Detection in Living Cells of Ca\textsuperscript{2+}-dependent Changes in the Fluorescence Emission of an Indicator Composed of Two Green Fluorescent Protein Variants Linked by a Calmodulin-binding Sequence

A NEW CLASS OF FLUORESCENT INDICATORS

(Received for publication, February 19, 1997)

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We have designed a novel fluorescent indicator composed of two green fluorescent protein variants joined by the calmodulin-binding domain from smooth muscle myosin light chain kinase. When (Ca\textsuperscript{2+})\textsubscript{4}-calmodulin is bound to the indicator (\(K_d = 0.4 \text{ nmol/L}\)), fluorescence resonance energy transfer between the two fluorophores is attenuated; the ratio of the fluorescence intensity measured at 505 nm to the intensity measured at 440 nm decreases 6-fold. Images of microinjected living cells demonstrate that emission ratios can be used to monitor spatio-temporal changes in the fluorescence of the indicator. Changes in indicator fluorescence in these cells are coupled with no discernible lag (<1 s) to changes in the cytosolic free Ca\textsuperscript{2+} ion concentration, ranging from below 50 nmol/L to ~1 \mu mol/L. This observation suggests that the activity of a calmodulin target with a typical 1 nmol/L affinity for (Ca\textsuperscript{2+})\textsubscript{4}-calmodulin is responsive to changes in the intracellular Ca\textsuperscript{2+} concentration over the physiological range. It is likely that the indicator we describe can be modified to detect the levels of ligands and proteins in the cell other than calmodulin.

The Ca\textsuperscript{2+}-binding protein calmodulin (CaM)\textsuperscript{1} is a key transducer of intracellular Ca\textsuperscript{2+} ion signals, largely through its Ca\textsuperscript{2+}-dependent activation of many enzyme activities (1–4). Yet little is known about the extent and kinetics of enzyme activation in vivo, mainly because of the difficulty of directly monitoring target activation in the cell. Mitra et al. (5) have recently reported that changes in fluorescence resonance energy transfer (FRET) between variants of green fluorescent protein (GFP) can be used to monitor cleavage at a protease site within a linker amino acid sequence. We have designed a similar fluorescent indicator protein in which the GFP variants are linked by a CaM-binding sequence. This indicator exhibits a large CaM-dependent change in its fluorescence emission due to disruption of FRET when calmodulin is bound to the linker sequence. This response can be monitored in living cells, where it closely follows changes in the intracellular Ca\textsuperscript{2+} concentration.

MATERIALS AND METHODS

Expression and Purification of Proteins—The vector for expression of FIP-CB\textsubscript{39A} is similar to the one described by Mitra et al. (5). The coding sequences for the BGFP (6) and RGFP (7) domains were produced by amplifying the GFP-encoding sequences in the BioBlue™ and BioYellow™ vectors obtained from PharMingen, Inc. (San Diego, CA). The vector pETIC, encoding a fluorescent indicator protein (FIP) control consisting of RGFP and BGFP domains joined by the linker sequence, GTSGSGSTGA, was generated first. The RGFP domain in pETIC is fused to the His\textsubscript{4}-tag/thrombin/S-tag/enterokinase leader sequence derived from pET30a (Novagen, Inc., Madison, WI). The C terminus of the BGFP domain is fused to an additional His\textsubscript{6} sequence, also derived from pET30a. The vector pETIC-1 encodes FIP-CB\textsubscript{39A}, which is identical to the FIP control, except that the linker has the sequence: GTSSRKRWKNKGHAVIDRAGLSSTGA. Boldface type denotes the CaM-binding sequence from avian smooth muscle myosin light chain kinase (8).

For expression, pETIC and pETIC-1 were transformed into Escherichia coli strain BL21(DE3). Cells containing pETIC or pETIC-1 were grown at 23 °C to an \(A_600\) of 0.6–0.8, and protein expression was induced by addition of isopropyl-1-thio-\beta-D-galactopyranoside to 0.5 mM. After incubating at 23 °C for ~40 h, cells were harvested. Control FIP and FIP-CB\textsubscript{39A} were purified using His\textsubscript{6} affinity chromatography essentially as described by Mitra et al. (5). Vertebrate CaM expressed in E. coli was purified as described previously (9). The concentrations of control FIP and FIP-CB\textsubscript{39A} were determined using an \(A_{490}\) of 89 \text{ mmol/L} \text{ cm}^{-1}. Concentrations of FIP-CB\textsubscript{39A} stock solutions were verified by titration with a standard CaM solution.

In Vitro Measurements of FIP-CB\textsubscript{39A} Fluorescence—Fluorescence measurements were performed using a Photon Technology International (Monmouth Junction, NJ) QuantaMaster™ photon counting spectrophotometer. Reaction volumes (3 ml) were incubated at 30 °C in a stirred cuvette. Excitation and emission slit widths were 5 nm. An excitation wavelength of 380 nm was used for in vitro measurements of FIP fluorescence. For most experiments, a buffer containing 25 \text{ mM} Tris, 0.1 \text{ mM} NaCl, and 300 \text{ mM} CaCl\textsubscript{2}, pH 7.5, was used. For experiments in which the free Ca\textsuperscript{2+} ion concentration was varied, a buffer containing 50 mM Tris, 0.1 \text{ mM} NaCl, 0.5 \text{ mM} MgCl\textsubscript{2}, and 3 \text{ mM} 1,2-bis(2-amino-5,5'-dibromophenoxy)ethane-N,N,N',N'-tetraacetic acid, pH 7.5, was used. Aliquots of standard CaCl\textsubscript{2} solutions were added to achieve various levels of free Ca\textsuperscript{2+} ion, which were calculated using the MaxChelator program (10). The FIP-CB\textsubscript{39A} fluorescence emission spectrum is essentially independent of the pH between 7.0 and 8.0, either in the presence or absence of bound (Ca\textsuperscript{2+})\textsubscript{4}-CaM.
Measurements of FIP-CB<sub>SM</sub> Fluorescence in Cells—Human embryonic kidney cells (HEK-293) stably transfected with an epitope-tagged thyrotropin-releasing hormone (TRH) receptor (11) were grown on glass coverslips to 60–80% confluence, rinsed in Hank’s balanced salt solution, and placed in a Sykes-Moore chamber maintained at 37 °C. Microinjections were performed on an Eppendorf Transjector 5246 equipped with a Micromanipulator 5171 using Femtotips from Eppendorf (Madison, WI). Microinjection solutions were centrifuged and filtered through 0.2-μm nitrocellulose filters and injected at pressures of 50–100 hectopascals for 0.1 s. Successful injections were visualized in brightfield and by observing at 530 nm the fluorescence of RGFP excited directly at 495 nm. After microinjection, cells were allowed to recover for at least 30 min.

Dynamic measurements were performed using a Dage CCD72 camera and Geniisys image intensifier system (Michigan City, IN) and IMAGE-1/AT analysis software from Universal Imaging (Media, PA). Fura-2 340/380 fluorescence excitation ratios were obtained as described previously (12). The fluorescence emission of FIP-CB<sub>SM</sub> and control FIP excited at 380 nm was measured at 500-ms intervals using a 510-nm emission filter. Still photographs were obtained with a Cohu CCD camera (San Diego, CA) and a 440A integrator (Colorado Video, Inc., Boulder, CO) and analyzed with Metamorph software from Universal Imaging. Cells were illuminated at 380 nm, and emitted light was collected for 10–20 s at 510 nm and then at 440 nm.

**FIG. 1.** CaM-dependent changes in the FIP-CB<sub>SM</sub> fluorescence emission spectrum. FIP-CB<sub>SM</sub> at a concentration of 1 μM was titrated with 0.2 μM increments of (Ca<sup>2+</sup>)<sub>4</sub>-CaM. The effect of (Ca<sup>2+</sup>)<sub>4</sub>-CaM on the FIP-CB<sub>SM</sub> emission spectrum is completely reversed by adding 5 mM EDTA. A scheme depicting the conformational change undergone by FIP-CB<sub>SM</sub> upon binding (Ca<sup>2+</sup>)<sub>4</sub>-CaM is also presented in the figure. The RGFP (R) and BGFP (B) domains in FIP-CB<sub>SM</sub> are joined by a linker sequence containing a CaM-binding domain. The fluorophores in the GFP domains are represented by shaded rectangles. The relative dimensions used for the GFP domains are based on the published crystal structures for GFP (14, 15). (Ca<sup>2+</sup>)<sub>4</sub>-CaM is depicted as two hemispheres, corresponding to two EF hand pairs, joined by the flexible central helix. Ca<sup>2+</sup> ions bound to the EF hands are depicted as filled circles.

**FIG. 2.** Characterization of CaM binding by FIP-CB<sub>SM</sub>. A, titration of a mixture of 8.2 nM FIP-CB<sub>SM</sub> and 200 nM CaM with increasing concentrations of free Ca<sup>2+</sup> ion. Data were fit to an equation of the form: F = α[L<sub>t</sub>/(L<sub>t</sub> + K<sub>a</sub>)], where F is the fractional saturation of FIP-CB<sub>SM</sub> with CaM, given in the figure as 1 − [(F − F<sub>min</sub>)/(F<sub>max</sub> − F<sub>min</sub>)], L<sub>t</sub> is the free Ca<sup>2+</sup> ligand concentration, K<sub>a</sub> is an apparent dissociation constant that depends upon the total amount of CaM, and α is the number of interacting sites, which equals 3.9 for the curve shown. This indicates that (Ca<sup>2+</sup>)<sub>4</sub>-CaM is the species bound by FIP-CB<sub>SM</sub>. F is the fluorescence measured at 505 nm, F<sub>max</sub> and F<sub>min</sub> are the values for F measured at maximal and minimal free Ca<sup>2+</sup> concentrations, and α is a correction factor allowing adjustment of the maximal fraction of FIP-CB<sub>SM</sub> bound to give the best fit. α values are between 1 and 1.1 for the curves shown in panels A and B. B, binding of (Ca<sup>2+</sup>)<sub>4</sub>-CaM to FIP-CB<sub>SM</sub> at concentrations of 1.4 and 1.0 nM. The data measured at a 1 nM FIP-CB<sub>SM</sub> concentration were fit to a standard single-site kinetic model as described previously (20). The data measured at a 1.4 nM FIP-CB<sub>SM</sub> concentration were fit to an equation of the form: F = α[P<sub>t</sub>/(L<sub>t</sub> + K<sub>d</sub>)] − [(P<sub>t</sub> + L<sub>t</sub> + K<sub>d</sub>)<sup>2</sup> − 4(P<sub>t</sub>)(L<sub>t</sub>(L<sub>t</sub> + K<sub>d</sub>))]/2(L<sub>t</sub>), where L<sub>t</sub> is the total CaM concentration and P<sub>t</sub> is the total FIP-CB<sub>SM</sub> concentration. In both cases a K<sub>d</sub> value of 0.4 nM was obtained. Emission spectra for titration of 1.4 nM FIP-CB<sub>SM</sub> with (Ca<sup>2+</sup>)<sub>4</sub>-CaM are shown in the inset.
RESULTS AND DISCUSSION

We have designed a fluorescent indicator protein containing two green fluorescent protein variants, with reported fluorescence excitation and emission maxima of 382 and 446 nm (BGFP) (6) and 495 and 509 nm (RGFP) (7), joined by an amino acid linker containing the CaM-binding sequence from smooth muscle myosin light chain kinase (8) (Fig. 1). We term this particular indicator FIP-CBSM. Excitation of the fluorophore in FIP-CBSM can be monitored in living cells, providing a view of free CaM (17).

CaM-dependent changes in FRET between the fluorophores in FIP-CBSM can be monitored in living cells, providing a view of free CaM in the cell. We have microinjected FIP-CBSM with or without equimolar CaM, into HEK-293 cells stably transfected with the Gq/11-coupled Ca2+–mobilizing receptor for TRH. We estimate an intracellular FIP-CBSM concentration in microinjected cells of 1 – 10 μM, similar to estimates for the intracellular concentrations of high-abundance CaM targets, such as smooth muscle myosin light chain kinase (18).

Injection of FIP-CBSM undoubtedly perturbs the balance between CaM and its targets. Coinjection of the indicator with equimolar CaM should help to restore it, but the exact mole ratio of CaM to its binding sites in the cell is unknown. The intracellular free Ca2+ ion concentration ([Ca2+]i) increases from below 50 nM, when cells are incubated in media containing BAPTA, to greater than 1 μM, when extracellular Ca2+ and ionomycin are added (data not shown). Corresponding fluorescence images show a clear Ca2+-dependent reduction in the F510/F440 ratios in cells injected either with FIP-CBSM alone or with FIP-CBSM and CaM. This indicates an increase in the fraction of FIP-CBSM bound to CaM (Fig. 3). A greater reduction in the F510/F440 ratio is evident in cells injected with both FIP-CBSM and CaM. The basal fluorescence intensity of the RGFP acceptor (Fig. 3, C and F) is brighter in some regions of the cell than others due to the distribution of the probe within the cells. Some cells were injected in the nucleus, some into the cytoplasm, and some into both nuclear and cytoplasmic regions. FIP-CBSM remained localized to the nucleus or cytoplasm.

A New Class of Fluorescent Indicators

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The maximum fractional reduction in the $F_{S10}$ of FIP-CBSM observed in cells was 30%, or about half of the maximal 65% CaM-dependent reduction in $F_{S10}$ measured in vitro (Fig. 1). Given the very different experimental systems used for measurements of fluorescence in vitro and in vivo, this difference in the maximal signals appears acceptable. The amount of probe injected was kept low to minimize perturbation of Ca$^{2+}$-CaM homeostasis. A consequence is that fluorescence filters with bandwidths of ~40 nm were required to obtain an adequate fluorescence signal. In contrast, the monochromator bandwidths used for in vitro measurements were about 3 nm. Some of the small discrepancy between in vitro and in vivo measurements of CaM-dependent changes in indicator fluorescence may therefore be attributable to the different optical systems used.

Our results clearly indicate that changes in [Ca$^{2+}$], ranging from below 50 nM to ~1 mM are coupled to changes in the $F_{S10}$ of FIP-CBSM. This suggests that the activity of a calmodulin target with a typical 1 nM affinity for (Ca$^{2+}$)$_4$-calmodulin is responsive to changes in the intracellular Ca$^{2+}$ concentration over the physiological range. It also suggests that physiological changes in [Ca$^{2+}$], are coupled to changes in the free (Ca$^{2+}$)$_4$-CaM concentration in the low nanomolar range. The free concentrations of (Ca$^{2+}$)$_4$-CaM occurring in the cell are therefore ~1000-fold less than the total concentration of CaM. Thus, the calmodulin concentration in the cell is limiting; essentially all the (Ca$^{2+}$)$_4$-CaM present in the cell must be bound to targets, as has been proposed based on studies of the intracellular mobility of tagged CaM (19). The very low physiological levels of free (Ca$^{2+}$)$_4$-CaM indicate that small changes in the affinity of a typical target should significantly affect its level of activity at a submaximal [Ca$^{2+}$], as recently demonstrated for smooth muscle myosin light chain kinase activity (18). The ability to monitor free (Ca$^{2+}$)$_4$-CaM levels in living cells provides an exciting new approach for dissecting the processes that link variations in [Ca$^{2+}$], to diverse cellular responses. Indeed, FIP-CBSM appears to represent a new class of ligand-dependent indicators that have the potential of reporting the levels of a variety of proteins and other ligands in the cell, depending upon the nature of the linker sequence.

**REFERENCES**


plasm in cells, depending upon its initial site of injection. The indicator, with a molecular mass of 63.4 kDa, would not be expected to move through nuclear pores, and such translocation was not observed. No systematic effort was made to compare the responses of the indicator in the nucleus and cytosol, although it is clear that similar responses occur in these compartments (Fig. 3). Ca$^{2+}$-dependent changes in the 510/440 emission ratio measured in cells suggest microheterogeneity in the response that may be artifactual, perhaps reflecting small changes in cell shape occurring during data collection. Investigations of a possible physiological basis for the apparent microheterogeneity are clearly a high priority.

To more precisely establish the kinetics and magnitude of the FIP-CBSM response to changes in [Ca$^{2+}$], the $F_{S10}$ of microinjected cells was measured at 500-ms intervals, while [Ca$^{2+}$]$_i$ was manipulated (Fig. 4). Reductions in $F_{S10}$ mirror increases in [Ca$^{2+}$], caused by addition of TRH, Ca$^{2+}$, and ionomycin with no discernible lag (<1 s) (Fig. 4). Measurements using Fura-2 indicate that basal [Ca$^{2+}$], in these cells is below 50 nM; it increases to 200–400 nM with TRH and to greater than 1 mM with the combination of external Ca$^{2+}$ and ionomycin. In cells injected with equimolar FIP-CBSM and CaM, the combination of Ca$^{2+}$ and ionomycin causes a 30% reduction in $F_{S10}$. This appears to represent the saturated response of the indicator, since it is reached and maintained when [Ca$^{2+}$], is still increasing (Fig. 4A). Treatment of cells injected with FIP-CBSM alone with TRH causes a ~6% reduction in $F_{S10}$; the combination of Ca$^{2+}$ and ionomycin causes a ~10% reduction (Fig. 4B). Thus, saturation of the FIP-CBSM response is not approached in these cells, indicating that the CaM concentration is limiting.

**FIG. 4.** Kinetics of CaM activation in living cells. Cells were microinjected with solutions containing 88 mM FIP-CBSM and 88 mM CaM (panel A), 88 mM FIP-CBSM alone (panel B), or 2.5 mM Fura-2 free acid (panel C). At the indicated times, 3 mM BAPTA, 4 mM TRH, 5 mM CaCl$_2$, and 500 nM ionomycin were added to the bathing solution. Based on measurements in cell populations using Fura-2, BAPTA decreases [Ca$^{2+}$], to below 50 nM, TRH increases it to 200–400 nM, and the combination of Ca$^{2+}$ and ionomycin increase it to >1 mM. Panels A and B, the average $F_{S10}$ emission intensity of 12–27 individual cells, expressed as $F/F_0$, with $F_0$ defined as the average of the first 10 values in the time course. The SEM for each time point averages 4%. Note the difference in scales for cells injected with equimolar FIP-CBSM and CaM or FIP-CBSM alone. Panel C, relative [Ca$^{2+}$], values expressed as average Fura-2 340/380 excitation ratios. In control experiments, cells were microinjected with a solution containing 88 mM control FIP, which lacks a CaM-binding sequence, either with or without equimolar CaM, show no detectable changes in $F_{S10}$ in response to externally applied BAPTA, TRH, CaCl$_2$, or ionomycin. Cells microinjected with Fura-2 free acid, and those injected with solutions containing Fura-2 + 88 mM CaM or Fura-2 + 88 mM CaM-binding peptide, show similar changes in Fura-2 fluorescence. The CaM-binding peptide used has the sequence: KERKKKNP1AVSAAAANPFRKK-amide, and is based on the CaM-binding domain in skeletal muscle myosin light chain kinase (21). The $F_{S10}$ have been adjusted so that the times of TRH addition are aligned.
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doi: 10.1074/jbc.272.20.13270

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