent diffusion constant, D, of $(0.78 \pm 0.18) \times 10^{-5}$ $cm^2\ s^{-1}$, which is similar to estimates for aqueous solutions (*11*). These experiments suggest that there is no obvious diffusion barrier in these cells and that injected kinase can be dissociated throughout the cell. Also arguing against unidirectional restriction of diffusion is the finding that cAMP injected into an axon diffuses both toward and away from the cell body.

After the injected cAMP had diffused throughout the cell, its concentration decayed in a spatially uniform manner over the next few minutes (Fig. 2, G and H), which suggests that local variations in sensitivity of FlCRhR to cAMP and activity of phosphodiesterases are insufficient to generate apparent gradients. Also, 0.5 mM IBMX, a broad-spectrum phosphodiesterase inhibitor, had little effect on the spatial gradients produced by bath serotonin and the diffusibility of injected cAMP, although the peak concentrations of cAMP achieved throughout the cell were increased and the rate of decline of [cAMP] was decreased (data not shown). These experiments confirmed that the [cAMP] gradients did not depend on the activity of IBMX-sensitive phosphodiesterases.

Nor were the gradients artifacts of isolated cells in culture. They were also observed in sensory neurons in freshly excised cell clusters bathed in serotonin, although the images were less sharp because of the greater thickness of the tissue and the more complicated geometry of the neurons in situ.

The simplest explanation for the gradients is the greater surface-to-volume ratio of the fine processes compared with the cell body. The cAMP is generated by adenylyl cyclase at the plasma membrane, whereas it is measured by FlCRhR and destroyed by phosphodiesterases in the interior volume of the cell. Some contribution from receptor and cyclase localization cannot be ex-

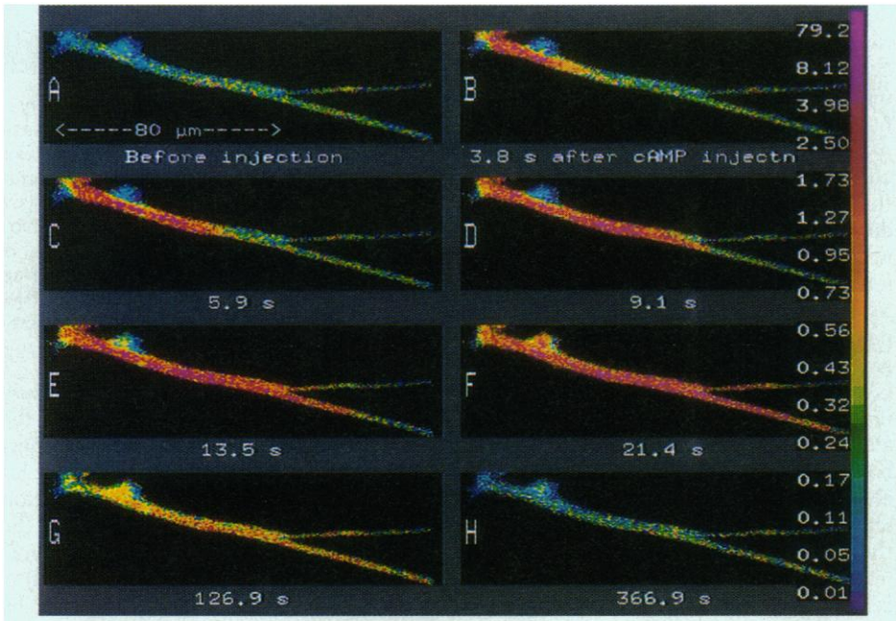
cluded. Whatever the cause, the functional compartmentation of [cAMP] at the distal processes puts the second messenger where it is most needed for short-term plastic changes.

To test whether the injection of FlCRhR at concentrations that probably equal or exceed the concentration of endogenous kinase (*9*, *12*) disrupted the normal cell physiology of these neurons, we used the injection pipette as an intracellular recording electrode to monitor the excitability of the cells during kinase injections and during the addition of serotonin. Injection of FlCRhR neither raised the excitability of the cells on its own nor buffered the normal increases in excitability induced by serotonin (*2*). Injection of free C subunits did lead to increases in excitability, as expected.

Optical sections through the cell body showed the nucleus as a zone that tended to exclude the holoenzyme (injected into the cytoplasm) as long as [cAMP] was not increased. As in previous work on mammalian cells (*3*, *4*), prolonged elevation of [cAMP] and dissociation of the holoenzyme caused the gradual translocation of the C subunit into the nucleus. Strong pharmacological stimulation led to an immediate increase in perinuclear [cAMP], followed over the next 2 hours by entry of the C subunit into the nucleus (Fig. 3, A through F). These images show the maximum extent of translocation observed; the brightness of the C subunit in the nucleus never exceeded that of the cytoplasm, even in experiments in which fluorescein-labeled C subunits were injected instead of R_2C_2 holoenzyme. Translocation of the C subunit in the same cell was reversible if cAMP was removed (Fig. 3G). Because the holoenzyme could be stimulated to undergo a second round of dissociation (Fig. 3H) and nuclear translocation (Fig. 3I), a large fraction of the protein remained intact and functional for many hours.

The major redistribution of the C subunit was obtained with pharmacological rather than physiological stimulants. A major question was whether serotonin alone, which gave only small increases in [cAMP] in the cytoplasm surrounding the nucleus (Fig. 1), could cause detectable shifts of the C subunit into the nucleus. Such relocation would be of obvious physiological utility for cAMP-dependent phosphorylation of transcription factors, such as CREB, which is known to be localized to the nucleus (*3*).

We quantitatively analyzed such translocation by measuring, at fluorescein wavelengths, the ratio of the average pixel intensity of the nucleus to that of the cytoplasm, which provided a measure of the concentration of the fluorescein-labeled C subunit in each of the two compartments. A prolonged exposure to serotonin alone was sufficient to stimulate a small gradual increase in the nucleus/cytoplasm intensity ratio in four of nine cells (*13*). A single 5-min application of serotonin elicited a transient increase in [cAMP], but translocation was undetectable in each of five cells. A training protocol of five short-term applications of serotonin, which produces long-term facilitation in vitro (*14*) in 50 to 70% of experiments (*15*), gave translocation in 3 of 11 cells (*13*). The addition of forskolin, plus IBMX to produce a much larger increase in perinuclear [cAMP], elicited a correspondingly faster rate and larger extent of translocation, which was easily measured (22 of 23 cells). The serotonin treatments alone are sufficient to cause the expression of *lacZ* reporter genes driven by cAMP response elements in only 50% of the sensory neurons of the pleural ganglion (*16*). Thus, the fraction of cells with measurable translocation elicited by serotonin is less than but still comparable with literature measures of gene expression and long-term facilitation. A reasonable interpretation might be that the amount of translocation necessary to turn on gene transcription is a small fraction of the maximum possible and may coincide with the minimum change detectable by the current optical technique. The correlation between peak kinase-activity ratio in the cytoplasm surrounding the nucleus with the eventual translocation achieved for various modes of stimulation is summarized in Fig. 4. Although there is a



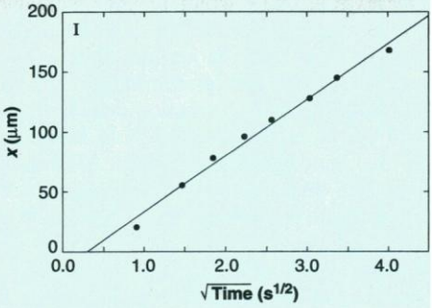


Fig. 2. Diffusion of cAMP within the processes of a cell. After acquisition of (**A**) and before (**B**), cAMP was microinjected into the cell body (upper left, just out of the field of view) with a 10-ms pressure pulse. The images (**C** through **H**) capture the diffusion of cAMP along the process of the neuron up to 170 μm from the injection pipette. Each image is the average of eight frames (0.267 s of acquisition). In the first 0.5 min, 60 images were acquired, 6 of which are shown here (A through F). (**I**) Calculation of the intracellular diffusion coefficient, *D*, of cAMP. This plot was constructed from the experiment shown in (A) through (H). Each point represents the time τ at which [cAMP] was increasing most steeply ($\partial^2[\text{cAMP}]/\partial t^2 = 0$) at the respective distance *x* from the injection pipette. The [cAMP] was averaged in each of eight circular spots, ~5 μm in diameter, for each of the 60 images. The time of steepest increase was chosen instead of the time to reach a predetermined concentration to minimize dependency on the amount of cAMP injected or accurate calibration of FlCRhR. A least squares linear regression of *x* versus $\sqrt{\tau}$ yielded the slope ($r = 0.99$). The diffusion coefficient was calculated with the relation $x = (6D\tau)^{1/2}$ from seven experiments in three different cells, which led to a value of $D_{[\text{cAMP}]} = (0.78 \pm 0.18) \times 10^{-5}$ cm^2 s^{-1} (mean ± SE). We derived the relation between *x*, *D*, and τ from the diffusional profile $[\text{cAMP}] = [\text{cAMP}]_0 \operatorname{erfc}[x(4Dt)^{1/2}]$ by setting its second time derivative to zero. This profile should describe simple one-dimensional diffusion (*24*) down the neuronal process from a source (the cell body) large enough to not be depleted by that diffusion, neglecting phosphodiesterase activity over this short time scale.

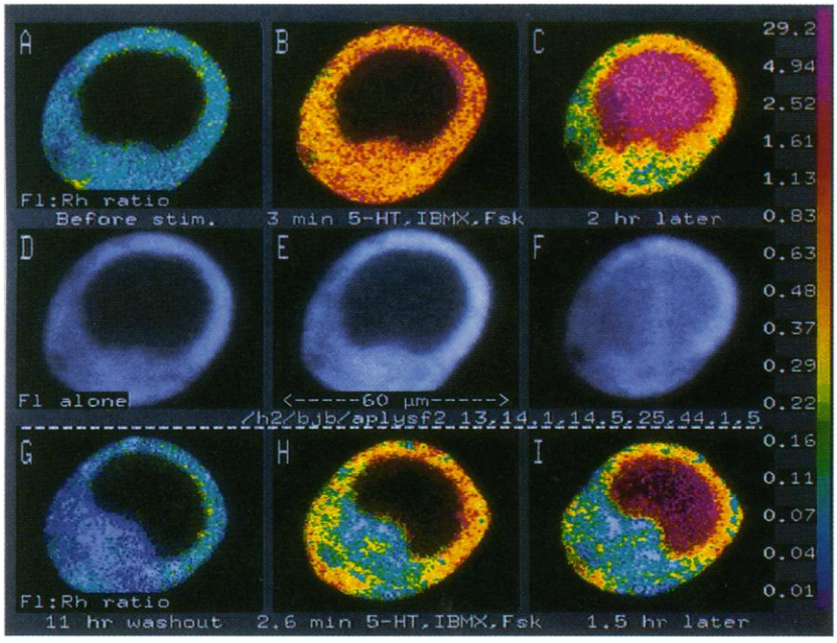


Fig. 3. Translocation of C subunit of cAMP-dependent protein kinase into and out of the nucleus. A single neuron was microinjected with FICRhR and imaged at a plane 20 to 30 μm above the cover slip. (**A**) The nucleus excluded the labeled protein and was therefore not fluorescent; the cytoplasm, however, was very bright and exhibited negligible concentrations of cAMP. (**B**) Soon after application of 25 μM forskolin, 0.5 mM isobutylmethylxanthine (IBMX), and 20 μM 5-HT to raise [cAMP], the fluorescence ratio increased in the cytoplasm, although the nucleus remained dim. (**C**) The nucleus became brightly fluorescent 2 hours later. This apparent high [cAMP] in the nucleus is an artifact generated by entry of C subunits and exclusion of R subunits, causing a fluorescein/rhodamine ratio higher than that caused by dissociated holoenzyme. (**D** through **F**) Single-wavelength images of fluorescein fluorescence (distribution of C subunit) from the corresponding images of (A) through (C). Confocal sections at different planes of focus (not shown) confirmed that the C subunit entered the nucleus and was not just concentrated around its periphery. The R subunits stayed mostly or completely within the cytoplasm, as determined by excitation of rhodamine alone (not shown) or by red pseudocolor of nucleus in C. (**G**) The same cell was washed and allowed to recover overnight. The emission ratio in the cytoplasm fell to low levels, indicating reconstitution of holoenzyme, and the nucleus was again relatively devoid of fluorescence. Blue patches probably represent degradation of protein still labeled with rhodamine. (**H**) After treatment with 25 μM forskolin and 0.5 mM IBMX, the fluorescence ratio increased, showing that, after more than 12 hours in a cell, much of the FICRhR was still responsive to changes in [cAMP]. (**I**) Nuclear translocation could be observed again after an additional 1.5 hours of stimulation.

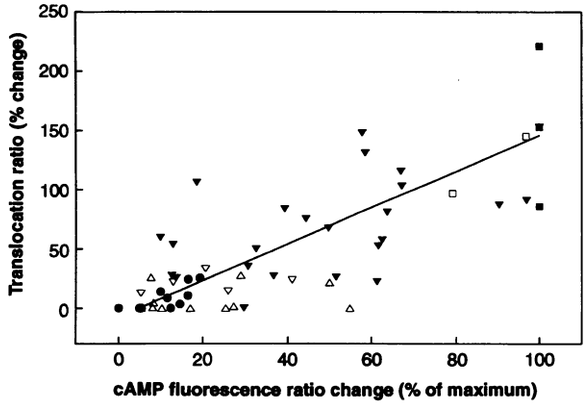


Fig. 4. Correlation between kinase activity and nuclear translocation of C subunit; summary of several experiments. (●) Addition of 5-HT (20 to 100 μM) for 1 to 1.5 hours; (▽) 5-HT (20 to 100 μM) and IBMX (0.5 mM) for 1 to 1.5 hours; (△) five applications of 5-HT (20 μM, 5 min each) and a wash (15 min) in between each addition; (▼) forskolin (25 μM) with IBMX (0.5 mM) and 5-HT (20 to 100 μM) for 30 min to 2 hours; (□) direct pressure microinjection of cAMP (1 mM in pipette) into the cell body repeatedly for 20 to 30 min; (■) direct pressure microinjection of fluorescein-labeled free C subunits of protein kinase into the cell body. The *x*-axis is the maximum increase in FICRhR ratio in the cytoplasm surrounding the nucleus as a percentage of the increase in ratio obtainable with saturating [cAMP]. The percentage change in the translocation ratio at the end of each experiment is plotted along the *y*-axis. Injection of free C subunits was considered equivalent to a maximal change in cAMP ratio. A least squares linear regression describes a line with positive slope significantly different than zero ($P < 0.001$, two-tailed t test) with correlation coefficient $r = 0.77$.

considerable amount of scatter, a fairly strong positive correlation is observed ($r = 0.77$). There is no obvious sign of any nonlinear or threshold relation between [cAMP] and translocation.

The steep spatial gradient in the response of [cAMP] to a uniformly applied agonist is direct evidence for functional compartmentation of this second messenger. Analogous compartmentation of Ca^{2+} has long been directly observable (*17*), whereas cAMP compartmentation has only been suspected (*18*). Also, cAMP has recently been shown to participate in mammalian models of learning and memory (*19*). In the present sensory neurons, the observed gradient puts the cAMP where it is most needed for short-term plasticity, at the tips of the processes where most of the presynaptic terminals are in vivo. The attenuation of the cAMP signal as it approaches the nucleus could serve as a filtering or integrating mechanism to ensure that only strong or repeated stimulations manage to release the C subunit to diffuse into the nucleus, phosphorylate transcription factors, and thereby affect gene expression. Current results on translocation of the C subunit are compatible with a simple model in which the R subunit and the intact holoenzyme are unable to enter the nucleus, whereas the free C subunit can gradually traverse nuclear pores in either direction. These results also demonstrate the spatial and temporal intricacies of cAMP-based signaling and the value of fluorescence imaging of live cells for the determination of the concentrations of free messengers with appropriate probe molecules.

REFERENCES AND NOTES

1. I. Kupfermann, V. Castellucci, H. Pinsker, E. Kandel, *Science* **167**, 1743 (1970); M. Brunelli, V. Castellucci, E. R. Kandel, *ibid.* **194**, 1178 (1976).
2. V. F. Castellucci *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7492 (1980); B. Hochner, M. Klein, S. Schacher, E. R. Kandel, *ibid.* **83**, 8410 (1986); D. A. Baxter and J. H. Byrne, *J. Neurophysiol.* **64**, 978 (1990).
3. E. A. Nigg, H. Hilz, H. M. Eppenberger, F. Dutley, *EMBO J.* **4**, 2801 (1985); J. L. Meinkoth, Y. Ji, S. S. Taylor, J. R. Feramisco, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9595 (1990); M. R. Montminy and L. M. Bilezikjian, *Nature* **328**, 175 (1987).
4. S. R. Adams, A. T. Harootunian, Y. J. Buechler, S. S. Taylor, R. Y. Tsien, *Nature* **349**, 694 (1991).
5. Primary cultures of sensory neurons were made from sensory clusters of pleural ganglia from *Aplysia californica* (50 to 100 g) as described [S. Schacher and E. Proshansky, *J. Neurosci.* **3**, 2403 (1983)]. Cells were used within 1 to 4 days in culture.
6. R. Y. Tsien, *Proc. R. Microscopical Soc.* **25**, S53 (1990).
7. Calibration of the emission ratio in terms of the concentration of free cAMP initially presented a problem because direct titrations of the holoenzyme in cuvettes had to be done at very low enzyme concentrations (nanomolar) so that we could conserve protein and ensure that the concentration of free cAMP was not too different from the known concentration of added cAMP. However, at such unphysiologically low protein

concentrations, some dissociation of R^{II} from C occurred even in the absence of cAMP. To control free cAMP in relatively concentrated (micromolar) solutions of enzyme, we confined the latter inside a microdialysis capillary (150 μm diameter, 9-kD cutoff). The capillary was placed in a solution of intracellular *Aplysia* buffer at high ionic strength (in mM: KOH 250, NaCl 25, $MgCl_2$ 5, glutamate 100, MOPS 300, pH = 7.3) on the stage of the microscope. During its exposure to various [cAMP] outside the capillary, the emission ratios were recorded. The half-maximal ratio change was obtained with 299 ± 53 nM free cAMP (mean ± SE, n = 5). This sensitivity is consistent with estimates in the literature for unlabeled type II kinase [F. Hofmann, J. A. Beavo, P. J. Bechtel, E. G. Krebs, *J. Biol. Chem.* **250**, 7795 (1975)].

8. L. H. P. Botelho, J. D. Rothermel, R. V. Coombs, B. Jastorff, *Methods Enzymol.* **159**, 159 (1988).
9. S. M. Greenberg, L. Bernier, J. H. Schwartz, *J. Neurosci.* **7**, 291 (1987).
10. L. Bernier, V. F. Castellucci, E. R. Kandel, J. H. Schwartz, *ibid.* **2**, 1682 (1982); K. A. Ocorr and J. H. Byrne, *Neurosci. Lett.* **55**, 113 (1985).
11. Values of $D_{[cAMP]}$ of 0.44 × 10^{-5}, 0.97 × 10^{-5}, and 0.33 × 10^{-5} cm^2 s^{-1} have been reported in water [M. Dworkin and K. H. Keller, *J. Biol. Chem.* **252**, 864 (1977)], agar [M. H. Cohen, D. J. Drage, A. Robertson, *Biophys. J.* **15**, 753 (1975)], and molluscan cytoplasm [R. C. Huang and R. Gillette, *J. Gen. Physiol.* **98**, 835 (1991)], respectively.
12. P. J. Bergold *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3788 (1990).
13. The means of pre- and post-stimulus periods (20 to 30 min each) for each cell were compared with the use of an unpaired Student's t test. Translocation was considered significant if $P <$ 0.001 for changes in the ratio of greater than 10%. Experiments with substantial base-line drift were considered uninterpretable and were discarded. We implemented the intensity ratio of fluorescein fluorescence to normalize the potential errors caused by changes in focus, cell shape, illumination intensity, and photobleaching. However, this ratio is not a perfect measure because changes in [cAMP] will artifactually affect the translocation ratio. Although the difference in detectability of C-subunit translocation between single and multiple administrations of serotonin (0 out of 5 and 3 out of 11 cases, respectively) is suggestive, statistical significance is not claimed.
14. N. Dale, S. Schacher, E. R. Kandel, *J. Neurosci.* **7**, 2232 (1987); K. P. Scholz and J. H. Byrne, *Science* **235**, 685 (1987); W. N. Frost, V. F. Castellucci, R. D. Hawkins, E. R. Kandel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 9266 (1985); P. G. Montarolo *et al.*, *Science* **234**, 1249 (1986).
15. B. Hochner and E. R. Kandel, unpublished data.
16. B.-K. Kaang *et al.*, *Neuron*, in press.
17. D. W. Tank, M. Sugimori, J. A. Connor, R. R. Llinás, *Science* **242**, 773 (1988); R. W. Tsien and R. Y. Tsien, *Annu. Rev. Cell Biol.* **6**, 715 (1990).
18. W. L. Terasaki and G. Brooker, *J. Biol. Chem.* **252**, 1041 (1977); J. S. Hayes and L. L. Brunton, *J. Cyclic Nucleotide Res.* **8**, 1 (1982); J. Barsony and S. J. Marx, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1188 (1990).
19. B. H. Morimoto and D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10835 (1991); D. M. Chetkovich, R. Gray, D. Johnston, J. D. Sweatt, *ibid.*, p. 6467; P. Greengard, J. Jen, A. C. Nairn, C. F. Stevens, *Science* **253**, 1135 (1991).
20. S. R. Adams, B. J. Bacskai, S. S. Taylor, R. Y. Tsien, in *Fluorescent Probes for Biological Activity of Living Cells*, W. T. Mason and G. Relf, Eds. (Academic Press, London, 1993), pp. 133–149.
21. R. L. Potter and S. S. Taylor, *J. Biol. Chem.* **254**, 2413 (1979).
22. J. D. Scott *et al.*, *ibid.* **265**, 21561 (1990).
23. The FICRhR was prepared from recombinant C subunits labeled with fluorescein isothiocyanate (*4*) and was combined with type II R subunits labeled with carboxytetramethylrhodamine *N*-hydroxysuccinimidyl ester (*20*). Such R^{II} subunits gave larger fluorescence ratio changes than the previously used R^{I} subunits (*4*). The R^{II} subunits were obtained either by isolation from porcine muscle (*21*) (gift from S. Taylor) or bacterial expression of the murine $R^{II}\alpha$ isoform (*22*) (gift from J. Scott), with similar results.
24. J. Crank, *The Mathematics of Diffusion* (Clarendon, Oxford, 1956).

31 July 1992; accepted 13 January 1993

Increased Frequency of Calcium Waves in *Xenopus laevis* Oocytes That Express a Calcium-ATPase

Patricia Camacho and James D. Lechleiter

When inositol 1,4,5-trisphosphate (IP_3) receptors are activated, calcium is released from intracellular stores in excitatory propagating waves that annihilate each other upon collision. The annihilation phenomenon suggests the presence of an underlying refractory period that controls excitability. Enhanced calcium–adenosine triphosphatase (ATPase) activity might alter the refractory period of calcium release. Expression of messenger RNA encoding the avian calcium-ATPase (SERCA1) in *Xenopus laevis* oocytes increased the frequency of IP_3-induced calcium waves and narrowed the width of individual calcium waves. The effect of SERCA1 expression on calcium wave frequency was dependent on the concentration of IP_3 and was larger at higher (1 μM) than at lower (0.1 μM) concentrations of IP_3. The results demonstrate that calcium pump activity can control IP_3-mediated calcium signaling.

Many hormones and neurotransmitters activate pathways leading to elevations in the intracellular concentration of IP_3 and the subsequent release of Ca^{2+} from intracellular stores (*1*). Cellular signaling information may be encoded in periodic Ca^{2+} oscillations, and much work has focused on defining the underlying basis of these oscillations (*2*). In the spatial domain, Ca^{2+} signaling exhibits a complex variety of patterns. In *Xenopus* oocytes, for example, Ca^{2+} release is initiated at multiple focal sites that generate broken, circular, and spiral waves of Ca^{2+} (*3*, *4*). These observations suggest that the Ca^{2+}-mobilizing machinery acts as an excitable medium (*3*) as it does in other systems such as the classical Belousov-Zhabotinsky reaction, aggregation in the slime mold *Dictyostelium discoideum*, and electrical activity in neuronal and cardiac cells (*5*).

In this theoretical framework of Ca^{2+} signaling, activation of the IP_3 receptor (IP_3R) constitutes the elementary excitatory event (*3*). An important feature of Ca^{2+} release as an excitable medium is the concept of a refractory period, defined as a collection of states in which Ca^{2+} release is inhibited to different degrees, depending on the time elapsed since the preceding wave of excitation (*3*, *6*). Two factors acting singly or in concert may set refractory states of Ca^{2+} release: high cytoplasmic Ca^{2+} concentrations, which inhibit IP_3R activation (*7*), or depletion of Ca^{2+} in the intracellular stores, which inhibits release (*8*). Calcium-ATPases remove Ca^{2+} from the cytoplasm either across the plasma membrane (PMCA family) or into the sarcoplasmic reticulum or endoplasmic reticulum (SERCA family) (*9*). After a Ca^{2+} wave, increased pump activity should reduce cytoplasmic Ca^{2+} concentrations more efficiently and should increase the net uptake of Ca^{2+} into the luminal stores. Increased Ca^{2+}-ATPase expression, however, should not greatly affect the basal concentration of cytosolic Ca^{2+} because in unstimulated cells the Ca^{2+} concentration is too low to activate much pumping activity (*10*).

Oocytes from albino *Xenopus laevis* were injected with SERCA1 mRNA encoding the avian fast twitch Ca^{2+}-ATPase (*11*) and assayed for IP_3-induced Ca^{2+} wave activity 48 to 72 hours later (*12*). To visualize changes in intracellular Ca^{2+}, we injected oocytes with the Ca^{2+} dye indicator Calcium Green I. Individual oocytes were then injected with IP_3 and immediately imaged with confocal microscopy (*13*). A single optical slice near the plasma membrane surface was recorded at 1-s intervals. IP_3 (~1 μM) initiated a wave of Ca^{2+} release across the oocyte (Fig. 1B), which produced an elevated cytoplasmic Ca^{2+} concentration in its wake (*3*, *14*). As the cytoplasmic concentration of Ca^{2+} decreased, pulsatile Ca^{2+} wave activity developed (Fig. 1A). A spatial-temporal stack of 400 consecutive images (Fig. 1C) shows the Ca^{2+} waves generated in response to application of IP_3. In these stacks, time is represented by the z axis (*3*). This oocyte exhibited one of the largest amounts of Ca^{2+} wave activity observed for control oocytes. The average number of Ca^{2+} waves per 400-s temporal stack was 25 ± 14 (mean ± SD, n = 17).

In contrast, the same concentration of

Department of Neuroscience, University of Virginia Health Sciences Center, Charlottesville, VA 22908.