Reprinted from Nature, Vol. 349, No. 6311, pp. [illegible]

Fluorescence ratio imaging of cyclic AMP in single cells

Stephen R. Adams, Alec T. Harootunian, Ying Ji Buechler, Susan S. Taylor & Roger Y. Tsien

Fluorescence ratio imaging of cyclic AMP in single cells

Stephen R. Adams*, Alec T. Harootunian*, Ying Ji Buechler†, Susan S. Taylor† & Roger Y. Tsien*‡

* Howard Hughes Medical Institute M-047 and
† Department of Chemistry M-054, University of California San Diego, La Jolla, California 92093, USA
‡ To whom correspondence should be addressed

FLUORESCENCE imaging is perhaps the most powerful technique currently available for continuously observing the dynamic intracellular biochemistry of single living cells[1]. However, fluorescent indicator dyes have been available only for simple inorganic ions such as Ca^{2+}, H^+, Na^+, K^+, Mg^{2+} and Cl^-. We now report a fluorescent indicator for the adenosine 3',5'-cyclic monophosphate (cAMP) signalling pathway. The sensor consists of cAMP-dependent protein kinase[2] in which the catalytic (C) and regulatory (R) subunits are each labelled with a different fluorescent dye such as fluorescein or rhodamine capable of fluorescence resonance energy transfer in the holoenzyme complex R_2C_2. When cAMP molecules bind to the R subunits, the C subunits dissociate, thereby eliminating energy transfer. The change in shape of the fluorescence emission spectrum allows cAMP concentrations and the activation of the kinase to be nondestructively visualized in single living cells microinjected with the labelled holoenzyme.

The C_α and R^I isoforms of the kinase subunits have been cloned and expressed at high levels in *Escherichia coli*[3,4]. We have now labelled C_α with fluorescein isothiocyanate and R^I with tetramethylrhodamine isothiocyanate (Fig. 1). After many trials, reaction conditions were found in which one or two dye molecules could be covalently linked to each subunit without affecting recombination into holoenzyme. In the holoenzyme, the dyes are close enough so that excitation of the fluorescein donor at 480–495 nm results in detectable emission from the rhodamine acceptor moiety as a result of resonance energy transfer[5–7] (Fig. 2*a*). Cyclic AMP liberates the C subunits, effectively increases the donor–acceptor distance to infinity, and thereby prevents energy transfer. Excitation of the fluorescein now gives brighter emission of that dye at 500–570 nm and less emission at the 570–620 nm wavelengths characteristic of rhodamine. Therefore cAMP changes the ratio of emission amplitudes at two wavelength bands; such ratioing cancels out intensity variations due to probe concentration, optical path length, and excitation intensity, and is highly desirable for microscopic imaging[1,8]. A proposed abbreviation for the labelled holoenzyme is FlCRhR (pronounced 'flicker'). The calibration curve for FlCRhR emission ratio versus free cAMP is essentially superimposable upon the curves describing kinase activation for either labelled or native enzyme (Fig. 2*b*), all being half maximal at about 90 nM cAMP with slightly positive cooperativity. Thus the labelling does not alter the affinity for cAMP or

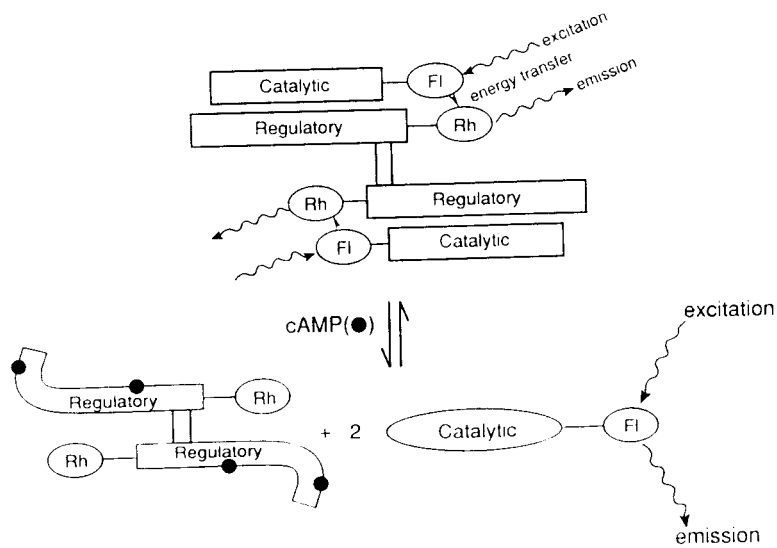

FIG. 1 Schematic diagram showing how to detect cAMP using fluorescence energy transfer[5–7,24] between two fluorophores, for example fluorescein and rhodamine, attached to the catalytic (C) and regulatory (R) subunits, respectively, of cAMP-dependent protein kinase. The protein was labelled as follows: recombinant C_α and $R^{I\alpha}$ subunits[3,4] (approximately 0.5–2 mg ml^{-1}) of mammalian cAMP-dependent protein kinase, were dialysed separately against 25 mM bicine, 0.1 mM EDTA for 4 h at pH 8.0, 0 °C. The catalytic subunit was labelled with 0.3 mM fluorescein 5'-isothiocyanate in the presence of 8 mM $MgCl_2$ and 5 mM ATP to prevent inactivation of kinase activity. The regulatory subunit was labelled with 0.5 mM tetramethylrhodamine isothiocyanate (isomer G). Both dye reagents were from Molecular Probes, Eugene, Oregon; labellings were allowed to proceed for 30 min at room temperature, then quenched by addition of 5 mM glycine for 10–15 min. The excess dye was removed by passing each protein solution through a Sephadex G-25 column (3 ml), eluting with 25 mM potassium phosphate pH 6.8, 2 mM EDTA, 5 mM mercaptoethanol and 10% glycerol. The first coloured band was collected. Covalent attachment of the dyes to the proteins was verified by gel electrophoresis under denaturing conditions. The dye:protein stoichiometries were determined by absorbance spectrophotometry to be 1.1 fluoresceins per catalytic subunit and 1.0 tetramethylrhodamines per regulatory subunit monomer, assuming extinction coefficients (in $10^3\ M^{-1}\ cm^{-1}$) of 65 and 11 for protein-bound fluorescein[25] at 495 and 280 nm, 72 and 18 for protein-bound tetramethylrhodamine[24] at 552 and 280 nm, 45 for catalytic at 280 nm, and 48 for regulatory at 280 nm. The subunits were then mixed at equal concentrations by weight, typically about 0.5 mg ml^{-1}, and dialysed against 25 mM potassium phosphate pH 6.8, 0.5 mM $MgCl_2$, 0.1 mM ATP, 5 mM mercaptoethanol, 5% glycerol for 3–5 days at 4 °C. Formation of holoenzyme was monitored by kinase assay[26]. A wide variety of other visible-wavelength fluorophores and linking groups (for example N-hydroxysuccinimides, iodoacetamides) have been tested on both isolated subunits and preformed holoenzyme, but the above procedure so far gives the best combination of yield, convenience, ability to reform holoenzyme, and optical sensitivity.

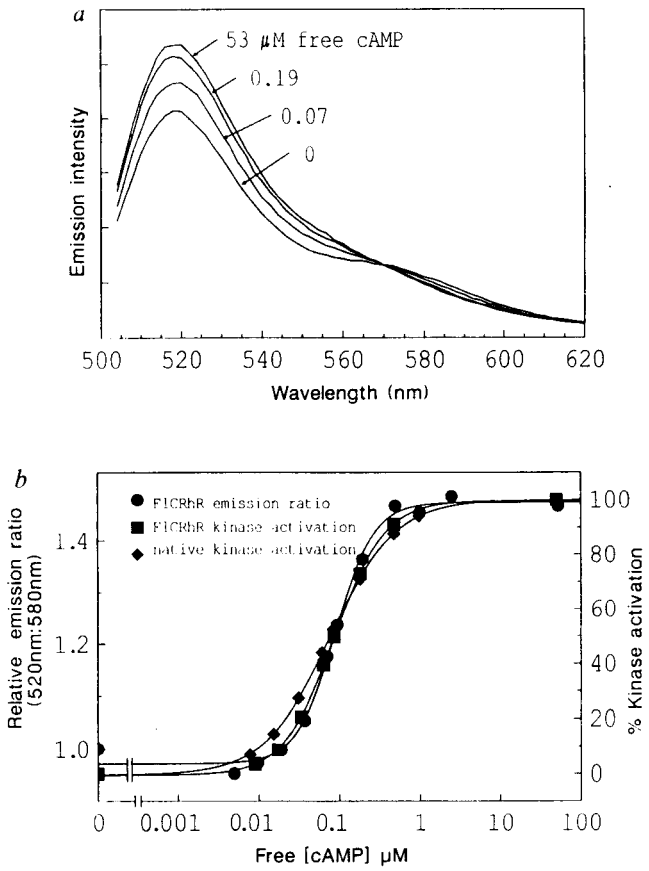

FIG. 2 Responses of FlCRhR to cAMP *in vitro*. *a*, Change in fluorescence emission spectrum of FlCRhR on titration with cAMP when the fluorescein is excited at 495 nm. The solutions contained 3 nM FlCRhR in 130 mM KCl, 5 mM $MgCl_2$, 3 mM ATP, 10 mM 3-(N-morpholino)propanesulphonate, pH 7.2, at 22 °C. Excitation and emission bandwidths were 1.8 and 4.6 nm, respectively. For clarity only two intermediate concentrations of cAMP are shown. Full saturation with cAMP increases the emission from fluorescein at 520 nm by about 31% and decreases the emission from rhodamine by about 13%, resulting in a 1.5-fold increase in ratio of 520 to 580 nm amplitudes in this batch of FlCRhR. The reason why the 520 nm amplitude changes by a larger percentage than the 580 nm is probably because the latter includes not only energy transfer but also spectral overlap, that is 580 nm emission from fluorescein and 490 nm excitation of rhodamine, signals insensitive to cAMP-induced dissociation of the holoenzyme. Control experiments with fluorescein-labelled C paired with unlabelled R showed no significant change in emission spectrum or quantum yield (0.42) on binding cAMP, though the kinase became activated. Therefore the ratio change required labelling of R. Likewise when the rhodamine in FlCRhR is directly excited at 540 nm, its emission spectrum and quantum yield are insensitive to cAMP. For this reason, emission ratioing at 520 nm versus ≥570 nm while exciting at 490 nm is more sensitive to cAMP (1.5-fold range of ratios) than excitation ratioing at 490 versus 540 nm excitation efficiencies while observing at ≥570 nm (1.13-fold range of ratios). The energy transfer efficiency of 0.31 in the holoenzyme corresponds to a distance between the fluorophores of 5.8 nm assuming random orientations for the transition moments. This distance is reasonable for the holoenzyme, as its Stokes radius is 4–5 nm (ref. 27) and the distance between the active sites in the two catalytic subunits exceeds 5 nm (ref. 2). *b*, Ratio of emissions at 520 nm to 580 nm (circles, left-hand scale), normalized to its value at zero cAMP, with the percentage activation of the labelled (squares) and unlabelled (diamonds) kinase (right-hand scale), all as a function of free cAMP. Kinase activity was measured as in ref. 26. Free cAMP was calculated from total cAMP by subtracting a small correction for bound cAMP, assuming that two molecules must bind to each R to release C. Curves were fitted by least-squares to the Hill equation; the concentrations for half-maximal activation were 88, 86 and 76 nM, and the Hill coefficients were 1.8, 1.4 and 1.1 for emission ratio, labelled kinase, and native kinase respectively. The internal differences in these sets of values are probably not significant because they are less than batch-to-batch variation in protein.

the activity of the freed C subunits. Ideally one would like yet higher efficiency of energy transfer in the holoenzyme to increase the overall range of emission ratios. Efforts are continuing in this direction, but FlCRhR is already good enough to permit many cell biological applications.

FlCRhR has been injected into smooth muscle (BC3H1) and fibroblast (REF-52) cell lines at estimated final concentrations approximating or exceeding intrinsic holoenzyme (0.2–2 μM)[9]. Ratioing of fluorescence emissions at 500–530 nm to ≥570 nm and digital image processing reveals the dissociation of FlCRhR, which can be related to the intracellular free cAMP concentration using Fig. 2*b*. Figure 3*a*, *b* shows recordings of the spatially averaged ratio from single BC3H1 cells. Before stimulation, the emission ratio was essentially stable at a level indicating that the FlCRhR was remaining intact without dissociation. Treatment of the cells with extracellular dibutyryl cAMP resulted in a sigmoidal rise in the fluorescence ratio (Fig. 3*a*). Supramaximal doses of β-adrenergic agonists produced faster increases in cAMP leading to 100% activation within 1 min (Fig. 3*b*). Removal of the agonist and blockage of the β-receptors with 100 nM propranolol caused a slow decrease in indicated cAMP concentration, which could then be increased again by direct activation of adenylyl cyclase with forskolin[10]. The reversibility of the emission ratio change was somewhat surprising and gratifying, since we had feared that considerable scrambling of exogenously labelled and native unlabelled subunits might occur. Such scrambling would have prevented reconstitution of energy transfer and detection of falling cAMP levels. In analogous experiments, treatment of FlCRhR-injected REF-52 fibroblasts with 10 μM prostaglandin E_1 (ref. 11) gave detectable rises in cAMP levels in 51 of 60 cells, two of which are shown in Fig. 3*c*. 5′-(*N*-ethylcarboxamido)adenosine, an adenosine analogue, (at 50 μM) gave similar increases in 9 of 10 cells respectively. Again these changes in emission ratio reversed on removal of agonist. The lack of FlCRhR response in some cells (see Fig. 3*c*, broken line) was not due to deterioration of probe molecules, as forskolin worked as expected, but may instead reflect genuine cell–cell heterogeneity analogous to that often seen in Ca^{2+} signalling[12,13]. Heterogeneity is most directly assessed by single-cell assays, and can strongly affect the interpretation of population measurements.

Figure 4 shows pseudocolour images of the emission ratio and subcellular localization of FlCRhR in a REF-52 fibroblast. Elevation of cAMP first dissociated FlCRhR and increased its emission ratio, whereupon the freed C subunit gradually translocated to the nucleus[14]. A new finding was that removal of cAMP stimulation could undo the nuclear localization and reconstitute holoenzyme and fluorescence energy transfer in the cytoplasm. These results were typical of both REF-52 and BC3H1 cells and could be explained by reversible diffusion of C subunits between cytoplasmic R^I and nuclear binding sites, where R^I wins the competition when cAMP is low. Also, the continued responsiveness of FlCRhR argues that major degradation was not occurring over the first 4 h after injection. It will be interesting to repeat such experiments with other isozymes of R and C, which may behave differently and give hints as to why so many isoforms exist[2].

A question that will need answering in each application is whether the injected kinase affects the cell physiology, either by buffering cAMP or by increasing its downstream efficacy. Past experience suggests that injection of holoenzyme has little effect whereas unbalanced regulatory or catalytic subunits respectively mimic inhibition or overstimulation of cAMP signalling[15–17]. This is a probable advantage of basing the sensor on holoenzyme rather than on another cAMP-binding protein such as R subunits alone or the cAMP receptor protein[18] of *E. coli*, which would divert cAMP to nonproductive binding sites. FlCRhR inherently covers the most interesting concentration range of cAMP because it has the same affinity for cAMP as the native target enzyme (Fig. 2*a*). Its use will be most

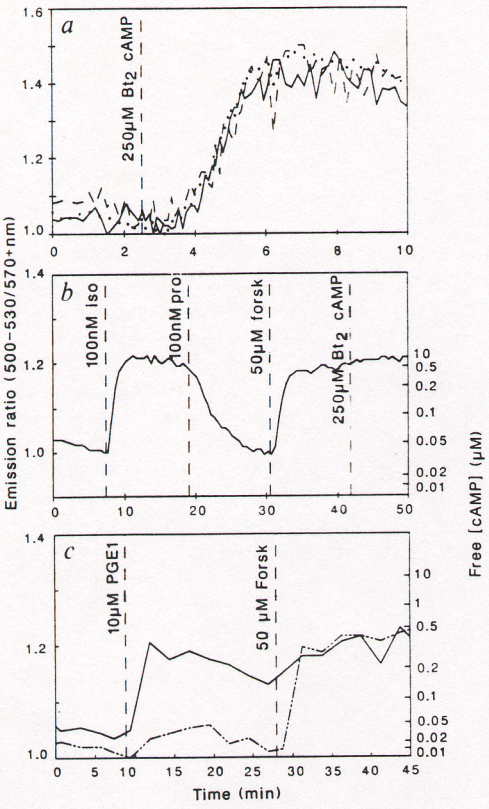

FIG. 3 Time courses of FICRhR emission ratio and cAMP in single cells. *a*, In three BC3H1[28] cells injected with FICRhR, 250 μM dibutyryl cAMP (Bt_2cAMP) causes a delayed increase in the ratios of fluorescence emission at 500–530 nm to ⩾570 nm. The three cells were simultaneously monitored by digital imaging microscopy[29] and are represented by solid, dashed, and dotted lines, respectively. The initial delay of about 2 min may reflect the time required for the cAMP analogue to cross the membrane and undergo hydrolysis of butyryl groups before becoming active. *b*, FICRhR response is reversible. In a single BC3H1 cell, the emission ratio increases rapidly in response to the β-adrenergic agonist isoproterenol[28] (100 nM). Similar increases were detected in every one of 50 cells exposed to 50–100 nM isoproterenol. The emission ratio then decreases when the isoproterenol is replaced by the β-antagonist propranolol (100 nM), and finally increases again when 50 μM forskolin is added to stimulate adenylyl cyclase directly[10]. The FICRhR is then fully saturated with cAMP, as Bt_2cAMP has no further effect. The right-hand ordinate axis represents an attempt to calibrate the ratio changes in terms of free cAMP concentrations. Ratios measured on the microscope are measured with a television camera and filters defining relatively broad bandwidths to maximize detection efficiency, so they differ quantitatively from those measured in a spectrofluorometer cuvet with a photomultiplier and monochromators with narrow bandwidth. This experiment was calibrated by assuming that the mean ratio obtained in parallel runs by coinjecting FICRhR with the cAMP antagonist R_p-adenosine cyclic 3′,5′-phosphorothioate[30] (BioLog Life Science Institute, La Jolla, California; 1 mM pipette concentration) represents zero cAMP, whereas the mean ratio obtained after 250 μM Bt_2cAMP corresponds to saturating cAMP. The Hill coefficient and cAMP concentration for half-maximal activation were taken from *in vitro* measurements on the same batch of FICRhR. *c*, Two adjacent REF-52 fibroblasts[29] (solid and dashed lines) respond differently to 10 μM prostaglandin E_1 (PGE_1). The cell represented by the dashed line subsequently elevates cAMP when challenged with 50 μM forskolin, showing that its adenylyl cyclase and FICRhR were functional. The cAMP calibration was calculated by a different protocol from *b*, in which the lowest ratio observed before stimulation was assumed to represent zero cAMP. The ratio representing saturating cAMP was assumed to be 1.33-fold higher, because when a parallel aliquot of this batch of FICRhR was sandwiched as a thin film between coverslips with 0 or 1 mM added cAMP, the emission ratios differed by a factor of 1.33. The two calibration procedures in *b* and *c* both have advantages and disadvantages and probably over- and underestimate free cAMP, respectively. FICRhR in REF-52 cells showed no response to 5% serum or 2 μM phorbol 12,13-dibutyrate (data not shown), indicating independence from other signalling pathways such as receptor tyrosine kinase, inositol phospholipid turnover, cytosolic Ca^{2+} spikes, and protein kinase C.

METHODS. Injection and imaging. BC3H1 and REF-52 cells in log phase growth were pressure-injected with 7.6–50 μM FICRhR, 25 mM potassium phosphate pH 7.36, 1 mM EDTA, 1 mM mercaptoethanol, and 5% glycerol. Injection volumes were 2–10% of cell volume. Digital images of the fluorescence were obtained on a system previously described[29], except that the excitation wavelength was fixed at 490 nm (4.6 nm bandpass) and two emission bands were sequentially sampled by alternately placing a 500–530 nm bandpass interference filter (Karl Feuer Optical Associates, Montclair NJ) and a 570 nm long-pass filter (EF570LP, Omega Optical, Brattleboro, Vermont) in front of the silicon intensified target camera (Dage-MTI, Michigan City, Indiana). Ratios are averages over the entire cell. Because of residual spatial non-uniformities in the filters, absolute ratios varied somewhat from one part of the field of view to another, so for mutual comparability the ratios for each cell have been normalized against the lowest ratio seen for that cell during the experiment. All data shown were obtained at 22–23 °C.

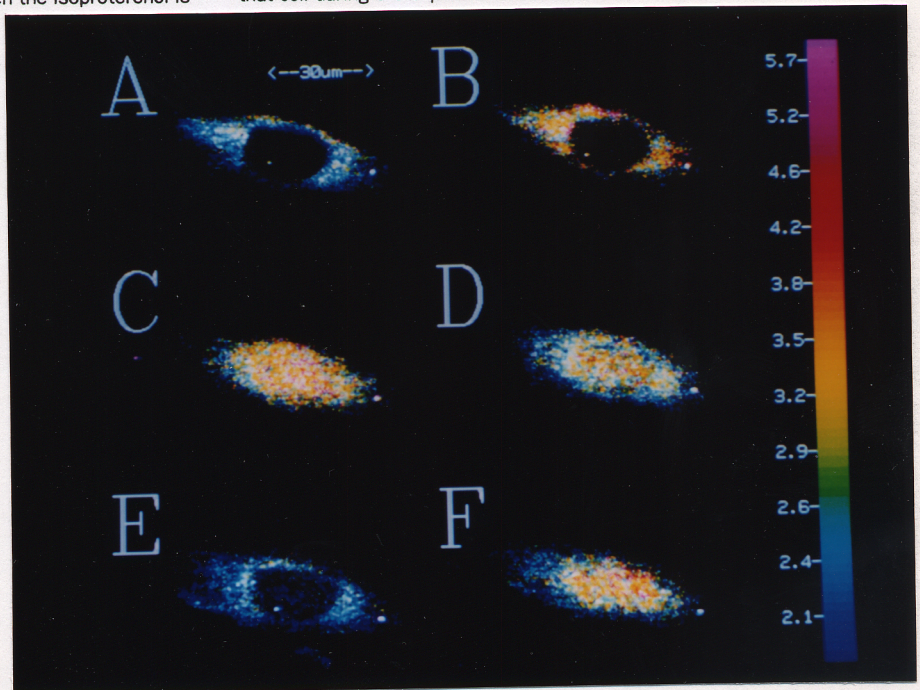

FIG. 4 Pseudocolour images of FICRhR emission ratio and subcellular localization in a single REF-52 fibroblast. Increasing ratios of 500–530 to ⩾570 nm intensities are coded in pseudocolour hues ranging through the spectrum from blue to red, as calibrated in the right hand colour scale. Pseudocolour brightness reflects the mean of the two emission intensities. *a*, Before stimulation, the cytoplasm shows a low ratio (blue), whereas the nucleus appears black because the holoenzyme is excluded. *b*, 5.5 min after addition of 2 μM prostaglandin E_1 (PGE_1), the emission ratio has risen (yellow pseudocolour) showing an increase in cAMP, but the nucleus is still mostly dark because it is only just beginning to take up catalytic subunit. *c*, 40.5 min after addition of PGE_1, the nucleus now contains a considerable amount of catalytic subunit, which by itself gives a higher ratio (redder pseudocolour) than when it is intermingled with rhodamine-labelled regulatory subunit. *d*, 7 min after removal of PGE_1 from the medium, emission ratios and cAMP levels are declining, though exit of catalytic subunit from the nucleus is far from complete. *e*, 66 min after removal of PGE_1, emission ratios (blue) and cAMP levels have recovered to baseline levels, and the nucleus has been cleared of catalytic subunit. *f*, 45 min after addition of 50 μM forskolin to activate adenylyl cyclase, emission ratios and cAMP are high and the nucleus has filled in again. The total elapsed time from injection of FICRhR was 4 h at this point. The absolute range of ratios indicated by the right-hand colour scale differs from those in Fig. 3 because the present values have not been normalized against their lowest value. These results were obtained at 22 °C; translocation of the catalytic subunit is faster at 37 °C.

advantageous when cells are scarce or heterogeneous, when high spatial and temporal resolution are required, or when cAMP or its kinase may be bound or compartmentalized so that traditional destructive measurements of total cAMP or activity ratio are inadequate[19–21]. Fluorescence resonance energy transfer is already known to be biologically useful in detecting colocalization of membrane components[5–7] and hybridization of nucleic acids[22]; one can now imagine further applications in intracellular signalling, such as detecting cyclic GMP using mutated[23] cAMP-dependent kinase, or analysing interactions between subunits of GTP-binding proteins, between calmodulin and its target enzymes, or between components of transcription-regulating complexes. □

Received 9 October; accepted 20 December 1990.

1. Tsien, R. Y. *A. Rev. Neurosci.* **12,** 227–253 (1989).
2. Taylor, S. S., Buechler, J. A. & Yonemoto, W. *A. Rev. Biochem.* **59,** 971–1005 (1990).
3. Slice, L. W. & Taylor, S. S. *J. biol. Chem.* **264,** 20940–20946 (1989).
4. Saraswat, L. D., Filutowicz, M. & Taylor, S. S. *J. biol. Chem.* **261,** 11091–11096 (1986).
5. Uster, P. S. & Pagano, R. E. *J. Cell Biol.* **103,** 1221–1234 (1986).
6. Herman, B. *Meth. Cell Biol.* **30,** 219–243 (1989).
7. Jovin, T. M. & Arndt-Jovin, D. J. *A. Rev. Biophys. biophys. Chem.* **18,** 271–308 (1989).
8. Bright, G. R., Fisher, G. W., Rogowska, J. & Taylor, D. L. *Meth. Cell Biol.* **30,** 157–192 (1989).
9. Beavo, J. W., Bechtel, P. J. & Krebs, E. G. *Proc. natn. Acad. Sci. U.S.A.* **71,** 3580–3583 (1974).
10. Seamon, K. B. & Daly, J. W. *Adv. Cycl. Nucl. Res.* **20,** 1–150 (1986).
11. Yamashita, T., Tsuda, T., Hamamori, Y. & Takai, Y. *J. biol. Chem.* **261,** 16878–16882 (1986).
12. Tsien, R. Y. & Poenie, M. *Trends Biochem. Sci.* **11,** 450–455 (1986).
13. Williams, D. A. & Fay, F. S. (eds) *Cell Calcium* **11,** 55–249 (1990).
14. Meinkoth, J. L., Ji, Y., Taylor, S. S. & Feramisco, J. R. *Proc. natn. Acad. Sci. U.S.A.* **87,** 9595–9599 (1990).
15. Riabowol, K. T. *et al. Nature* **336,** 83–86 (1988).
16. Clegg, C. H., Correll, L. A., Cadd, G. C. & McKnight, G. S. *J. biol. Chem.* **262,** 13111–13119 (1987).
17. Büchler, W. *et al. Eur. J. Biochem.* **188,** 253–259 (1990).
18. Wu, F., Nath, K. & Wu, C.-W. *Biochemistry* **13,** 2567–2572 (1974).
19. Terasaki, W. L. & Brooker, G. *J. biol. Chem.* **252,** 1041–1050 (1977).
20. Hayes, J. S. & Brunton, L. L. *J. Cycl. Nucl. Res.* **8,** 1–16 (1982).
21. Murray, K. J., Reeves, M. L. & England, P. J. *Molec. cell. Biochem.* **89,** 175–179 (1989).
22. Cardullo, R. A., Agrawal, S., Flores, C., Zamecnik, P. C. & Wolf, D. E. *Proc. natn. Acad. Sci. U.S.A.* **85,** 8790–8794 (1988).
23. Shabb, J. B., Ng, L. & Corbin, J. D. *J. biol. Chem.* **265,** 16031–16034 (1990).
24. Johnson, D. A., Voet, J. G. & Taylor, P. *J. biol. Chem.* **259,** 5717–5725 (1984).
25. Haugland, R. P. *Handbook of Fluorescent Probes and Research Chemicals*, 32 (Molecular Probes, Eugene, Oregon, 1989).
26. Cook, P. F., Neville, M. E. jr, Vrana, K. E., Hartl, F. T. & Roskoski, R. jr *Biochemistry* **21,** 5794–5799 (1982).
27. Taylor, S. S. & Stafford, P. H. *J. biol. Chem.* **253,** 2284–2287 (1978).
28. Hughes, R. J., Boyle, M. R., Brown, R. D., Taylor, P. & Insel, P. A. *Molec. Pharmac.* **22,** 258–266 (1982).
29. Tsien, R. Y. & Harootunian, A. T. *Cell Calcium* **11,** 93–109 (1990).
30. Botelho, L. H. P., Rothermel, J. D., Coombs, R. V. & Jastorff, B. *Meth. Enzym.* **159,** 159–172 (1988).

ACKNOWLEDGEMENTS. This work was supported by the NIH and HHMI.