Calmodulin levels are dynamically regulated in living vascular smooth muscle cells

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Hulvershorn, Justin, Cynthia Gallant, C.-L. Albert Wang, Chantal Dessy, and Kathleen G. Morgan. Calmodulin levels are dynamically regulated in living vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 280: H1422–H1426, 2001.—The total unbound calmodulin (i.e., not bound to target proteins) level in living smooth muscle cells from the ferret portal vein was monitored with a low-affinity, calmodulin-binding peptide tagged with an environmentally sensitive fluorophore. GS17C, a previously characterized peptide, from the calmodulin-binding domain of caldesmon was tagged with iodoacetyl nitrobenz-2-oxa-1,3-diazole (NBD) or, as a negative control, with iodoacetyl fluorescein isothiocyanate. Increases in NBD-GS17C fluorescence were detected by using confocal microscopy when chemically loaded cells were stimulated with solutions of elevated [K+] or the calcium ionophore 4-bromo-A-23187 to elicit increases in intracellular Ca2+ concentration ([Ca2+]i) quantified by fura 2. Increases in peptide fluorescence were detected in response to a phorbol ester in the absence of calcium ionophore and, furthermore, that phosphorylation of protein kinase C substrates may increase the level of available calmodulin in living smooth muscle cells.

caldesmon; fluorescence; calcium

Calmodulin (CaM) is an ubiquitous Ca-binding protein that plays a key role in the regulation of the function of all cell types, including smooth muscles. It has been reported that the total CaM content of tracheal and venous smooth muscle is ~40 μM (10, 16, 22). It is also assumed, however, that only a small fraction of this amount is available to bind to targets during changes in cell intracellular Ca2+ concentration ([Ca2+]i). Widely differing values have been estimated for the level of total unbound CaM (both Ca-free and Ca-saturated forms, not bound to target protein). Tansey et al. (20) reported that half of the endogenous CaM could be extracted (and presumably was not in a bound state) from cultured tracheal smooth muscle cells that were exposed to a Triton-glycerol skinning procedure. However, in the same study, fluorescence recovery after photobleaching (FRAP) experiments indicated that only about 14% of the CaM may be available to bind target proteins. Ruegg et al. (17) estimated that 10%, or roughly 3 μM CaM, was not bound to targets in rehydrated smooth muscle fibers that had been lyophilized. Luby-Phelps et al. (9) estimated that at most 5% was not bound to targets, based not only on extraction from resting, permeabilized cultured tracheal smooth muscle cells, but also on FRAP kinetics. Romoser et al. (14), by using fluorescence resonance energy transfer (FRET) analysis of a relatively high-affinity CaM-binding domain linked to two green fluorescent protein moieties, concluded that the unbound concentration of Ca-CaM under physiological conditions in HEK293 cells was only 1/1,000 of the total CaM concentration. Extrapolating to the smooth muscle system, this would predict an unbound concentration of Ca-CaM in the smooth muscle cell of only 39 nM. Similarly, Persechini and Cronk (12) recently used altered versions of the myosin light chain kinase CaM-binding domain linked to two green fluorescent protein moieties, concluded that the unbound concentration of Ca-CaM under physiological conditions in HEK293 cells was only 1/1,000 of the total CaM concentration. Extrapolating to the smooth muscle system, this would predict an unbound concentration of Ca-CaM in the smooth muscle cell of only 39 nM. Similarly, Persechini and Cronk (12) recently used altered versions of the myosin light chain kinase CaM-binding domain linked to two green fluorescent protein moieties, concluded that the unbound concentration of Ca-CaM under physiological conditions in HEK293 cells was only 1/1,000 of the total CaM concentration. Extrapolating to the smooth muscle system, this would predict an unbound concentration of Ca-CaM in the smooth muscle cell of only 39 nM. Similarly, Persechini and Cronk (12) recently used altered versions of the myosin light chain kinase CaM-binding domain linked to two green fluorescent protein moieties, concluded that the unbound concentration of Ca-CaM under physiological conditions in HEK293 cells was only 1/1,000 of the total CaM concentration. Extrapolating to the smooth muscle system, this would predict an unbound concentration of Ca-CaM in the smooth muscle cell of only 39 nM. Similarly, Persechini and Cronk (12) recently used altered versions of the myosin light chain kinase CaM-binding domain linked to two green fluorescent protein moieties, concluded that the unbound concentration of Ca-CaM under physiological conditions in HEK293 cells was only 1/1,000 of the total CaM concentration.

The issue of the unbound Ca-CaM level in the differentiated, contractile smooth muscle cell is important,
given that several CaM-binding proteins in the smooth muscle cell that have been postulated to play important regulatory roles, such as the actin-binding protein caldesmon (CaD) and Ca-CalM-dependent protein kinase II (2), have dissociation constant (K_d) values for CaM at or above the current estimates of total unbound CaM levels. The reported K_d for whole CaD range from 4.3 × 10^{-7} to 5 × 10^{-8}, depending on the method of preparation. The reported K_d for Ca-CalM-dependent protein kinase II varies among isoforms but ranges between 20 and 100 nM at saturating calcium (2, 8, 18).

In the current study, we used as a probe of total unbound CaM levels, a relatively low-affinity, CaM-binding peptide (K_d, 8 × 10^{-7} M) (21) tagged with an nitrobenz-2-oxa-1,3-diazole-4-yl (NBD) group, in a manner that changed fluorescence intensity upon binding CaM. The peptide GS17C was derived from the gizzard sequence (Gly^{651}-Ser^{667} with an added COOH terminal cysteine for labeling) of a relevant smooth muscle protein CaD. We specifically chose a peptide with a relatively low, near-micromolar affinity for CaM for two reasons: 1) to probe the total unbound CaM concentration in these cells without significantly modifying the endogenous levels, and 2) to explore the possibility that sufficient free Ca-CalM is present in these cells to interact with relatively low-affinity CaM-binding targets such as CaD. On loading the peptide into intact, differentiated smooth muscle cells, we found that Ca-CalM is detectable with this probe. Furthermore, we found that the measured signal is regulated by activation of protein kinase C (PKC) under conditions where the free [Ca^{2+}] does not change and, hence, the amount of available CaM appears to be a dynamic variable.

**MATERIALS AND METHODS**

**Tissue preparation and loading.** Ferrets were anesthetized with chloroform in a ventilation hood, and the portal vein from each ferret was quickly removed to an oxygenated physiological saline solution. All procedures were approved by the Institutional Animal Care and Use Committee. The endothelium was removed by gentle abrasion with a rubber policeman. Longitudinal strips were prepared and attached to a force transducer as previously described (11). The muscles were placed in a series of previously published (11) EGTA, ATP-containing loading solutions at 2°C, substituting 1 μM NBD-GS17C for aequorin, and kept overnight in 50% DMEM (catalog no. 31053-028, GIBCO-BRL) and 50% FSS with 1% penicillin-streptomycin. No significant change occurred in the amplitude of the contraction to 96 mM KCl or in basal tone before and after loading. Muscles were removed from the baths and secured by the ends with Supergel (Quick Gel, Loctite, Rocky Hill, CT) to a slide for confocal measurements.

**Fura 2 calcium measurements.** Freshly isolated portal vein cells were loaded with 1 μM fura 2 acetoxymethyl ester (AM) in PSS for 30 min as previously described. Signals were collected with a previously described apparatus (4). The objective lens used was a Nikon Fluor ×40 (numerical aperture 1.4). An in vitro calibration method was used, correcting ratios for viscosity as has been previously described (13).

**Confocal microscopy.** Images were obtained using a Leica TCS 4D confocal laser scanning microscope equipped with an
Argon-Krypton laser and a Leica ×16 water-immersion, infinity-corrected objective, using a 60-μm pinhole. The microscope was controlled using SCANware v5.1a run on a MS-DOS-based microcomputer. A laser line at 488 nm was used for excitation with a 530 ± 15 nm band-pass emission filter. The average pixel intensity was determined for each scan using National Institutes of Health Image software.

**Synthesis of GS17C.** GS17C (sequence: NH$_2$-GVRNIKS-MWEKGNVFSS-C-CONH$_2$) was synthesized as previously described. Both amino acid composition and NH$_2$-terminal sequence analyses were carried out to ascertain the correctness of the peptide synthesis. The [GS17C] was determined spectrophotometrically using an extinction coefficient of 5,600 cm$^{-1}$·M$^{-1}$ at 280 nm. For labeling, the peptide was first reduced with dithiothreitol (DTT), dialyzed, and reacted with either N,N'-dimethly-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD) or 5-iodoacetamido- fluorescein (IAF) (Molecular Probes; Eugene, OR) for 5 h at room temperature; the reaction was then terminated by addition of excess DTT, followed by exhaustive dialysis. A molar extinction coefficient of 25,000 cm$^{-1}$·M$^{-1}$ at 480 nm was used for IANBD (15).

**RESULTS**

**NBD-GS17C detects changes in [CaM].** Figure 1A illustrates the in vitro fluorescence of 1.5 μM NBD-tagged GS17C peptide (NBD-GS17C) in a solution of (in mM) 25 HEPES (pH 7.5), 100 KCl, and 1 CaCl$_2$. There is a sizable increase in fluorescence on addition of saturating CaM (4 μM) to the peptide (compare trace 2 with trace 1 in Fig. 1). Fluorescence returns to baseline on addition of 5 mM EDTA (compare trace 3 with trace 2), followed by another increase in fluorescence upon the addition of 10 mM Ca$^{2+}$ (compare trace 4 with trace 3). In comparison, when GS17C is labeled with fluorescein (F-GS17C), EDTA and mM Ca-containing solutions result in a 10% or less change in fluorescence (Fig. 1B). GS17C also contains an actin-binding domain; however, the addition of actin to NBD-GS17C has little or no effect on its fluorescence (Fig. 1C).

Figure 1D shows the dependence of fluorescent intensity of NBD-GS17C on the concentration of CaM (0–20 μM). The measurements were made in the presence of 200 nM [Ca$^{2+}$], a physiological free-Ca concentration for resting smooth muscle cells, physiological ionic strength (150 mM KCl), and a physiological free [Mg$^{2+}$] (0.5 mM). The fluorescence increases in a concentration-dependent fashion that can be fit with a straight line over the expected physiological range (0–5.0 μM) (Fig. 1E).

**NBD-GS17C is uniformly loaded into intact smooth muscle tissue.** Peptides were introduced into intact smooth muscle cells by using a chemical-loading procedure originally described by Sutherland et al. (19) for the introduction of obelin into cardiac muscle and first developed for smooth muscle in this laboratory to load the photoprotein aequorin into the same cell type as that used in the present study (11). We used FITC-tagged dextrans and confocal analysis to investigate the general applicability of this method. Using dextrans of increasing size, we found that molecules up to 150 kDa could be loaded intracellularly (Fig. 2A). The mean pixel intensity (MPI) was quantitated in at least five different locations within the same focal plane in each tissue sample. For each sample the MPI was not significantly different among locations within the same optical section (not shown). The dextrans were all labeled with 0.01 moles FITC per mole glucose. Thus each molecule of 70-kDa dextran contained more FITC than each molecule of 40-kDa dextran, explaining the higher signal for 70-kDa dextran-loaded muscles in Fig. 2A. The lower signal with the 150-kDa dextran presumably reflects a lower loading efficiency. The MPI was also calculated from 10 consecutive optical sections for each sample, and no significant difference was observed in the signal coming from different optical sections of the tissue (Fig. 2B). These results confirmed...
that this method can be used to produce uniform loading of a peptide into cells within a multicellular vascular strip.

NBD-GS17C fluorescent intensity varies with cellular \([Ca^{2+}]_i\). Figure 3A (squares) shows the time course of changes in fluorescence intensity, normalized to maximal fluorescence, in response to 96 mM KCl and also in response to 50 \(\mu\)M of the Ca ionophore 4-bromoA-23187, from a single confocal section of a portal vein loaded with NBD-GS17C. Both maneuvers also increased \([Ca^{2+}]_i\), as expected. The ionophore increased \([Ca^{2+}]_i\), presumably by creating Ca pores and KCl increased \([Ca^{2+}]_i\), presumably by depolarizing the cell and opening voltage-dependent Ca channels. Note that when the preparation was washed with normal PSS after application of KCl, fluorescence stabilized at baseline values. Figure 3B displays the average quantitative values of peak changes in fluorescence intensity from four separate experiments, normalized to resting fluorescence. Statistically significant increases were seen with both KCl and ionophore (\(P < 0.005\) by unpaired \(t\)-test).

NBD-GS17C fluorescent intensity varies with PKC activation. As can be seen from Fig. 3, A and B, when 3 \(\mu\)M 12-deoxyphorbol 13-isobutyrate (DPBA), a phorbol ester and activator of PKC, was added to NBD-GS17C-loaded preparations, there was a consistent increase in fluorescence. This result was somewhat surprising, because we have previously reported (7) that DPBA causes no significant increase in \([Ca^{2+}]_i\) when added to vascular smooth muscle cells from the ferret aorta. Furthermore, DPBA contracts permeabilized ferret aorta cells clamped at constant, resting Ca \(^{2+}\) levels (3). In the present study, we confirmed the lack of a change in Ca \(^{2+}\) directly in vascular cells isolated from the ferret portal vein, by using the fluorescent Ca indicator fura 2. A typical time course of the changes in fura 2 ratio values is shown in Fig. 3A (circles). In cells from three different preparations, resting \([Ca^{2+}]_i\), was calculated to be 160 \(\pm\) 8 nM. After 2 min exposure to 96 mM KCl, \([Ca^{2+}]_i\) was 400 \(\pm\) 46 nM. After 20 min exposure to 3 \(\mu\)M DPBA, \([Ca^{2+}]_i\) was 170 \(\pm\) 17 nM, a number not significantly different from the resting value. Thus the increase in fluorescence in the presence of phorbol ester cannot be due to an increase in \([Ca^{2+}]_i\), but rather appears to be due to an increase in the available Ca-CaM concentration. Such an increase could be a direct or indirect result of phorbol ester-induced release of CaM from PKC substrates upon phosphorylation.

Fluorescent signals are specific to NBD-GS17C and are CaM dependent. Control experiments were performed with F-GS17C, for which 10% or less Ca-CaM dependent increases in fluorescence are seen in vitro (Fig. 1B). The fluorescent intensity of cells loaded with F-GS17C, in the presence or absence of KCl, DPBA, or ionophore, is expressed as a percentage of basal fluorescence and compared with that from NBD-GS17C on the same scale in Fig. 3B. Little or no change in the signal was seen in the muscles loaded with F-GS17C. Additionally, no detectable change in autofluorescence was seen upon addition of the agonists to unloaded portal vein strips (data not shown).

Control experiments were also performed with NBD-GS17C in the presence of the CaM blocker calmidazolium. The same protocol as that illustrated in Fig. 3A was performed in preparations pretreated with, and in the continued presence of, 10 and 20 \(\mu\)M calmidazolium (Fig. 3B). The results are expressed as changes in fluorescent intensity as a percentage of baseline fluo-

![Fig. 3. In vivo fluorescence. A: time course of changes in: average pixel fluorescent intensity for NBD-GS17C, normalized to the maximal ionophore signal in a typical experiment (squares); and, fura-2 ratios (circles). KCl was added at time 0, removed at 15 min; phorbol was added at 30 min, Ionophore was added at 75 min and EGTA was added at 100 min. B: values of means \(\pm\) SE of mean peak fluorescence intensities (relative to resting value) under defined conditions. NBD-GS17C values represent means of 4 experiments, 10 \(\mu\)M calmidazolium of 3 experiments, 20 \(\mu\)M calmidazolium and F-GS17C of 2 experiments.](http://ajpheart.physiology.org/Downloadedfrom/2017/06/10.1152/ajpheart.00058.2017)
rescence. As can be seen, the fluorescence under these conditions was decreased, with a greater decrease at 20 μM compared with that at 10 μM calmidazolium.

**DISCUSSION**

With the use of a relatively low-affinity, CaM-binding peptide from the CaD sequence as a probe in intact, fully differentiated smooth muscle cells, the main finding of the present study was that the fluorescent signal was detectable and was observed to vary in living smooth muscle cells upon stimulation. Because the probe comes from the CaD sequence, this strongly suggests that there is sufficient available Ca-CaM in smooth muscle cells to bind endogenous CaD. This finding has major implications for the role of CaD in smooth muscle signal transduction.

Considerable controversy has surrounded the suggestion (e.g., see review in Ref. 6) that CaD is a physiologically important modulator of smooth muscle contractility. In vitro, CaD inhibits actin-activated myosin ATPase activity. This inhibition can be reversed in vitro by either the addition of Ca-CaM or phosphorylation by PKC or calcium-calcium-dependent kinase (CaMKII). In vivo, it appears that CaD is primarily phosphorylated by mitogen-activated protein kinase (MAPK) but thus far, there has been no clear demonstration that MAPK phosphorylation of CaD can reverse its inhibition of actomyosin ATPase activity in vitro. The significance of regulation of CaD by CaM has been called into question because of the relatively low affinity for CaM. In the present study, however, we have shown that a signal caused by binding and dissociation of CaM to and from the CaM-binding domain of CaD indeed occurs in the intact cell under physiological conditions, pointing to a functionally relevant role of this interaction between CaD and CaM.

The fact that the phorbol ester DPBA increased the signal of the probe, even though Ca indicator studies confirmed the lack of a change in [Ca2+] in these cells under the same conditions, strongly suggests that the total unbound CaM levels in these cells is regulated. Others (1, 5) have reported that the CaM-binding affinity of abundant cellular proteins such as myristoylated alanine-rich C kinase substrate (MARCKS) and related proteins, can be modulated in vitro by PKC-mediated phosphorylation. We assume that a similar phosphorylation event occurs in these smooth muscle cells to result in a change in the total unbound CaM level. An alternative interpretation that cannot be ruled out is that a PKC-mediated phosphorylation of CaM might change its affinity for the peptide or target proteins. To the best of our knowledge, however, there is no precedent in the literature for such a phosphorylation of CaM by PKC.

In summary, these results strongly suggest that the level of unbound CaM in smooth muscle cells is a variable and, furthermore, that the total unbound intracellular CaM levels may be sufficient to regulate the activity of CaD.

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