A genetically encoded, fluorescent indicator for cyclic AMP in living cells

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Cyclic AMP controls several signalling cascades within cells, and changes in the amounts of this second messenger have an essential role in many cellular events. Here we describe a new methodology for monitoring the fluctuations of cAMP in living cells. By tagging the cAMP effector protein kinase A with two suitable green fluorescent protein mutants, we have generated a probe in which the fluorescence resonance energy transfer between the two fluorescent moieties is dependent on the levels of cAMP. This new methodology opens the way to the elucidation of the biochemistry of cAMP *in vivo*.

Signalling through cyclic 3′,5′-adenosine monophosphate (cAMP) remains one of the most important and exciting areas of study in cell biology and physiology[1]. Alterations in intracellular cAMP levels govern fundamental metabolic, electrical, cytoskeletal and transcriptional responses within cells. The main effector of cAMP is protein kinase A (PKA), an holotetramer composed of two regulatory and two catalytic subunits[1–4]. Activation of PKA by cAMP results in the dissociation of the catalytic subunits from the regulatory subunits. Then, the catalytic subunits both phosphorylate cytoplasmic substrates and diffuse into the nucleus, where they trigger some of the long-term effects of cAMP[5]. The common method used to measure total cAMP levels is radioimmunoassay of cell lysates. The only approach by which to image the dynamics of free cAMP in single living cells is based on microinjection of fluorescein-labelled catalytic subunits and rhodamine-labelled regulatory subunits, and relies on fluorescence resonance energy transfer (FRET)[6]. FRET is a physicochemical phenomenon whereby the excited-state energy of a donor fluorophore is directly transferred to an acceptor molecule, which can then emit its own characteristic fluorescence[7]. Dissociation of PKA subunits by cAMP disrupts FRET between the fluorescein donor and rhodamine acceptor and increases the donor's emission at the expense of the acceptor[6]. This approach is technically highly demanding (it requires *in vitro* labelling, purification, and microinjection of the holotetramer, which has a relative molecular mass of 170,000) and is not applicable to all cell types. We now show that appropriate mutants of green fluorescent protein[8] (GFP) fused to the PKA subunits can be used as donor and acceptor chromophores and generate cAMP-sensitive FRET. This system is entirely genetically encoded and offers the potential for much wider applications than were available with previous techniques.

Results

Generation and expression of GFP-tagged PKA. We generated a large number of chimaeras in which GFP mutants were fused to either end of several isoforms of PKA catalytic and regulatory subunits. In some of the constructs a peptide linker was inserted between GFP and the enzyme subunit. Among all the constructs tested, the two fusions that have given the best results so far in intact cells are C-S65T and RII-EBFP, in which an improved mutant[8] of GFP (mutation S65T) and a blue GFP mutant[8] (EBFP; mutations F64L/Y66H/Y145F) were linked to the carboxy termini of the mouse catalytic α-subunit or the rat regulatory RII β-subunit, respectively, through a linker peptide of 11 amino acids that includes the immunodominant epitope of the influenza virus (Fig.

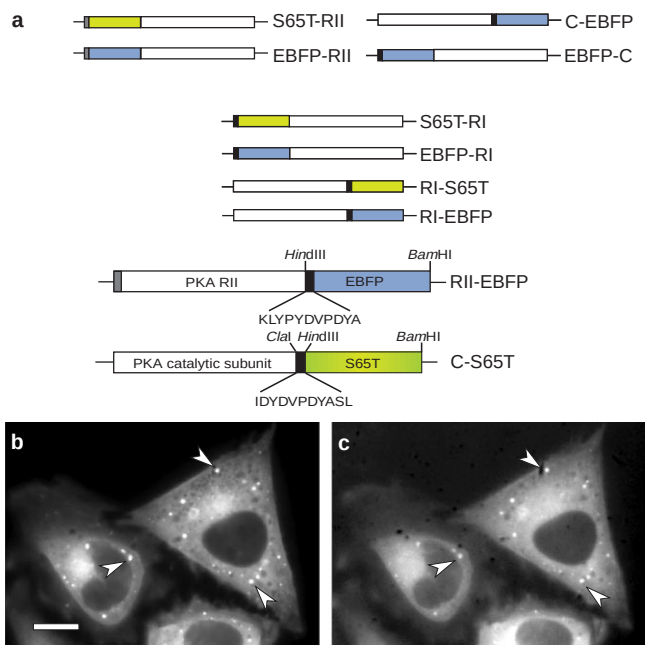

Figure 1 **Construction and expression of GFP-tagged PKA. a**, Representation of some of the GFP-tagged PKA subunits tested. Black box, peptide linker; grey box: six-histidine tag; RI, isoform I of the regulatory subunit. For RII-EBFP and C-S65T the amino-acid sequence of the linker is shown; C, mouse catalytic α-subunit of PKA. **b**, **c**, CHO cells were co-transfected with RII-EBFP and C-S65T. In **b**, fluorescence images were taken using a filter set specific for S65T (475-nm excitation and 545-nm emission). **c**, The same cells imaged with a filter set specific for EBFP (380-nm excitation and 460-nm emission). Arrowheads indicate some of the bright spots where the two chimaeras co-localize. Scale bar represents 10 μm.

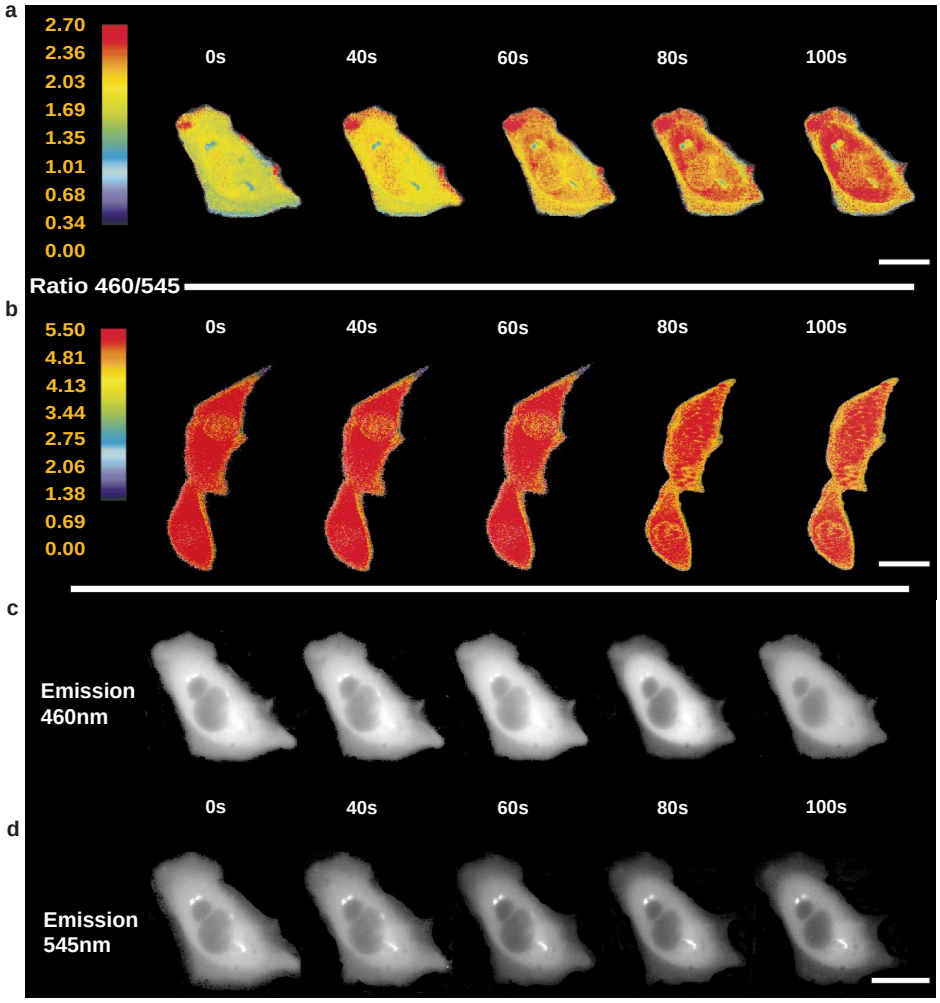

Figure 2 **Fluorescence changes induced by increasing cAMP levels in cells transfected with GFP-tagged PKA. a**, **b**, Pseudocolour emission ratio images (460-nm/545-nm ratio) before (time 0 s) and at different time points after the addition of 25 μM forskolin plus 1 mM dbcAMP. CHO cells were transiently transfected with **a**, C-S65T + RII-EBFP, or **b**, S65T-RII + EBFP-C (control constructs). **c**, **d**, Fluorescence images of cells transfected with C-S65T + RII-EBFP were taken at 380-nm excitation and **c**, 460-nm emission or **d**, 545-nm emission. The fluorescence emission intensities shown in **c**, **d** were used to calculate the ratio values shown in **a**. **a**, The reduced changes in FRET (observed upon increases in cAMP levels) at the periphery of the cell are artefacts resulting from an inadequate signal-to-noise ratio within the thinnest regions. We analysed at least 50 CHO cells transfected with GFP-tagged PKA and with control constructs and obtained a consistent response. Comparable results were obtained with COS-7 and HEK cell lines. A clear change in ratio was still evident after the addition of only 10 μM forskolin. Scale bar represents 10 μm.

1a). We transfected the complementary DNAs into various stable cell lines and into primary cultures of striated muscle (heart and skeletal). Transfection with RII-EBFP alone consistently resulted in cytoplasmic staining with nuclear exclusion. A variable number of small fluorescent aggregates were observed in all cell types tested. Transfection with C-S65T alone resulted in more variable results. In about 50% of transfected cells, either the morphology was grossly altered (by the appearance of long cytoplasmic processes) or/and the nucleus was clearly labelled. In the remaining cells the morphology was unaltered and C-S65T was excluded from the nucleus. These morphological alterations probably result from the overexpression of a catalytically active subunit of PKA without a concomitant increase in the level of endogenous regulatory subunits. In fact, we observed similar alterations in cells overexpressing an untagged catalytic PKA subunit or in cells treated for several hours with agents that increase the cellular level of cAMP. When RII-EBFP and C-S65T were transiently coexpressed, the fusion proteins localized in the cytoplasm and were clearly and consistently excluded from the nucleus (Fig. 1b, c). No obvious alteration in cell morphology was observed. Most cells also showed a few bright spots, which were positive for both the RII-EBFP and the C-S65T fusions (Fig. 1b, c, arrowheads), indicating a co-localization of the two fluorescent subunits.

GFP-tagged PKA generates cAMP-sensitive FRET. A first indication that FRET between the tagged PKA subunits occurs was obtained by co-transfecting CHO cells with C-S65T and RII-EBFP, exciting at 380 nm, the optimum for EBFP, and collecting emissions at 460 and 545 nm for EBFP and S65T, respectively. In unstimulated cells the ratio of 460-nm to 545-nm emissions was typically between 1.5 and 2.5 (Fig. 2a, Fig. 3a, time 0 s). In control cells co-transfected with EBFP and S65T not fused to anything, or with S65T-RII and EBFP-C (that is, chimaeras in which GFP was fused at the amino terminus of the RII β-subunit or catalytic α-subunit, respectively), the 460-nm/545-nm ratio in basal conditions was typically between 4 and 5 (Fig. 2a, Fig. 3a, time 0 s). This difference in ratio indicates that, in the holoenzyme, EBFP and S65T may be brought together by fusion to the C termini of the RII β-subunit and catalytic α-subunit, respectively.

To confirm the existence of FRET between C-S65T and RII-EBFP, we measured the fluorescence emission intensity of the donor RII-

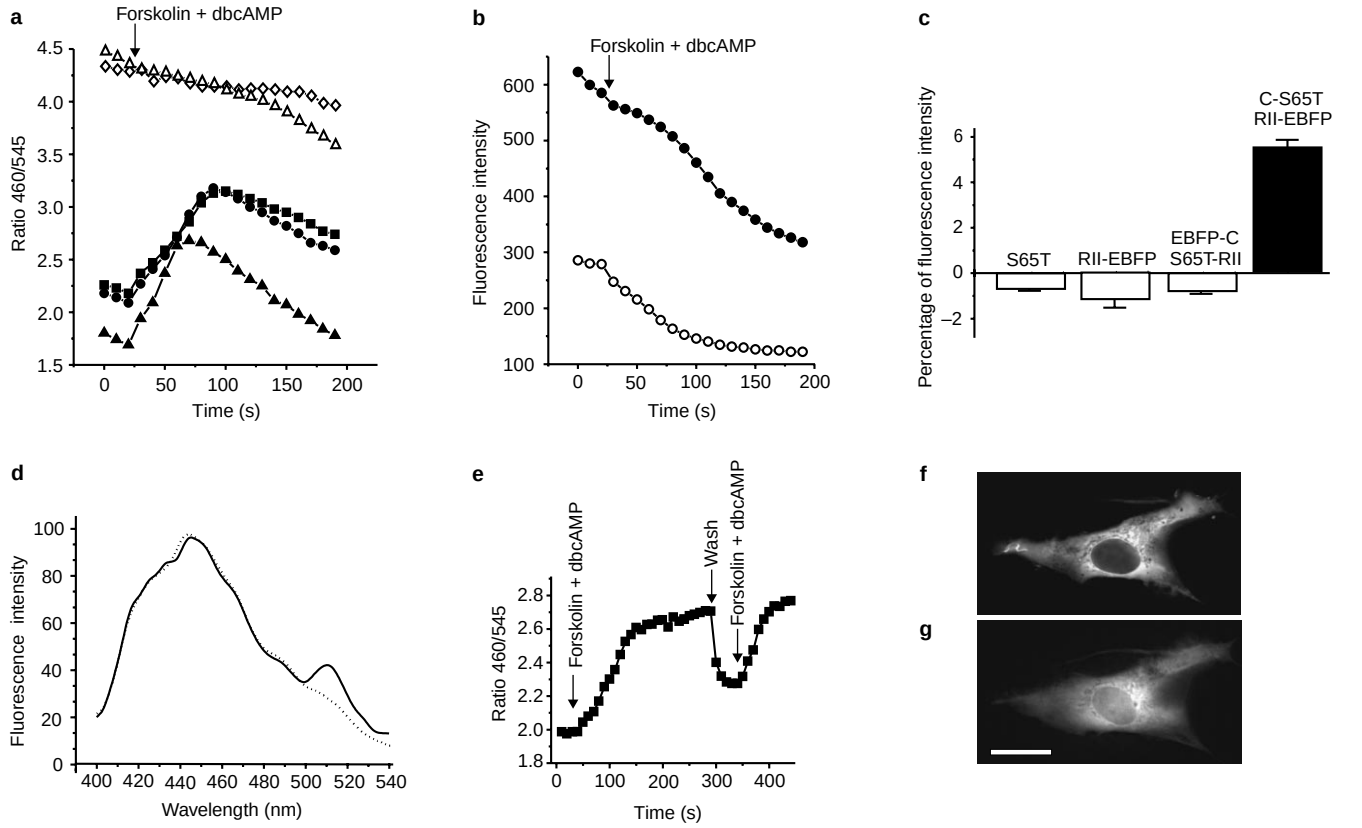

Figure 3 **Kinetics of changes in the fluorescence emission ratios from GFP-tagged PKA subunits. a**, Forskolin and dbcAMP acutely increase the 460-nm/545-nm emission ratio produced from C-S65T and RII-EBFP, consistent with dissociation of the subunits and loss of FRET, without affecting control cells. Filled symbols indicate three independent CHO cells co-transfected with C-S65T + RII-EBFP. Open diamonds indicate CHO cells co-transfected with S65T + EBFP, and open triangles indicate CHO cells co-transfected with the control constructs EBFP-C + S65T-RII. The baseline ratio and the downward drift of the controls result from bleaching of the EBFP. **b**, Kinetics of fluorescence intensities in cells transfected with C-S65T + RII-EBFP, recorded after addition of forskolin and dbcAMP. Open symbols: excitation 380 nm, emission 545 nm. Filled symbols: excitation 380 nm, emission 460 nm. These values were used to calculate the ratios shown in **a** (filled squares). **c**, Effect of photobleaching of the acceptor fluorophore GFP on fluorescence emission of the donor fluorophore EBFP. COS-7 cells were co-transfected with C-S65T + RII-EBFP (black bar) or the indicated control constructs (white bars). Fluorescence emission at 460 nm was recorded (excitation 380 nm, integration time 200 ms) before and after a 2-min illumination of the cells at 475±20 nm. Changes in blue emission are plotted as the percentage of initial blue fluorescence. Values shown represent means ± s.e.m. (n=25). **d**, Emission spectra of a population of cells transfected with C-S65T + RII-EBFP (solid line) or EBFP-C + S65T-RII (dotted line). Excitation was 380 nm. The spectra were normalized; the background fluorescence of untransfected cells and the fluorescence due to the direct excitation of S65T at 380 nm were then subtracted. **e**, CHO cells co-transfected with C-S65T + RII-EBFP were treated with 25 μM forskolin + 1 mM dbcAMP, washed and treated for a second time with the same stimulus. Data are corrected for the bleaching effect as calculated on the basis of the baseline drift before the stimulus. **f**, **g**, Localization of the chimaera C-S65T before (**f**) and 45 min after (**g**) the addition of 25 μM forskolin and 1 mM dbcAMP. CHO cells were co-transfected with the constructs C-S65T and RII-EBFP and stacks of fluorescence images were acquired at the optimal wavelength for S65T (excitation 475±20 nm excitation, emission 545±15 nm) and computationally deblurred. **f** and **g** show the same focal plane. Scale bar represents 10 μm.

EBFP before and after bleaching of the acceptor C-S65T. Selective excitation at 475 nm for 2 min reduced the green fluorescence emission of C-S65T by ~80%. Complete bleaching of C-S65T was unpractical in our experimental conditions because of partial excitation of RII-EBFP at 475 nm and the consistent bleaching of the donor upon exposure for longer than 2 min. Figure 3c shows that, in CHO cells co-transfected with C-S65T and RII-EBFP, selective photobleaching of C-S65T resulted in a 5% increase in blue fluorescence emission. In control cells transfected with RII-EBFP alone, a 2-min excitation at 475 nm reduced the blue fluorescence emission by 1%. About the same reduction in blue fluorescence emission was detected in cells transfected with the control constructs EBFP-C and S65T-RII. We also tested whether any photochemical products of photobleached GFP could fluoresce in the EBFP spectral region, thus giving rise to an apparent increase in the fluorescence of the donor. After a 2-min excitation at 475 nm of cells transfected with GFP only, no increase in blue fluorescence was detected.

Finally, we recorded the emission spectrum of a population of cells co-transfected with C-S65T and RII-EBFP (excitation 380 nm). We observed a clear shoulder peaking at 511 nm. No such shoulder was present when we analysed cells co-transfected with the controls EBFP-C and S65T-RII (Fig. 3d). On the basis of these results we conclude that FRET occurs between C-S65T and RII-EBFP, and we estimate its efficiency to be in the range 5–8%.

To determine whether FRET was sensitive to changes in cAMP levels, we treated transfected cells with cAMP-raising agents. As shown in Figs 2a, 3a, forskolin and dibutyryl cAMP (dbcAMP) acutely increased the 460-nm/545-nm ratio produced from C-S65T and RII-EBFP, consistent with dissociation of the subunits and loss of FRET, without affecting the control cells expressing cytosolic GFP and EBFP or the chimaeras EBFP-C and S65T-RII (Figs 2b, 3a). The increase in ratio was evident a few seconds after addition of the stimulus, reached a plateau level in 1–2 min (Fig. 3a), and reflected both a small increase in the 460-nm EBFP emission and a

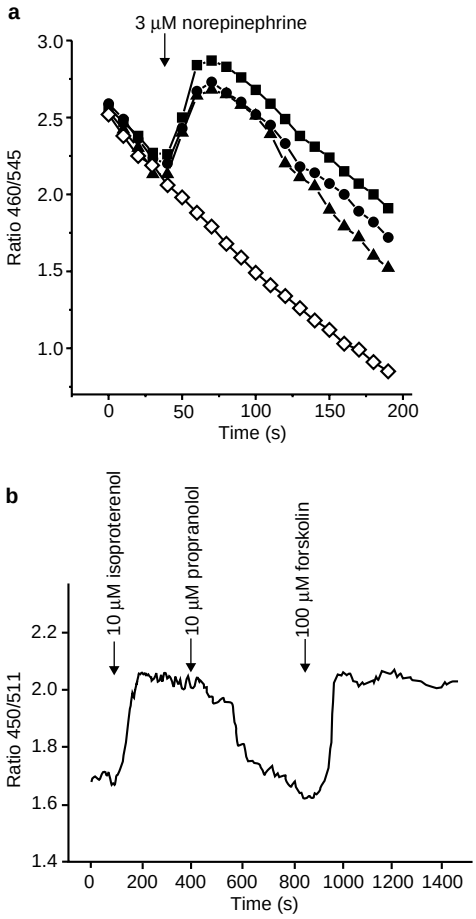

Figure 4 **Effect of norepinephrine on COS-7 cells co-transfected with C-S65T and RII-EBFP. a**, 3μM norepinephrine induced a rapid and transient change in the fluorescence emission ratio in cells co-transfected with C-S65T + RII-EBFP (filled symbols; the different symbols represent the kinetics of ratio changes in three independent cells). Open diamonds indicate the effect of 3μM norepinephrine on cells pretreated with 3μM propranolol before the addition of norepinephrine. **b**, Kinetics of ratio changes in a population of COS-7 cells co-transfected with C-S65T + RII-EBFP and treated successively with isoproterenol, propranolol and forskolin. Emission intensities were collected at 450 and 511 nm; excitation was at 380 nm. Background values (from untransfected cells) were subtracted before calculation of the ratio values. The signal was not corrected for the minor direct excitation of S65T at 380 nm. These results are from a typical experiment out of at least five independent trials.

more marked reduction in the 545-nm S65T emission (Fig. 3b). This confirms that the change in ratio is due to disruption of FRET. Similar results were observed when the same experiment was carried out using a population of transfected cells in a fluorimeter cuvette. Addition of forskolin caused an increase in the 460-nm/511-nm ratio, because of an increase in the 460-nm peak and a reduction of fluorescence at 511 nm (see below). It should be noted that, at the single-cell level, the fluorescence emission ratio declines with time both in cells transfected with cytosolic EBFP and GFP and in cells transfected with the tagged PKA subunits. This decrease in the ratio is due to the high sensitivity of EBFP to photobleaching, whereas the signal resulting from the small, but measurable, direct excitation of GFP (and the fluorescence of endogenous chromophores) is more resistant. Accordingly, the ratiometric calculation can only partially compensate for the effect of bleaching. Figure 3e shows that the change in the 460-nm/545-nm ratio induced by the cAMP-raising agents was reversible upon removal of the drugs, and a new challenge with forskolin plus dbcAMP resulted in a second rise in the fluorescence emission ratio. A prolonged increase in cAMP levels also caused a dissolution of the bright fluorescence spots (if present) and slow diffusion of the catalytic subunit to the nucleus (Fig. 3f, g). The diffusion of C-S65T to the nucleus was slowly reversed by withdrawal of the cAMP-raising stimulus. A similar redistribution of the catalytic subunit was observed in cells co-transfected with S65T-RII and EBFP-C, a combination that does not give rise to FRET.

GFP-tagged PKA can sense cAMP changes induced by physiological stimuli. Forskolin and dbcAMP are crude pharmacological tools with which to induce rises in cAMP. We next investigated whether the methodology was sufficiently sensitive to reveal changes in cAMP levels induced by a more physiological stimulus, that is, by receptor stimulation of COS-7 cells, which express β-adrenergic receptors. 3μM norepinephrine induced a rapid and transient change in the fluorescence emission ratio in cells co-transfected with C-S65T and RII-EBFP (Fig. 4a, filled symbols). When the same cells were pretreated with 3μM propranolol, a β-adrenergic-receptor antagonist, this effect was completely abolished (open symbols). Within the time resolution of this experiment (1 ratio image every 10 s), there was no appreciable lag between the addition of the receptor agonist and the change in FRET. A better time resolution was obtained in cell suspension; in this case, the change in ratio started less than 1 s after addition of isoproterenol. This effect was reversed by propranolol and a second rise in ratio was induced by addition of forskolin (Fig. 4b). Pretreatment of cells with propranolol completely blocked the effect of isoproterenol (data not shown).

Overexpression of GFP-tagged PKA does not significantly alter cAMP signalling. Overexpression of PKA might alter the cAMP-dependent signalling pathway and/or other physiological responses, although it is hard to predict theoretically whether the inhibitory effect of increased cAMP buffering or the excitatory effect of added effectors would dominate. Past experience with fluorescein- and rhodamine-labelled holoenzyme indicated that the net effect was inhibitory but was detectable only at very high levels of PKA microinjection[9]. We determined the expression levels of endogenous and EBFP- or GFP-tagged PKA by western blotting, using antibodies specific for GFP and the catalytic, RI and RII subunits of PKA. The tagged catalytic subunit was expressed at a level about fourfold that of the endogenous subunit, while RII was hardly detectable in COS-7 cells. Levels of endogenous RI were unaffected by transfection. The ratio between C-S65T and RII-EBFP was, on average, 1.5. Several lines of evidence indicate that co-transfection with equal amounts of cDNA encoding the tagged regulatory and catalytic subunits does not grossly alter the overall physiological behaviour of the cells. First, cell morphology is unaltered. Second, receptor-induced increases in Ca^{2+} levels, measured with recombinant aequorin, in both the cytoplasm and the mitochondrial matrix are unaffected. Third, spontaneous contraction in primary cultures of cardiac myocytes is not appreciably altered. Fourth, in CHO cells stably transfected with a β-lactamase reporter gene[10] under the control of a cAMP-response element (CRE), the percentage of β-lactamase-positive cells monitored by flow cytometry was 1–2% without stimulation and 56–63% in the presence of 10 mM forskolin, in cells transiently transfected with C-S65T and RII-EBFP or with empty vector. Transfection efficiency, monitored by C-S65T fluorescence, was ~60%.

Discussion

Our aim was to generate a genetically encoded sensor for cAMP that could be used to measure dynamic changes in amounts of this second messenger both at the single-cell level and in populations of cells. For this purpose, we fused catalytic and regulatory subunits of PKA to GFP mutants with excitation and emission spectra suitable for FRET. Upon transfection, a molecularly homogeneous probe

was generated *in situ*, and fluctuating levels of cAMP could be monitored in 'real time' as changes in emission ratios. The construction of a GFP-tagged PKA capable of FRET was not a trivial task. We tested several constructs, some of which included linker peptides, but only C-S65T and RII-EBFP were found to be efficient partners for cAMP-sensitive FRET. This difficulty is explained by the extreme sensitivity of FRET to the distance and reciprocal orientation of the fluorophores[6]. It was particularly difficult to create a functional catalytic-subunit–GFP chimaera, perhaps because the N and C termini of the native catalytic subunit are, respectively, myristoylated and buried[11]. Of particular interest is the fact that C-S65T did not give FRET when co-transfected with a chimaera in which EBFP was fused at the C terminus of an RI subunit. This could reflect a significant structural diversity between the RI and RII isoforms and a consequent different topography in the holoenzyme. Fusion of two GFPs to a large enzyme complex such as PKA may easily result in an inactive protein[12]. The fact that transfection with C-S65T alone was detrimental to the cells, and that co-transfection with RII-EBFP restored a normal phenotype, indicated that these tagged subunits are functionally intact. In addition, the demonstration that the activation of a CRE-dependent gene is not appreciably altered in cells overexpressing the tagged PKA subunits further confirms that the cAMP signalling pathway is intact in the co-transfected cells. Our results show that C-S65T and RII-EBFP can give cAMP-sensitive FRET (Figs 2, 3) and that this methodology is sufficiently sensitive to pick up physiological changes in cAMP levels (Fig. 4). The lack of an appreciable lag phase between receptor stimulation and FRET changes (Fig. 4) indicates that the affinity of the GFP-tagged PKA for cAMP may be similar to that of the endogenous PKA.

We have not yet investigated whether or not the overexpressed, tagged PKA binds to PKA-anchoring proteins (AKAPs), as the endogenous regulatory subunits do[13]. This key aspect of the cAMP signalling pathway, that is, the kinetics of dissociation of PKA subunits in relevant places in the cell, will be an exciting prospect for future study. To allow monitoring of dynamic changes in cAMP amounts throughout cells, the non-localized distribution of the tagged PKA in the cytoplasm is appropriate so that it can report the diffusion of the second messenger from its site of generation deep into the cytoplasm. As with indicators for other second messengers, such as Ca^{2+}, the determination of very localized signals will require specific targeting strategies, which now become conceivable with genetically encoded indicators.

In conclusion, our results indicate that direct imaging of cAMP and PKA activation is now possible in a wide variety of cells, and open the way to the study of this key cellular function in whole organisms. Further improvements should result from the use of GFP mutants with even better spectral properties[8] or of alternative methods for *in situ* protein labelling[14]. Last, but not least, from a methodological point of view, our data show that FRET between genetically encoded labels can be used to image dynamic protein–protein interactions in a large enzyme complex. □

Methods

Construction of GFP-tagged PKA.

The constructs RII-EBFP and C-S65T were generated by fusing the blue (EBFP) or the bright green (S65T) mutants of GFP to the C termini of the regulatory RII or the catalytic subunits of PKA, respectively. EBFP was amplified by polymerase chain reaction (PCR) using the forward primer 5′-CACCATGAAGCTTTATCCTTATGATGTTCCTGATTATGCCGTGAGCAAGGGC and the reverse primer 5′-TTAGGATCCCTTGTACAGCTCGTCCATGCC and cloned, as a *Hind*III/*Bam*HI fragment, in-frame at the 3′ end of the rat regulatory subunit type II-β of PKA. S65T was amplified by PCR using the forward primer 5′-ATCGATTATGATGTTCCTGATTATGCAAGCTTAATGAGTAAAGGAGAAGAACTTTTC and the reverse primer 5′-GCGGGATCCTTATTTGTATAGTTCTTC and cloned as a *Cla*I/*Bam*HI fragment in-frame at the 3′ end of the mouse catalytic subunit, type α, of PKA. For both fusions, the forward primers used to amplify GFP mutants included the linker sequence. Both constructs were generated in a CMV-based vector for transient expression in eukaryotic cells.

Cell cultures.

CHO, COS-7 and HEK293 cells were plated onto flamed glass coverslips or plates and grown to 60–80% confluency at 37 °C and in 5% CO_2 in DMEM medium (Sigma). Cells were transfected with the FuGENE-6 transfection reagent (Boehringer). Briefly, 24-mm glass coverslips (for imaging experiments) or 100-mm Petri dishes (for cuvette fluorescence experiments) were transfected with 1.5 μg or 5 μg DNA for each construct, 20 μl or 50 μl 10 mM Tris (pH7.4) and 1 mM EDTA (pH8.0), 100 μl or 600 μl DMEM medium and 6 μl or 30 μl FuGENE, respectively. After a 15-min incubation at room temperature, the mixture was added to the cells. Transfected cells were analysed 36 h later. For imaging, glass coverslips with adherent cells were transferred to the experimental chamber containing HEPES-buffered saline supplemented with 1 gl^{-1} glucose, with the addition of forskolin (Sigma) and dbcAMP (Sigma) or norepinephrine (Sigma) as indicated. The cells were kept at 37 °C throughout the experiment.

Imaging of single cells.

Cells were imaged on a Zeiss Axiovert microscope connected with a cooled charge-coupled-device (CCD) camera (Princeton Instruments, Trenton, NJ). For dual-emission ratio imaging, we used a 380HT15 excitation filter, a 425DCLP dichroic mirror and two emission filters (460/50 for EBFP and 545RDF35 for S65T) alternated by a filter changer (Lambda 10-2, Sutter Instruments, San Rafael, CA). Interference filters were obtained from Omega Optical and Chroma Technologies (Brattleboro, VT). Fluorescence images were background-corrected and analysed with MetaFluor software (Universal Imaging, West Chester, PA). Integration time was 200 ms per image and images were taken every 10 s.

Population experiments.

Transfected cells from a 100-mm Petri dish were washed with PBS, trypsinized and resuspended in 2 ml PBS containing 1 mM Ca^{2+} and 1 mg ml^{-1} glucose, placed in a quartz cuvette and kept in suspension with a stir-bar. Experiments were performed in a Perkin-Elmer Spectrofluorometer LS50B. Emission spectra (excitation 380 nm, emission 400–550 nm) were collected and 450/511 emission ratios calculated using the FL WinLab software (Perkin-Elmer).

CRE-dependent gene expression.

CHO cells stably transfected with a β-lactamase reporter gene[10] under the control of a cAMP-response element (3.5×10^5 cells per well on a 6-well plate) were transfected with 3 μg each of the C-S65T and RII-EBFP constructs (1:1 ratio) using lipofectamine (Gibco). The plasmids were mixed with 36 μg lipids in 0.5 ml Opti-MEM and incubated at room temperature for 30 min. The DNA–lipid mixture was then added onto the cells. The empty vector was transfected as a negative control using the same conditions (mock-transfected control). 40 h after transfection, cells (those transfected with fluorescent proteins and mock-transfected controls) were stimulated with 10 μM forskolin for 5 h (control cells for both conditions were left unstimulated). Cells were collected using cell-dissociation buffer (Gibco). Half of the cells from each well were loaded with CCF2/AM for fluorescence-activated cell sorting (FACS) analysis of CRE-dependent β-lactamase expression, and half were left without exogenous fluorescent labelling for analysis of fluorescent protein expression. FACS analysis was carried out using Becton Dickinson FACS Vantage and FACS Vantage SE flow cytometers. Flow-cytometric analysis of fluorescent protein expression was done using 60 mW of 351–364-nm laser emission (Coherent Enterprise II argon laser) for excitation of RII-EBFP, with 440/10-nm and 530/30-nm emission filters (RII-EBFP direct emission and C-S65T FRET emission, respectively), and using 80 mW of 488-nm laser emission (Coherent Enterprise II argon laser) with a 530/30-nm emission filter for direct excitation of the C-S65T. Flow-cytometric analysis of CRE-dependent β-lactamase expression in cells loaded with CCF2 was carried out using 45 mW of 413-nm laser emission (Coherent Innova 302C krypton laser) and 460/50-nm and 535/40-nm fluorescence emission filters.

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