The Relationship between the Free Concentrations of Ca\(^{2+}\) and Ca\(^{2+}\)-calmodulin in Intact Cells

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Using stably expressed fluorescent indicator proteins, we have determined for the first time the relationship between the free Ca\(^{2+}\) and Ca\(^{2+}\)-calmodulin concentrations in intact cells. A similar relationship is obtained when comparing the free Ca\(^{2+}\) concentration with the total calmodulin concentration. We conclude that the fluorescent indicator proteins are efficiently activated throughout the cell, but efficient resonance energy transfer; FIP-CA, Fluorescent Indicator Protein-Calmodulin phosphodiesterase, and cGMP-gated ion channels (2–5). Targets of interest include the global relationship between the concentrations of free Ca\(^{2+}\) and Ca\(^{2+}\)-calmodulin in intact cells using stably expressed indicator proteins.

EXPERIMENTAL PROCEDURES

Indicators were expressed under control of a cytomegalovirus promoter/enhancer using the pcDNA3.1 or pcDNA6 vectors supplied by InvitroGen (Carsilbad, CA). HEK-293 cells were transfected using LipofectAMINE according to the instructions of the manufacturer (Life Technologies, Gaithersburg, MD). Mixed-clonal populations of stably transfected cells used in experiments were grown under drug selection (G418 or Zeocin) for at least 6 weeks. The CaM-binding sequences in the indicators are altered versions of the avian smooth muscle myosin light chain kinase sequence: RRRWQKTGHA10VRAIGRL (17). According to this numbering scheme, the sequences in the indicators have the following changes: FIP-CBSM-38 and FIP-CA37, R1Q, R2Q; FIP-CBSM-39, R1Q, R2Q, R12Q, R16Q; and FIP-CBSM-41, R2Q. In vitro cooperativity coefficients and Kd values for the indicators were determined in buffered saline as described previously (18, 19). Values for the Ca\(^{2+}\) indicator were also determined in cells; the lack of a reliable buffering system for CaM precluded doing this with the CaM indicators.

Indicator responses were monitored in individual cells using a standard microscope photometry setup with dual photomultiplier tubes (Photon Technology Int., Monmouth Junction, NJ). The emitted light from single cells was isolated using an adjustable diaphragm, and emission intensities were determined by photon counting using 200 msec integration times. Excitation light at 430 nm was supplied by a monochromator. A 455DCLP microscope dichroic was used, and emitted light was distributed to the two photomultipliers using a 510DCLP dichroic cube fitted with D535/25 and D480/40 bandpass filters. Dichroics and filters were obtained from Chroma Technologies (Brattleboro, VT). All measurements were made in cells expressing n5 µM indicator as assessed by comparing the fluorescence intensities of cells with the intensities of indicator standards. Cells were incubated in a buffered saline solution containing 141 mM NaCl, 5 mM KCl, 1 mM MgSO\(_4\), 10 mM HEPES, 10 mM glucose, pH 7.4, and added CuCl\(_2\) and/or Ca\(^{2+}\)-chelators as indicated. Free (Ca\(^{2+}\)-) and Ca\(^{2+}\)-calmodulin concentrations were calculated from indicator emission ratios using an equation of the form,

\[
\frac{[L]_{free}}{[L]_{lig}} = K_r \frac{R - R_{\text{min}}}{R_{\text{max}} - R}
\]

(Eq. 1)

where \(L\) is Ca\(^{2+}\) or (Ca\(^{2+}\)-)CaM, \(K_r\) is the ligand concentration when the emission ratio (R) is midway between its maximum (R\(_{\text{max}}\)) and minimum (R\(_{\text{min}}\)) possible values and n is the cooperativity coefficient for the response. \(K_d\) is related to the true titration midpoint (K) according to the following equation,

\[
K_d = K_r \left( \frac{S_d}{S_a} \right)^n
\]

(Eq. 2)

where \(S_d\) and \(S_a\) are the indicator emission intensities at 535 nm when ligand-free or ligand-saturated, respectively. The \(S_d/\)S\(_{\text{max}}\) value is 1.8 for FIP-CBs and 1.5 for FIP-CAs.

RESULTS AND DISCUSSION

The indicator proteins used in this study are similar to those we have described previously (18, 19). The main difference is in the green fluorescent protein variants used to generate ligand-dependent fluorescence resonance energy transfer (FRET), which have been replaced by the ECFP and EYFP variants described by Miyawaki et al. (20). The advantages of these are
that FRET can be monitored as the 480/535 emission ratio, and the ECFP donor fluorophore can be excited at a longer wavelength of 430 nm. To estimate physiological (Ca^{2+})_{i}-CaM values, we have made measurements in cells expressing indicators with different affinities, which were generated by varying the CaM-binding linker sequence between the GFP variants. The Ca^{2+}-indicator protein used in some experiments is similar to the CaM indicators, but incorporation of the complete amino acid sequence of CaM renders it directly responsive to changes in the free Ca^{2+} concentration (18). Stably transfected cells were produced that express one of three different CaM indicators, FIP-CBSM-41 (K_d = 2 nM), FIP-CBSM-38 (K_d = 45 nM), and FIP-CBSM-39 (K_d = 400 nM), or the Ca^{2+} indicator, FIP-CA_{37} (K_d = 0.6 μM). The CaM indicators are freely distributed but FIP-CA_{37} is confined to the cytoplasm, probably because of its higher molecular weight (data not shown). The morphology and growth kinetics are similar for control and indicator-expressing HEK-293 cells.

To determine R_{max}, the 480/535 emission ratio for fully liganded indicator, in cells expressing the CaM indicators, the cells were permeabilized with 50 μM β-escin, followed by addition of 10 μM CaM, which diffuses into the cells producing the maximum possible indicator response. The indicator response is specific to Ca^{2+}-liganded CaM and is reversed by a high-affinity CaM-binding peptide (Fig. 1A). To determine R_{perm}, the emission ratio corresponding to the free (Ca^{2+})_{i}-CaM concentration produced at a saturating free Ca^{2+} concentration, cells were permeabilized with 15 μg/ml α-toxin (α-hemolysin) in a buffered saline solution containing 1.3 mM added CaCl_2 (Fig. 1B). There is no loss of indicator fluorescence from α-toxin permeabilized cells, and addition of 20 μM CaM or 50 μM CaM-binding peptide has no effect on the indicator response, demonstrating that the cells are not permeable to proteins or even small peptides. Indicator emission ratios identical to R_{perm} are produced in cells incubated with 5 μM iomyycin and 5 mM CaCl_2. Because the FIP-CA_{37} response is CaM-independent, R_{perm} is equivalent to R_{max}, and we refer only to the latter value (Fig. 1B). To determine R_{min}, the emission ratio for ligand-free indicator, cells were permeabilized with α-toxin in 3 mM BAPTA (Fig. 1B). Indicator emission ratios identical to R_{min} are observed in intact resting cells. R_{min}/R_{perm} values are consistently 2 for the CaM indicators and 1.6 for the Ca^{2+} indicator. This difference is because of a higher R_{min} value for the Ca^{2+} indicator (Fig. 1B), which is expected based on a comparison of the in vitro emission spectra for the two types of indicator (data not shown).

The free (Ca^{2+})_{i}-CaM concentrations produced in cells expressing each CaM indicator were calculated from R_{perm} values as described under “Experimental Procedures.” A value of 2.3 ± 0.5 nM was determined for cells expressing FIP-CBSM-41, and values of 23 ± 2 nM and 45 ± 4 nM were determined for cells expressing FIP-CBSM-38 and FIP-CBSM-39. A plot of these values versus the apparent K_d values for the corresponding indicators suggests that the physiologically global maximum free (Ca^{2+})_{i}-CaM concentration is ~45 nM (Fig. 1B, inset). We have found that R_{perm} corresponds to only 60% of the full indicator response in cells expressing FIP-CBSM-41, which has a 2 nM K_d value, and is significantly less than this in cells expressing the lower-affinity indicators (Fig. 1B). If we assume that the native CaM-binding proteins in the cell have K_d values for (Ca^{2+})_{i}-CaM that are ~2 nM, this observation suggests that on a molar basis CaM-binding proteins outnumber CaM by a factor of ~2.

To define the relationship between the intracellular concentrations of free Ca^{2+} and (Ca^{2+})_{i}-CaM in greater detail, we used dibromo-BAPTA to control the free Ca^{2+} concentration in α-toxin permeabilized cells (21). Effective control of intracellular free Ca^{2+} is demonstrated by the FIP-CA_{37} responses in cells incubated at different calculated free Ca^{2+} concentrations (Fig. 2A). A cooperativity coefficient of 1.7 and an apparent K_d value of 0.9 μM were derived from these data; respective in vitro values obtained with the same Ca^{2+} buffering system are 1.8 and 0.6 μM. The FIP-CBSM-41 or FIP-CBSM-38 responses and corresponding free (Ca^{2+})_{i}-CaM concentrations are presented in Fig. 2, B and C, respectively. Data for the two indicators are fit by binding curves with cooperativity coefficients of 2.6 and apparent K_d values of 1 and 1.1 μM, respectively. Because these indicators bind (Ca^{2+})_{i}-CaM, not Ca^{2+}, the apparent K_d values are actually the free concentrations of Ca^{2+} producing half-maximal free (Ca^{2+})_{i}-CaM concentrations. Despite the 20-fold difference in the affinities of the two indicators, the apparent K_d values determined in cells expressing them are similar, which points to a physiological value of ~1 μM for this parameter (Fig. 2C).

Based on the observation that Ca^{2+} is bound more tightly to the C-terminal EF hand pair in CaM than to the N-terminal pair, it has been proposed that CaM targets might be associated with (Ca^{2+})_{i}-CaM at resting free Ca^{2+} concentrations.
The Free Concentration of Ca$^{2+}$-calmodulin in Intact Cells

Binding of a tryptic fragment of CaM containing only the C-terminal EF hand pair produces about half the maximal indicator response in vitro seen with intact CaM (data not shown). Hence, if there were significant levels of indicator complexes involving just the C-terminal EF hand pair of CaM in resting cells, they would have been detected.

To investigate the relationship between the free concentrations of Ca$^{2+}$ and (Ca$^{2+}$)$_4$-CaM under more physiological conditions, we stably expressed FIP-CB$_{SM-38}$ or FIP-CA$_{37}$ in HEK-293 cell line expressing the receptor for thyrotropin releasing hormone (TRH), which exhibits reproducible transients in intracellular free Ca$^{2+}$ in response to this agonist (19, 23). We determined time courses for the concentrations of free Ca$^{2+}$ and free (Ca$^{2+}$)$_4$-CaM produced after addition of TRH (Fig. 3B), and combined the time-aligned transients to produce the relationship in Fig. 3A. The cooperativity coefficient and apparent $K_d$ values derived from these data are 2.7 and 0.9 M, essentially identical to the values derived from data measured in α-toxin-permeabilized cells expressing the CaM indicator. The maximum free (Ca$^{2+}$)$_4$-CaM concentration of 16 M is slightly less than the value determined in permeabilized cells. This may reflect a lack of spatial alignment in the indicator signals, because FIP-CA$_{37}$ is cytoplasmic and FIP-CB$_{SM-38}$ is freely distributed in the cell.

These studies represent the first quantitative evaluations of the relationship between the concentrations of free Ca$^{2+}$ and (Ca$^{2+}$)$_4$-CaM in cells. We find that no detectable Ca$^{2+}$-liganded CaM is produced in the cell below a free Ca$^{2+}$ concentration of 0.2 M. A maximum free (Ca$^{2+}$)$_4$-CaM concentration of ~45 M is produced at a free Ca$^{2+}$ concentration of 3 M, and a half-maximal concentration is produced when the free Ca$^{2+}$ concentration is 1 M. The total concentration of CaM-binding sites appears to exceed the total concentration of CaM by a factor of ~2, consistent with investigations of the mobility of labeled CaM that suggest its concentration in the cell is matched or exceeded by the concentration of CaM-binding sites (24). Given a total CaM concentration of ~10 M (25–27), only a minute fraction of the (Ca$^{2+}$)$_4$-CaM produced is free in the cell. This underscores the importance of directly determining the free (Ca$^{2+}$)$_4$-CaM concentration, as it clearly cannot be deduced from the total CaM concentration. The free Ca$^{2+}$ concentration producing a half-maximal free (Ca$^{2+}$)$_4$-CaM concentration in the cell is 20-fold less than the concentration required to half-saturate pure CaM in vitro (28). This demonstrates that thermodynamic coupling between Ca$^{2+}$ and target binding in CaM-target complexes is of crucial importance in the cell as it keeps free (Ca$^{2+}$)$_4$-CaM concentrations low and shifts their dependence on free Ca$^{2+}$ into the physiological concentration range (29–31). Furthermore, our results suggest that, if resting global intracellular free Ca$^{2+}$ concentrations are maintained below 0.2 M little or no global activation of CaM targets should occur.

Our observations indicate that high-affinity calmodulin targets ($K_d$ ~ 10 M) are efficiently activated throughout the cell, but efficient activation of low-affinity targets ($K_d$ ~ 100 M) occurs only where free (Ca$^{2+}$)$_4$-CaM concentrations can be locally enhanced. Interestingly, most low-affinity CaM targets that have been identified are permanently or transiently associated with the plasma membrane, including the CaM-dependent adenylate cyclases (15, 32). We hypothesize that there are at least two mechanisms that could produce a local enhancement in the free (Ca$^{2+}$)$_4$-CaM concentrations in this region: 1) diffusional recruitment of CaM because of local increases in free Ca$^{2+}$, and 2) concentration of CaM by plasma membrane-associated CaM-binding proteins like neuromodulin or neurogranin, which have been proposed to function as CaM sinks (33–35). Recent evidence suggests that store-operated Ca$^{2+}$ entry, which appears to produce local elevations in the free Ca$^{2+}$ concentration at the plasma membrane, is necessary to activate expressed CaM-dependent adenylate cyclase activities in HEK-293 cells, consistent with a requirement for diffusional recruitment of CaM (36–38). And CaM-dependent cyclase activities in neurons are associated with neurogranin and/or neuromodulin, implying that the putative CaM sink proteins may help to produce the free (Ca$^{2+}$)$_4$-CaM concentrations needed to activate cyclase activity (15, 39). Mechanisms similar to these may operate in other regions of the cell. For example, Deisseroth et al. (40) have recently reported that translocation of CaM into the nucleus correlates with Ca$^{2+}$-dependent phosphorylation of CREB in hippocampal neurons, suggesting that enhanced free (Ca$^{2+}$)$_4$-CaM concentrations may be required to activate a necessary protein kinase activity in the nucleus. Our observations suggest that spatial variations in the free (Ca$^{2+}$)$_4$-CaM concentrations that can be produced in different regions of the cell may play an important role in shaping the functional response to a Ca$^{2+}$-mediated signal.
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