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Stephen R. Adams*, Alec T. Haroutunian*, 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. However, fluorescent indicator dyes have been available only for simple inorganic ions such as Ca**, H**, Na**, K**, Mg** and Cl**. We now report a fluorescent indicator for the adenine 3',5'-cyclic monophosphate (cAMP) signalling pathway. The sensor consists of cAMP-dependent protein kinase 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•C•. When cAMP molecules bind to the R subunit, 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• and R1 isomers of the kinase subunits have been cloned and expressed at high levels in Escherichia coli. We have now labelled C• with fluorescein isothiocyanate and R1 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 (27) (Fig. 2a). 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. A proposed abbreviation for the labelled holoenzyme is FICRhR (pronounced 'licker'). The calibration curve for FICRhR emission ratio versus free cAMP is essentially superimposable upon the curves describing kinase activation for either labelled or native enzyme (Fig. 2b), 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 transfer5,6,7,24 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 and R subunits3,4 (approximately 0.5–2 mg ml**1) of mammalian cAMP-dependent protein kinase, were dialysed separately against 25 mM bicarbonate, 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 MgCl2 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 mercaptopethanol 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 3.0 tetramethylrhodamines per regulatory subunit monomer, assuming extinction coefficients (in 104 M**1 cm**1) of 65 and 11 for protein-bound fluorescein22 at 495 and 280 nm, 72 and 18 for protein-bound tetramethylrhodamine23 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 MgCl2, 0.1 mM ATP.
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 FICHR has already gone far enough to permit many cell biological applications.

FICHR has been injected into smooth muscle (BC3H1) and fibroblasts (REF-52) cells at essentially cAMP concentrations approximating or exceeding intrinsic holoenzyme (0.2–2 μM). Ratios of fluorescence emissions at 500–530 nm to >570 nm and digital image processing reveals the dissociation of FICHR, which can be related to the intracellular free cAMP concentration using Fig. 2b. Figure 3a, 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 FICHR was remaining intact without dissociation. Treatment of the cells with extracellular dibutyryl cAMP resulted in a sigmoidal rise in the fluorescence ratio (Fig. 3a). Supramaximal doses of β-adrenergic agonists produced faster increases in cAMP leading to 100% activation within 1 min (Fig. 3b). Removal of the agonist and blockade 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 adenyl cyclase with forskolin. 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 FICHR-injected REF-52 fibroblasts with 10 μM prostaglandin E1 (ref. 11) gave detectable rises in cAMP levels in 51 of 60 cells, two of which are shown in Fig. 3c. 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 FICHR response in some cells (see Fig. 3c, 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 Ca2+ signalling14,15. 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 FICHR in a REF-52 fibroblast. Elevation of cAMP first dissociated FICHR and increased its emission ratio, whereupon the freed C subunit gradually translocated to the nucleus16. 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 and nuclear binding sites, where R wins the competition when cAMP is low. Also, the continued responsiveness of FICHR argues that major degradation was not occurring over the first 4 h after injection. It will be interesting to repeat such experiments with other isoforms of R and C, which may behave differently and give hints as to why so many isoforms exist.

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 mimicked inhibition or overstimulation of cAMP signalling11,17. 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 protein1 of E. coli, which would divert cAMP to nonproductive binding sites. FICHR inherently covers the most interesting concentration range of cAMP because it has the same affinity for cAMP as the native target enzyme (Fig. 2a). Its use will be most
FIG. 3 Time courses of FICRR emission ratio and cAMP in single cells. a. In three BC3H1 cells injected with FICRR, 250 μM dibutyryl cAMP (DB-cAMP) causes a delayed increase in the ratio of fluorescence emission at 500–530 nm to >570 nm. The three cells were simultaneously monitored by digital imaging microscopy29 and are represented by solid, dash, 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 by butyryl groups before becoming active. b. FICRR response is reversible. In a single BC3H1 cell, the emission ratio increases rapidly in response to the β2-adrenergic agonist isoproterenol26 (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 β2-adrenergic propranolol (100 nM), and finally increases again when 50 μM forskolin is added to stimulate adenyl cyclase directly.30 The FICRR is then fully saturated with cAMP, as B2-cAMP 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 cAMP and a cAMP antagonist, P2-adrenergic cyclic 3'5'-phosphorothioate29 (BioLog Life Science Institute, La Jolla, California; 1 mM pipette concentration) represents zero cAMP, whereas the mean ratio obtained after 250 μM B2-cAMP 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 FICRR. c. Two adjacent REF-52 fibroblasts29 (solid and dashed lines) respond differently to 10 μM prostaglandin E1 (PGE1). The cell represented by the dashed line subsequently elevates cAMP when challenged with 50 μM forskolin, showing that its adenyl cyclase and FICRR were functional. The cAMP calibration was calculated by a different protocol from that 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 FICRR was scanned 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 a and c both have advantages and disadvantages and probably over- and underestimate free cAMP, respectively. FICRR in REF-52 cells showed no response to 5% serum or 2 μM phospholipid 12,13-dibutyrinate (data not shown), indicating independence from other signalling pathways such as receptor tyrosine kinase, intracellular phospholipid turnover, cytosolic Ca2+ 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 FICRR. 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 described29, 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–

FIG. 4 Pseudocolour images of FICRR emission ratio and subcellular localization in a single REF-52 fibroblast. Increasing ratios of 500–530 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 E1 (PGE1), 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 PGE1, the nucleus now contains a considerable amount of catalytic subunit, which by itself would give a higher ratio (redder pseudocolour) than when it is intermingled with rhodamine-labelled regulatory subunit. d. 7 min after removal of PGE1, the emission ratios and cAMP levels are declining, though exit of catalytic subunit from the nucleus is far from complete. e. 65 min after removal of PGE1, 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 elevate adenyl cyclase, emission ratios and cAMP are high and the nucleus has filled in again. The total elapsed time from injection of FICRR 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, translation 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. Fluorescence resonance energy transfer is already known to be biologically useful in detecting colocalization of membrane components and hybridization of nucleic acids; one can now imagine further applications in intracellular signalling, such as detecting cyclic GMP using mutated 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.


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