Intracellular coupling via limiting calmodulin

Quang-Kim Tran, DJ Black and Anthony Persechini

From the Division of Molecular Biology & Biochemistry,
School of Biological Sciences,
University of Missouri-Kansas City,
5007 Rockhill Road, Kansas City, MO 64110-2499, USA.

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Corresponding author:
Anthony Persechini, Ph.D.,
Tel.: 1-816-235-5972; Fax: 1-816-235-5595
E-mail: persechini@umkc.edu
Summary - Measurements of cellular Ca\textsuperscript{2+}-calmodulin concentrations have suggested that competition for limiting calmodulin may couple calmodulin-dependent activities. Here we have directly tested this hypothesis. We have found that in endothelial cells the amount of calmodulin bound to nitric oxide synthase and the catalytic activity of the enzyme both are increased \(~ \times 3\) fold upon changes in the phosphorylation status of the enzyme. Quantitative immunoblotting indicates that the synthase can bind up to 25\% of the total cellular calmodulin. Consistent with this, simultaneous determinations of the free Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-calmodulin concentrations in these cells performed using indo-1 and a fluorescent calmodulin biosensor (K\textsubscript{d} = 2 nM) indicate that increased binding of calmodulin to the synthase is associated with substantial reductions in the Ca\textsuperscript{2+}-calmodulin concentrations produced, and an increase in the [Ca\textsuperscript{2+}]\textsubscript{50} for formation of the calmodulin-biosensor complex. The physiological significance of these effects is confirmed by a corresponding 40\% reduction in calmodulin-dependent plasma membrane Ca\textsuperscript{2+} pump activity. An identical reduction in pump activity is produced by expression of a high-affinity (K\textsubscript{d} = 0.3 nM) calmodulin biosensor, and treatment to increase calmodulin binding to the synthase then has no further effect. This suggests that the observed reduction in pump activity is due specifically to reduced calmodulin availability. Increases in synthase activity thus appear to be coupled to decreases in the activities of other calmodulin targets through reductions in the size of a limiting pool of available calmodulin. This exemplifies what is likely to be a ubiquitous mechanism for coupling among diverse calmodulin-dependent activities.
The Ca\(^{2+}\)-binding protein calmodulin (CaM) is involved in essentially all aspects of cellular function through its many target proteins, which include adenylyl cyclases and phosphodiesterases (1), numerous protein kinases (2), the protein phosphatase calcineurin (3), nitric oxide synthase (4), the plasma membrane Ca\(^{2+}\) pump (5,6), and several ion channels (7). Measurements of the Ca\(^{2+}\)-CaM concentrations produced in living cells (8,9) have suggested that the intracellular pool of CaM is limiting, i.e., the concentration of available CaM in the cell is less than the concentration of CaM-binding sites (9,10). This has led us to propose that competition for a limiting pool of CaM likely constitutes a pervasive mechanism for coupling among CaM-dependent activities (11). In this study we have directly tested this hypothesis and have found that in endothelial cells increases in the CaM-binding ability of nitric oxide synthase (eNOS) are correlated with significant reductions in the free Ca\(^{2+}\)-CaM concentrations produced and in CaM-dependent activity of the plasma membrane Ca\(^{2+}\) pump (PMCA).

**EXPERIMENTAL PROCEDURES**

*Cell culture and transfection* - Bovine aortic endothelial cells (BAECs) were purchased from Coriell Institute for Medical Research (Repository No. AG04762A, Camden, NJ), and cultured in Ham's F-12 medium containing 10% fetal bovine serum. Cells were used between 6-10 passages. Cells were plated onto glass coverslips and grown until sub-confluence for transfection. pcDNA3.1 vectors encoding CaM biosensors was then transfected into the cells using Targefect F-1 (Targeting System, Santee, CA).

*Western blotting and immunoprecipitation* - Sub-confluent BAECs were stimulated with 1 \(\mu\)M ionomycin in the presence of 1 mM CaCl\(_2\) with or without pretreatment with 50 \(\mu\)M FSK and 0.5mM IBMX. After 3 minutes, cells were lysed and homogenates were processed as described elsewhere (12). Anti-eNOS antibody and anti-CaM antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA); antibodies against phospho Thr-497 and phospho Ser-1179 (rabbit polyclonal IgG) were purchased from Upstate Biotech (Lake Placid, NY). Blotting was performed as per manufacturers' instructions.

*Measurement of NO production* - Sub-confluent BAECs were washed with normal HEPES-buffered saline (NHBS; composition in mM: 141 NaCl, 1 CaCl\(_2\), 1 MgSO\(_4\), 5 KCl, 10 HEPES
and 10 glucose) and incubated at room temperature for 30 minutes in the presence or absence of 50 μM forskolin and 0.5 mM IBMX with either 100 μM L-NAME or 100 μM L-arginine. This medium was then removed and cells were stimulated by the addition of 0.5 mL of a buffer containing 1 μM ionomycin, in addition to the above concentrations of L-NAME or L-arginine, and forskolin/IBMX. After 5 min, the supernatant was collected and treated to reduce nitrate to nitrite using a procedure involving catalysis with cadmium metal (Nitralyzer II, World Precision Instruments). Nitrite was then converted to NO and measured using an NO-specific electrode as described by the manufacturer (ISO-NOP MARK II, World Precision Instruments).

**Simultaneous measurement of free Ca\(^{2+}\) and Ca\(^{2+}\)-CaM concentrations** - Biosensor ECFP and EYFP fluorescence was collected using 480/40M and 535/30M emission filters, while indo-1 fluorescence was collected using 405/30M and 485/25M filters (Chroma Technology, Brattleboro, VT). Alternating 340 (indo-1) and 435 (biosensor) excitation light was provided by a DeltaRAM rapid-switching monochromator (PTI International) coupled with a custom-made 410/30M-460LP microscope polychroic (Chroma Technology, Brattleboro, VT). There is no detectable spillover between indo-1 and biosensor channels in this system. Alternating indo-1 (405/485) and biosensor (480/535) emission ratios were determined at 1-second intervals. Sub-confluent BAECs transiently expressing CaM biosensors were incubated with indo-1/AM (6 μM) and the specified pharmacological agents or equal volumes of vehicle control (DMSO) for 30 minutes. Free Ca\(^{2+}\) concentration was estimated from indo-1 emission ratios using a 460 nM K\(_d\) value determined in BAECs by comparison with the response of a CaM-based Ca\(^{2+}\) biosensor (9). Free Ca\(^{2+}\)-CaM concentrations were determined from biosensor emission ratios as described previously and are “effective” values for free (Ca\(^{2+}\))\(_4\)-CaM, as they are calculated based on biosensor K\(_d\) values for this fully liganded CaM species (9). Thermodynamic coupling effects and differences in the mechanisms controlling CaM-binding to different targets make it difficult to precisely define the CaM species in equilibrium with a CaM-biosensor complex under all conditions in the cell, hence we prefer the generic “Ca\(^{2+}\)-CaM” designation. Nevertheless, calculated effective values are useful for comparative purposes, and as a means of evaluating, based on its affinity for (Ca\(^{2+}\))\(_4\)-CaM, whether a given target is likely to compete successfully for CaM in the cell.
Competitive binding assay for eNOS and BSCaM₂ – Equimolar BSCaM₂ and eNOS in a buffer containing 25 mM Tris-HCl, 3 mM BAPTA, 0.1 M KCl, 1 mM MgCl₂, 0.1 mM CaCl₂ and 0.1 mg/ml BSA, pH 7.4 were titrated with CaM until the maximal BSCaM₂ fluorescence response was obtained. Titration data were fit to a quadratic equation for competitive binding to extract the Kₐ value for CaM binding to eNOS.

RESULTS AND DISCUSSION

For several reasons, we chose to use endothelial cells for our initial investigations of coupling among calmodulin target activities. First, endothelial nitric oxide synthase (eNOS) is a CaM-binding protein of undisputed physiological importance (13), whose catalytic activity is increased up to 20-fold by Ca²⁺-CaM (4). Second, CaM binding to eNOS can be manipulated experimentally, as it is known to be influenced by in vivo phosphorylation at one or more residues (14). Thr-497 (Thr-495 in the human sequence) in the putative CaM-binding domain appears to be a particularly important determinant of CaM-binding affinity. Dephosphorylation at this site is associated with a significantly increased CaM-binding ability of eNOS, whereas phosphorylation occurs constitutively, and is correlated with decreased binding (12,14). Conditions that cause Thr-497 dephosphorylation have generally been found to also produce phosphorylation at Ser-1179, so this site may also play a role in controlling CaM binding to the synthase (15). The focus of this study is not on eNOS phosphorylation *per se*, but is instead on how physiologically relevant changes in the phosphorylation status of eNOS affect CaM availability in the endothelial cell.

To increase CaM binding to eNOS in BAECs, we used combined treatment with forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX), which has previously been shown to mimic, albeit in a more sustained manner, agonist-evoked Thr-497 dephosphorylation and Ser-1179 phosphorylation (15). In our hands, FSK/IBMX treatment produces a ~3-fold increase in the amount of CaM bound to eNOS in cell homogenates (Fig. 1A). Western blotting indicates a similar 2.3-fold decrease in Thr-497 phosphorylation, with a concomitant 2.5-fold increase in Ser-1179 phosphorylation. To verify that FSK/IBMX treatment increases CaM-dependent eNOS
activity, nitric oxide (NO) production was measured. This activity is also increased ~3-fold, and is completely inhibited by the NOS inhibitor L-NAME (Fig. 1B).

Changes in CaM availability are seen as changes in the apparent free Ca\(^{2+}\)-CaM concentrations produced at comparable free Ca\(^{2+}\) concentrations. Therefore, we have simultaneously measured both free Ca\(^{2+}\) and free Ca\(^{2+}\)-CaM concentrations produced in BAECs in single cells using indo-1 and CaM biosensors. We have previously developed fluorescence biosensors to monitor dynamic changes in Ca\(^{2+}\)-CaM concentrations in living cells (8,9). In this study, the ability to simultaneously monitor free Ca\(^{2+}\) and free Ca\(^{2+}\)-CaM has allowed us for the first time to precisely assess their relationship under different experimental conditions. Figures 2A and 2B contain pseudo-color fluorescence ratio images of cells transiently expressing a 2 nM K\(_d\) CaM biosensor (BSCaM\(_2\)) and also loaded with indo-1. All the cells take up indo-1 (Fig. 2A), and generally ~20% express BSCaM\(_2\) (Fig. 2B). Under control conditions, ionomycin rapidly increases the apparent free Ca\(^{2+}\)-CaM concentration from below 0.1 nM to ~8 nM (Fig. 2C). Pretreatment with FSK/IBMX causes a ~3-fold reduction in the peak free Ca\(^{2+}\)-CaM concentrations produced in response to ionomycin, with a slight increase in the peak free Ca\(^{2+}\) concentrations (Fig. 2D). Hence, the observed reduction in free Ca\(^{2+}\)-CaM is due to reduced CaM availability, not a decrease in free Ca\(^{2+}\) concentration. The mean Ca\(^{2+}\) concentration producing 50% of the peak BSCaM\(_2\) fractional response ([Ca\(^{2+}\)]\(_{50}\) value) is increased from 295 ± 18 nM to 502 ± 84 nM (p < 0.05, n = 6) by FSK/IBMX treatment (Fig. 2E). Blocking eNOS activity with 100 \(\mu\)M L-NAME does not alter the effects of FSK/IBMX, indicating that increased NO production is not a contributing factor. To verify that changes in CaM availability could account for the observed changes in both maximal biosensor response and Ca\(^{2+}\) sensitivity, we have determined in vitro how formation of the CaM-BSCaM\(_2\) complex at a fixed CaM concentration is affected by increasing amounts of a CaM-binding peptide (nPEP) (16) (Fig. 2F).

At a low peptide concentration, the Ca\(^{2+}\) sensitivity of the biosensor response is reduced, followed at higher concentrations by decreases in both the Ca\(^{2+}\) sensitivity and magnitude of the response. Thus, changes in CaM availability are sufficient to explain the observed changes in both the magnitude and Ca\(^{2+}\) sensitivity of BSCaM\(_2\) response in BAECs (Fig. 2E).
A potential difficulty with the results presented so far is that the biosensor may itself reduce CaM availability enough to exaggerate the effects of FSK/IBMX treatment. To address this potential problem, and to confirm the physiological significance of the observed changes in CaM availability, we have investigated the effect of FSK/IBMX and biosensor expression on plasma membrane Ca\(^{2+}\) pump (PMCA) activity. Among the major routes for cytosolic Ca\(^{2+}\) removal, only the PMCA has been shown to depend directly on Ca\(^{2+}\)-CaM, which is bound with an apparent dissociation constant of 4-10 nM and increases PMCA activity up to 10-fold (17). To determine PMCA activity we first inhibited the SERCA pump with thapsigargin and the Na\(^+\)-Ca\(^{2+}\) exchanger by replacing Na\(^+\) in cell buffers with an equimolar amount of N-methyl-D-glucamine. L-NAME was also applied as a precaution against NO-dependent effects. Under these conditions PMCA activity is directly proportional to the Ca\(^{2+}\) extrusion rate (18,19). Since PMCA activity is itself Ca\(^{2+}\)-dependent, extrusion rates were determined for cells grouped on the free Ca\(^{2+}\) concentration (300-600 nM) at the start of each extrusion time course (Fig. 3A). Relaxation times (\(\tau\)) for Ca\(^{2+}\) extrusion from individual cells were then estimated by fitting the first 50 seconds of extrusion time courses to a mono-exponential (Fig. 3A). A similar approach has been used to assess PMCA activity in endothelial cells (18,19).

As seen in Fig. 3B and 3D, BSCaM\(_2\) expression does not alter PMCA activity, but FSK/IBMX treatment reduces it by \(\sim\) 40% in both wild-type and BSCaM\(_2\)-expressing BAECs. This effect may also partly explain the slight increase in peak free Ca\(^{2+}\) concentrations observed in cells treated with FSK/IBMX. We reasoned that if the effect of FSK/IBMX on PMCA activity is due specifically to reduced CaM availability it should be reproduced by a CaM antagonist. We therefore expressed a CaM biosensor with a 0.3 nM \(K_d\) for Ca\(^{2+}\)-CaM (BSCaM\(_{0.3}\)) in BAECs. This high-affinity biosensor reduces PMCA activity to essentially the same extent as does FSK/IBMX treatment of wild-type BAECs (Fig. 3C and 3D). Peak free Ca\(^{2+}\)-CaM concentrations are reduced to \(\sim\) 2 nM in cells expressing BSCaM\(_{0.3}\) (Fig. 4A and 4B), compared with \(\sim\) 8 nM in cells expressing BSCaM\(_2\). Most important, subsequent FSK/IBMX treatment has no further effect on PMCA activity (Fig. 3C and 3D) or CaM availability (Fig. 4). Interestingly, FSK/IBMX treatment of cells expressing BSCaM\(_2\) also reduces free Ca\(^{2+}\)-CaM to \(\sim\) 2 nM (Fig. 2C), indicating that this is below what is needed for significant PMCA activation, and most likely accounting for the lack of any additional effect of FSK/IBMX in cells expressing
BSCaM_{0.3}. Consistent with this, published values for the apparent K_{d} value for CaM binding to the pump range from 4 to 10 nM (17). Although a reduction in the amount of CaM bound to the pump due to reduced CaM availability appears to adequately explain our results, other factors, notably phosphorylation, also can significantly affect PMCA activity (20-24). However, even allowing for such effects, our observations indicate that FSK/IBMX treatment affects PMCA activity in BAECs by reducing the free Ca^{2+}-CaM concentrations that are produced. This confirms that the changes in CaM availability reported by BSCaM_{2} correspond with physiologically significant changes in the activities of CaM targets in endothelial cells.

Increased CaM binding to eNOS is correlated with significant reductions in both the Ca^{2+} sensitivity and maximum level of BSCaM_{2} response. These changes represent coupling between increases in eNOS activity and decreases in the activities of other CaM targets, such as the PMCA. In this context, it should be emphasized that the response of BSCaM_{2} itself is also representative of a CaM target insofar as the response to a particular free Ca^{2+} concentration is concerned. A simple distributive mechanism is indicated, in which additional CaM is bound to the synthase at the expense of other CaM-binding proteins in the cell. Consistent with this model, quantitative immunoblotting indicates total eNOS and CaM concentrations in BAEC homogenates of 5.6 ± 0.6 and 25.9 ± 1.5 pmol/mg total protein, respectively (data not shown). Thus eNOS can bind up to 25% of total CaM in BAECs. Using a competitive binding assay we have determined an in vitro K_{d} value of 0.2 nM for the complex between unphosphorylated eNOS and (Ca^{2+})_{4}-CaM. This value indicates a major role for eNOS in controlling CaM availability, since FSK/IBMX treatment reduces availability over a range in the free Ca^{2+}-CaM concentrations that determines binding to targets with K_{d} values > 1 nM, such as the PMCA and BSCaM_{2}, but not over the lower concentration range that determines binding to those with subnanomolar dissociation constants close to that of eNOS, such as BSCaM_{0.3}.

CaM availability is a function of the affinities, Ca^{2+} sensitivities and concentrations of all CaM-binding proteins in the cell. A high-affinity CaM-binding protein, such as BSCaM_{0.3}, sees a larger pool of available CaM than does a lower-affinity protein. A CaM-target complex with high Ca^{2+} sensitivity sees a larger pool than does one with a lower sensitivity, because the former can interact with CaM under conditions where the latter cannot. In addition, all else being equal,
an abundant CaM-binding protein will have a greater impact on CaM availability than one lower in abundance. The findings presented here demonstrate that signaling through CaM is shaped by the extensive network of cellular CaM-binding proteins, and is therefore modulated by changes in their characteristics. Even allowing for the participation of other CaM-binding proteins in the response to FSK/IBMX, our results unequivocally demonstrate that such modulation occurs, and that physiologically relevant changes in the CaM-binding characteristics of eNOS contribute to it. Phosphorylation-dependent regulation of CaM-binding is a common theme among CaM targets. It is now evident that this type of regulation can modulate CaM availability, broadly affecting the network of CaM targets and amplifying the effect of an initial regulatory phosphorylation or dephosphorylation event. From a practical standpoint, if signaling involving CaM is under study it is important to evaluate both free Ca$^{2+}$ and CaM availability, reflected in biosensor occupancy or measured free Ca$^{2+}$-CaM.
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The abbreviations used are: CaM, calmodulin; eNOS, endothelial nitric oxide synthase; PMCA, plasma membrane Ca\(^{2+}\) pump; BAECs, bovine aortic endothelial cells; NHBS, normal HEPES-buffered saline; FSK, forskolin; IBMX, 3-isobutyl-1-methyl xanthine; L-NAME, N\(\eta\)-Nitro-L-Arginine Methyl Ester; DMSO, dimethyl sulfoxide; BSCaM\(_2\) and BSCaM\(_{0.3}\), fluorescent CaM biosensors with apparent dissociation constants for CaM of 2 nM and 0.3 nM, respectively.
FIGURE LEGENDS

FIG. 1. Effects of treatment with FSK (50 μM) and IBMX (0.5 mM) on eNOS phosphorylation, CaM binding and catalytic activity. Results are expressed as the percentage of control values. Panel A, Immunoblots of anti-eNOS immunoprecipitates performed using anti-eNOS and anti-CaM antibodies, and of whole cell homogenates performed using phosphospecific antibodies as described in Methods. Columns represent densitometric values for immunoblots of control (cross-hatched) and treated (filled) samples, respectively (n = 5). Panel B, Effects of FSK/IBMX on NO production in BAECs (n = 8). Data for treated and untreated cells in the presence and absence of L-NAME (100 μM) are presented. IM, 1 μM ionomycin.

FIG. 2. Changes in CaM availability in BAECs. Pseudo-color emission ratio images of indo 1-loaded BAECs (Panel A) also expressing BSCaM2 (Panel B). Time courses of free Ca\(^{2+}\)-CaM (Panel C) and Ca\(^{2+}\) (Panel D) simultaneously determined in control (filled black circles, n = 12) and FSK/IBMX-treated cells (filled red circles, n = 6). Panel E, The relationship between the fractional saturation of BSCaM2 and free Ca\(^{2+}\) concentration in control (filled black circles) and FSK/IBMX-treated BAECs (filled red circles). Open circles and open triangles represent the mean (n = 12) of free Ca\(^{2+}\) and BSCaM2 fractional responses in control and treated cells, binned over three 200-nM intervals in free Ca\(^{2+}\) concentration. Representative data from a single experiment are presented for control and FSK/IBMX treated cells. As the free Ca\(^{2+}\) concentration remained elevated for a prolonged period after addition of ionomycin, the data presented do not cover the entire span of the BSCaM2 fluorescence response. Panel F, In vitro fractional response of BSCaM2 (1.5 μM) as a function of free Ca\(^{2+}\) in the presence of 5 μM CaM and different concentrations of nPEP. (□) 0 nPEP; (△) 3 μM nPEP; (▼) 4 μM nPEP; (▼) 6 μM nPEP; and (■) 10 μM nPEP.

FIG. 3. Effects of changes in CaM availability on PMCA activity in wild-type (WT) and BSCaM2- or BSCaM0.3-expressing BAECs. (Panel A) The protocol used to determine PMCA activity in BAECs. TGN, thapsigargin. L-NAME (100 μM) was applied throughout to prevent any effect of NO on PMCA activity. (Panels B and C) Ca\(^{2+}\) extrusion time courses in wild-type
and BSCaM₂-expressing (B) or BSCaM₀.₃-expressing (C) BAECs under control conditions and after treatment with FSK/IBMX. Time courses are the mean (n = 12) of data taken from cells in which [Ca²⁺] was between 300-600 nM at the onset of extrusion measurements. Circles and triangles represent, respectively, wild-type and biosensor-expressing BAECs in control condition (open) or under pretreatment with FSK/IBMX (filled). (Panel D) Individual time courses were fit to a mono-exponential. Histograms are the mean (n = 12) τ values determined from this analysis. Asterisks indicate statistically significant difference (p < 0.05, two-sample unpaired Student’s t-test) between control (cross-hatched) and experimental (filled) values.

FIG. 4. FSK/IBMX treatment does not affect CaM availability in BAECs expressing a 0.3 nM K₅ CaM biosensor (BSCaM₀.₃). Mean (n = 8) time courses for free Ca²⁺-CaM (Panel A) and Ca²⁺ (Panel B) concentrations in control (●) and FSK/IBMX-treated (○) BAECs expressing BSCaM₀.₃. (Panel C) Relationship between BSCaM₀.₃ fractional saturation and free Ca²⁺ under control (●) and treated (○) conditions. There is no difference in [Ca²⁺]₅₀ value between control and FSK/IBMX-treated BAECs expressing BSCaM₀.₃. Superimposed open circles and triangles are as in Fig. 2, panel E, n = 8.
Figure 3.

A

\[ \text{Free Ca}^{2+} (\text{nM}) \]

- \( \text{Ca}^{2+}\)-free
- \( 0.2 \text{ mM BAPTA} \)
- \( 1 \mu\text{M TGN} \)
- Wash
- \( 2 \text{ mM CaCl}_2 \)
- \( \text{Ca}^{2+}\)-free
- \( 5 \text{ mM BAPTA} \)
- Nominally Na\(^+\)-free

B

Free \( \text{Ca}^{2+} \) vs. time after washout (s)

C

Free \( \text{Ca}^{2+} \) vs. time after washout (s)

D

Extrusion + value (s)

- BSCaM
- BSCaM
- FSK+IBMX

|          | - | - | + | + | - | + |
|----------|---|---|---|---|---|---|
| BSCaM\(_{e,3}\) | - | - | - | - | + | + |
| BSCaM\(_{2}\)  | - | - | + | + | - | - |
| FSK+IBMX      | - | + | - | + | - | + |
Figure 4.
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