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Biologically Active Fluorescent Derivatives of Spinach Calmodulin That Report Calmodulin Target Protein Binding†

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ABSTRACT: Spinach calmodulin (CaM) has been labeled at cysteine-26 with the sulfhydryl-selective probe 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS) to produce MIANS-CaM. The interaction of MIANS-CaM with CaM binding proteins was studied by fluorescence enhancement accompanying the protein–protein interactions. MIANS-CaM bound to smooth muscle myosin light-chain kinase with a K_d of 9 nM, causing a 4.6-fold fluorescence enhancement. Caldesmon bound with a K_d of 250 nM, causing a 2-fold fluorescence enhancement. Calcineurin (CaN) bound to MIANS-CaM with a K_d <5 nM, causing an 80% increase in fluorescence. On the other hand, binding of the CaM antagonist drugs prenylamine and calmidazolium or the potent peptide antagonist melittin did not alter MIANS fluorescence. MIANS-CaM activated brain cGMP phosphodiesterase and CaN as effectively as unlabeled CaM. Spinach CaM was also labeled with three other sulfhydryl reagents, 6-acryloyl-2-(dimethylamino)naphthalene, (2,5-dimethoxy-4-stilbenyl)maleimide, and rhodamine X maleimide. CaN bound to the highly fluorescent rhodamine X maleimidyl-CaM with a K_d of 1.4 nM, causing a 25% increase in polarization. Both MIANS-CaM and rhodamine X-CaM were used to monitor the Ca^{2+} dependence of the interaction between CaM and CaN. Half-maximal binding occurred at pCa 6.7–6.8 in the absence of Mg^{2+}, or at pCa 6.3 in the presence of 3 mM Mg^{2+}. In both cases, the dependence of the interaction was cooperative with respect to Ca^{2+} (Hill coefficients of 1.7–2.0). Use of these fluorescent CaMs should allow accurate monitoring of CaM interactions with its target proteins and perhaps their localization within the cell.

Calmodulin (CaM)[1] is a highly conserved protein which interacts with a large number of target proteins in a Ca^{2+}-dependent manner [for reviews, see Johnson and Mills (1986), Cox et al. (1984), and Manalan and Klee (1984)]. Vertebrate CaM contains no cysteine, no tryptophan, and two tyrosine residues (Dedman et al., 1977). In order to study the binding of CaM to target proteins having dissociation constants in the nanomolar range, it is necessary to attach highly fluorescent labels to the CaM. However, since vertebrate CaM lacks cysteine, it is difficult to achieve such labeling in a specific manner. IAEDANS has been used to label vertebrate CaM at methionines (Olwin et al., 1984), but it was found necessary to affinity purify the fluorescently labeled CaM to obtain reliable results since small amounts of unlabeled CaM markedly interfered with kinetic determinations. DANS-CaM has been used to monitor interactions with CaM's target proteins but was not sufficiently fluorescent to determine dissociation constant in the low nanomolar range and its fluorescence was affected by Ca^{2+} and CaM antagonist drugs (Kincaid et al., 1982; Malencik & Anderson, 1982; Johnson & Wittenauer, 1983). Spinach CaM has recently been sequenced and shown to contain a single cysteine residue at position 26 (Lukas et al., 1984). Since it appears to activate some target proteins in a manner identical with vertebrate CaM (Watterson et al., 1980), it represents an ideal candidate for specific fluorescent labeling with sulfhydryl-selective fluorescent probes.

In this paper, we describe the preparation and properties of 2-(4-maleimidoanilino)naphthalene-6-sulfonate (MIANS) spinach CaM and its interactions with three target proteins: smooth muscle myosin light-chain kinase, smooth muscle

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[1] Abbreviations: MOPS, 4-morpholinepropanesulfonic acid; CaM, calmodulin; MLCK, chicken gizzard smooth myosin light-chain kinase; CaD, caldesmon; PDE, phosphodiesterase; CaN, calcineurin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; App(NH)p, 5′-adenylyl imidodiphosphate; MIANS-CaM, 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid derivatized calmodulin; DANS-CaM, 5-(dimethylamino)naphthalene-1-sulfonyl chloride derivatized calmodulin; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)-aminomethane.

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caldesmon, and brain CaN. In addition, several other fluorescent spinach CaMs have been prepared and their spectral characteristics determined. One of these, the highly fluorescent rhodamine X maleimide derivative, has been used to monitor binding to CaN in the low nanamolar range using polarization.

Experimental Procedures

Materials and Methods. 2-(4-Maleimidoanilino)-naphthalene-6-sulfonic acid (MIANS), 6-acryloyl-2-(dimethylamino)naphthalene (acrylodan), (2,5-dimethoxy-4-stilbenyl)maleimide (DMSM), rhodamine X-maleimide, and 2′-(*N*-methylanthraniloyl)-cGMP were obtained from Molecular Probes (Eugene, OR). All other chemicals were reagent grade. Fractogel-DEAE was obtained from Rannin Instrument Corp., and phenyl-Sepharose CL-4B was obtained from Sigma Chemical Co. CaN and cyclic nucleotide PDE were purified by the method of Sharma et al. (1983), through the CaM–Sepharose step. Myosin light-chain kinase and caldesmon were purified as described previously (Ngai et al., 1984; Clark et al., 1984). Hill plots of the polarization data were determined by plotting log $[\Delta P/(\Delta P_\infty - \Delta P)]$ as a function of $[Ca^{2+}]$. ΔP_∞ was changed iteratively to obtain the best fit. Since there is no change in fluorescence intensity with binding, the change in polarization (ΔP) is directly proportional to binding as described by Weber (1952).

Purification of Spinach CaM and Fluorescent Labeling. Spinach CaM was purified according to method of Watterson et al. (1980), with the following modifications. DEAE purification was done on Fractogel-DEAE using 10 mM MOPS, pH 7.0, instead of Tris, pH 8.0, on DEAE-Sephadex. The eluted CaM was then purified on phenyl-Sepharose CL-4B as described by Gopalakrishna and Anderson (1982) in 10 mM MOPS, pH 7.0. The sample was concentrated on a small Fractogel-DEAE column and eluted with 0.5 M NaCl + 10 mM MOPS, pH 7.0 [all buffers contained 200 μM phenylmethanesulfonyl fluoride (PMSF)]. This step removed the EGTA as well as concentrated the sample. This sample was incubated with a 4-fold molar excess of either MIANS, acrylodan, DMSM, or rhodamine X-maleimide at room temperature for 2 h in the presence of 2 mM EGTA. To remove unreacted reagent, the labeled sample was then diluted 1:5 with 10 mM MOPS, pH 7.0, and placed on a small Fractogel-DEAE column. The column was washed briefly with 2 mL of 10 mM MOPS, pH 7.0, and the labeled sample eluted with 0.5 M NaCl, 10 mM MOPS, pH 7.0, and 200 μM PMSF.

The incorporations under these conditions were 1.0 mol/mol for MIANS (assuming E_{320} = 20 000 M·cm^{-1}), 1.0 mol/mol for acrylodan (E_{360} = 12 900 M·cm^{-1}; Prendergast et al., 1983), 0.6 mol/mol for DMSM (E_{364} = 25 000 M·cm^{-1}), and 0.8 mol/mol for rhodamine X-maleimide (E_{590} = 85 000 M·cm^{-1}).

Assay of Cyclic Nucleotide Phosphodiesterase and CaN. CaN was assayed according to the fluorescent assay of Anthony et al. (1986). PDE was assayed by using 2′-(*N*-methylanthraniloyl)-cGMP as a substrate, and its hydrolysis was followed by monitoring the fluorescence decrease observed when excited at 280 nm with emission at 450 nm (Johnson et al., 1987).

Fluorescence Titrations. Titrations of MIANS-CaM with MLCK, caldesmon, and CaN were carried out at room temperature in buffer containing 10 mM MOPS, pH 7.0, 90 mM KCl, and 1 mM $CaCl_2$. For Scatchard analysis, binding was corrected to free target protein concentrations: [free] = [total target protein] − $(\Delta F/\Delta F_{max})$[CaM], where ΔF represents the increase of [MIANS-CaM + target protein] relative to that of [MIANS-CaM] alone and ΔF_{max} the increase at infinite target protein concentration. Excitation and emission wavelength pairs of 320, 440 nm and 550, 610 nm were used for MIANS and rhodamine X derivatives, respectively. Polarization (P) was determined by using the equation $P = (V_v - L_v)/(V_v + L_v)$ where V_v and L_v are the fluorescence intensities measured with polarizers parallel and perpendicular to the vertically polarized exciting beam, respectively. Fluorescence polarizations were corrected for the unequal transmission intensities due to dispersion at the emission monochromator grating. Ca^{2+} titrations were carried out in 200 mM MOPS (pH 7.0), 90 mM KCl, and 2 mM EGTA to prevent pH changes. Free Ca^{2+} concentration was calculated by using the following logarithmic association constants for metals and H^+ to EGTA: H^+ to $EGTA^{4-}$, 9.46; H^+ to $HEGTA^{3-}$, 8.85; H^+ to H_2EGTA^{2-}, 2.68; H^+ to H_3EGTA^{1-}, 2.0; Ca^{2+} to $EGTA^{4-}$, 11.0; Ca^{2+} to $HEGTA^{3-}$, 5.32; Mg^{2+} to $EGTA^{4-}$, 5.21; and Mg^{2+} to $HEGTA^{3-}$, 3.37. Contamination $[Ca^{2+}]$ was 1 μM as determined by atomic absorption spectroscopy. Tb^{3+} titrations were carried out in the phosphorescence mode with excitation at 230 nm and emission at 543 nm, using 100 nM CaM. Delay time was 1 ms, and gate time was 1 ms. All measurements were carried out in a Perkin-Elmer LS5 spectrofluorometer, with a S-5 photomultiplier, except polarization measurements which were carried out in a Perkin-Elmer 650-10S, with an S-10 photomultiplier.

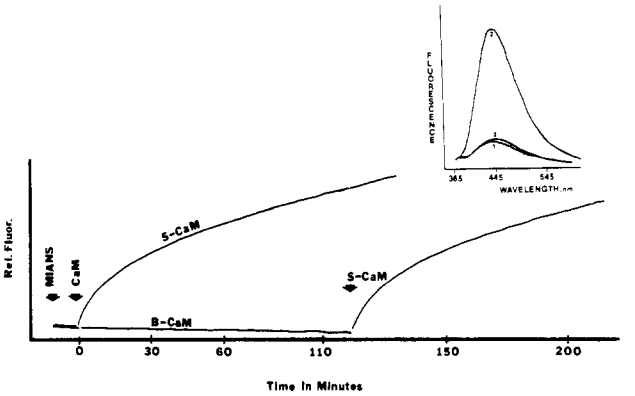

FIGURE 1: Reaction of MIANS with spinach and bovine brain CaM. Fluorescence was monitored at 440 nm with excitation at 320 nm. Spinach or brain CaMs (3 μM) were added as indicated to MIANS (10 μM) in 10 mM MOPS (pH 7.0), 90 mM KCl, and 2 mM EGTA. The inset shows the fluorescence emission spectra of (1) MIANS alone, (2) MIANS incubated 120 min with spinach CaM, and (3) MIANS incubated 120 min with bovine brain CaM. Subsequent addition of spinach CaM to the brain CaM–MIANS solution resulted in reactivity as indicated.

Results

To determine whether the reaction of MIANS was specific for cysteine-26 on spinach CaM, we compared the reactivity of MIANS toward spinach and bovine brain CaMs. The reaction of MIANS with sulfhydryl groups produces a large increase in fluorescence which is useful as an index of sulfhydryl recativity (Gupte & Lane, 1979). Figure 1 shows that spinach, but not bovine brain, CaM reacts with MIANS. Subsequent dialysis of each protein indicated that bovine brain CaM was not labeled while spinach CaM was labeled with 1:1 stoichiometry. It is unlikely that there is any reaction of MIANS with residues other than cysteine-26, since brain CaM contains no cysteine but is otherwise highly homologous with spinach CaM (Lukas et al., 1984). Amino acid analysis of MIANS-CaM revealed a complete absence of cysteine, with a small peak eluting prior to aspartic acid, near the expected position of *S*-2-succinylcysteine (Smyth et al., 1964), consistent with MIANS labeling of the single cysteine of spinach CaM. In the absence of Ca^{2+} (2 mM EGTA), the reaction is 5–

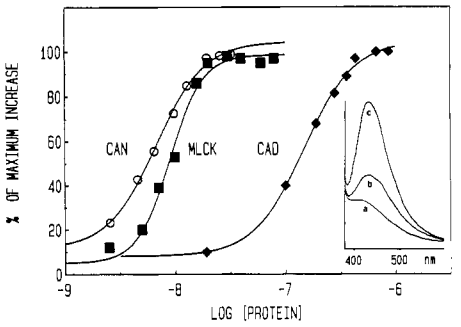

FIGURE 2: Titrations of MIANS-CaM (10 mM) with CaN (○), MLCK (■), and 50 nM MIANS-CaM with caldesmon (♦). Concentrations represent total target protein added. Buffer was 10 mM MOPS, 90 mM KCl, and 1 mM $CaCl_2$, pH 7.0. The inset shows the fluorescence emission spectra of buffer (a), 10 nm MIANS-CaM (b), and 10 nm MIANS-CaM + 40 nm MLCK (c).

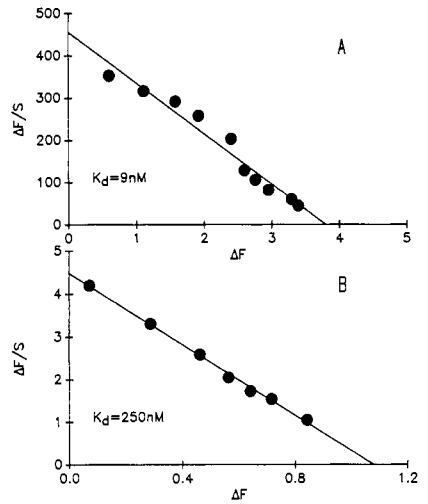

FIGURE 3: Scatchard analysis of MLCK and caldesmon binding to MIANS-CaM. Total [MIANS-CaM] was 5 nM with MLCK (A) and 50 nM with caldesmon (B). The correlation coefficients for MLCK and caldesmon were 0.979 and 0.999, respectively. ΔF represents the increase of MIANS-CaM + target protein concentration relative to that of MIANS-CaM concentration alone, i.e., $\Delta F = 1$ = 100% increase in fluorescence.

10-fold faster than in the presence of 1 mM Ca^{2+} (data not shown).

Figure 2 shows titrations of MIANS-CaM with CaN, MLCK, and caldesmon. The inset shows the fluorescence spectra of 10 nM MIANS-CaM compared to buffer alone and also its spectra in the presence of a 4-fold molar excess of MLCK. Figure 3A shows a Scatchard plot of 5 nM MIANS-spinach CaM with smooth muscle MLCK. Free MLCK was determined by assuming a 1:1 stoichiometry of CaM/kinase. MLCK produced a 4.6-fold fluorescence increase with a K_d of 9 nM. Caldesmon binding to MIANS-CaM (Figure 3B) produced a 2-fold increase in fluorescence, but its affinity was much lower (K_d = 250 nM). CaN produced an 80% fluorescence increase with a $K_d < 5$ nM. MLCK, CaN, and caldesmon produced no fluorescence change in the absence of Ca^{2+} (2 mM EGTA). Melittin, a small peptide which binds to CaM with high affinity (Cox et al., 1985), produced no change in fluorescence at concentrations up to 3 μM. In addition, the binding of the CaM antagonist drugs calmidazolium (2 μM) and prenylamine (100 μM) did not affect MIANS fluorescence. The presence of prenylamine and calmidazolium could, however, inhibit the increase in fluorescence produced by MLCK and CaN.

The most important criterion for a useful fluorescently labeled CaM is that the protein maintains its biological activity.

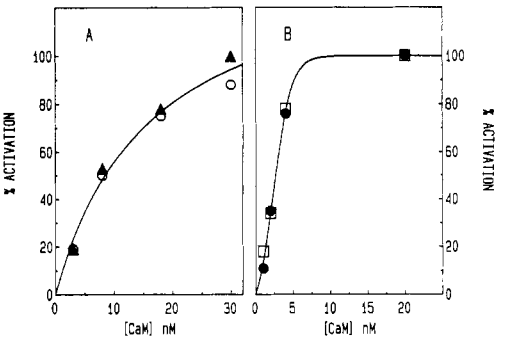

FIGURE 4: (A) Activation of CaN (18 nM) by unlabeled (▲) and MIANS-labeled (○) spinach CaM. Reaction was carried out in 50 mM Tris-HCl, pH 7.7, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 200 μM EGTA using 4-methylumbelliferyl phosphate as substrate. (B) Activation of cGMP PDE by unlabeled (□) or MIANS-labeled (●) spinach CaM. Reaction was done in 10 mM MOPS (pH 7.0), 90 mM KCl, 5 mM $MgCl_2$, and 1 mM $CaCl_2$, containing 8 μM 2′-(*N*-methylanthraniloyl)-cGMP. Both CaMs produced a 24-fold activation.

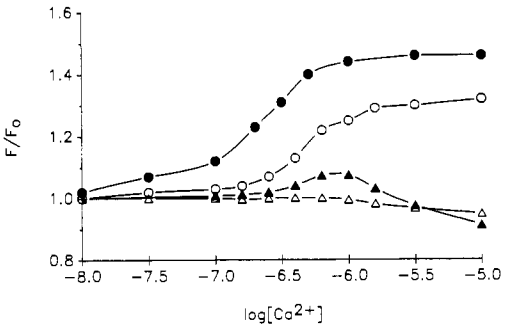

FIGURE 5: Calcium titrations of 500 nM MIANS-CaM alone (▲), or in the presence of 3 mM Mg^{2+} (△) and 20 nM MIANS-CaM + 40 nM CaN alone (●), or in the presence of 3 mM Mg^{2+} (○). Buffer was 200 mM MOPS, 2 mM EGTA, and 90 mM KCl, pH 7.0.

MIANS-labeled CaM activated CaN as effectively as unlabeled spinach CaM when 4-methylumbelliferyl phosphate (Anthony et al., 1986) was used as substrate (Figure 4A). The labeling of CaM with MIANS did not result in any decrease in its ability to activate cGMP PDE (Figure 4B). Thus, MIANS-labeled CaM was as effective as unlabeled CaM in activating both CaN and PDE. In addition, the labeling of spinach CaM did not alter its ability to bind terbium. A comparison of terbium binding to MIANS-labeled spinach CaM, unlabeled spinach CaM, and bovine brain CaM indicated no signficant difference in the binding of terbium among these species when direct binding of terbium to calmodulin is followed by excitation at 230 nm (data provided to reviewers).

MIANS-CaM undergoes small but reproducible changes upon binding Ca^{2+}. Figure 5 shows the calcium dependence of MIANS-CaM fluorescence. A 5% increase is observed which peaks at pCa 6.0, followed by a 10% decrease which is essentially complete at pCa 5.0. In the presence of 3 mM Mg^{2+}, Ca^{2+} caused essentially no change in CaM fluorescence. Ca^{2+} titrations of MIANS-CaM in the presence of CaN showed that half-maximal binding of CaM to CaN occurred at pCa 6.71 in the absence of Mg^{2+}, or at pCa 6.33 in the presence of 3 mM Mg^{2+} (Figure 5). In both cases, the Ca^{2+} dependence exhibited cooperativity [Hill coefficients of 1.7 (r = 0.995) and 1.9 (r = 0.996), respectively].

Several other sulfhydryl-selective fluorescent probes were also used to label spinach CaM. Figure 6 shows the relative fluorescence of MIANS-CaM, DMSM-CaM, acrylodan-CaM, and rhodamine X maleimide-CaM. Quantum yields of the derivatives were 0.05, 0.55, 0.16, and 0.25, respectively.

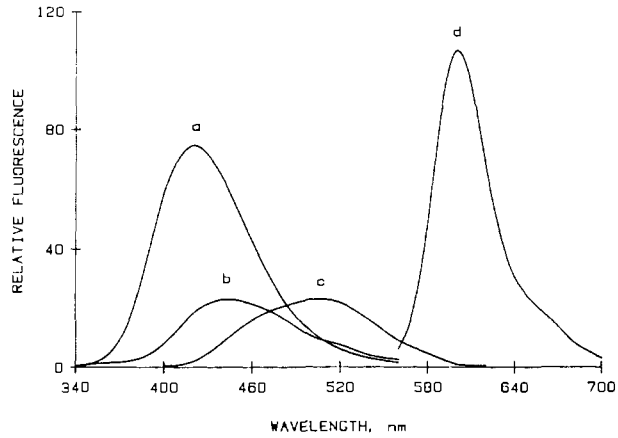

FIGURE 6: Fluorescence emission spectra of (a) DMSM, (b) MIANS, (c) acrylodan, or (d) rhodamine X maleimide labeled spinach CaM. Excitation was at 320 nm for DMSM and MIANS, 390 nm for acrylodan, and 550 nm for rhodamine X maleimide. Spectra were done with a 50 nM sample of each labeled CaM, and spectra were corrected for Raman and background fluorescence.

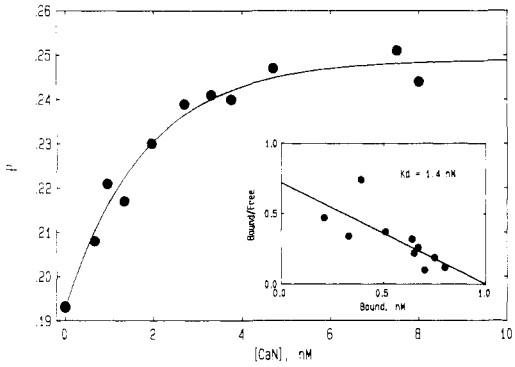

FIGURE 7: Polarization (P) changes in [rhodamine X-CaM] with CaN binding. 1 nM rhodamine X-CaM was titrated with the indicated total concentrations of CaN in 10 mM MOPS, 90 mM KCl, and 1 mM $CaCl_2$, pH 7.0. The inset shows a scatchard plot of CaN binding to CaM, with [CaN] corrected to free [CaN] using a 1:1 stoichiometry. Bound was determined from the change in polarization which is directly proportional to binding.

Matched absorbances at the excitation wavelength of 320 nm and integration of correction emission spectra were used to determine quantum yields. Quinine sulfate (Q_F = 0.70 in 0.1 N H_2SO_4) with matched absorbance at 320 nm was used as quantum standard (Secrist et al., 1972). Although all derivatives are highly fluorescent, DMSM-CaM and rhodamine X maleimide-CaM are more fluorescent than MIANS-CaM and acrylodan-CaM. Acrylodan-CaM, rhodamine X-CaM, and DMSM-CaM, like MIANS-CaM, were as effective as unlabeled CaM in stimulating cGMP PDE.

Since the binding of CaN to 10 nM MIANS-CaM was essentially stoichiometric (Figure 2), the binding of CaN to 1 nM rhodamine X-CaM was investigated. CaN titrations of 1 nM rhodamine X-CaM produced a 25% increase in rhodamine X-CaM polarization which was half-maximal near 1.5 nM (Figure 7). The insert to Figure 7 shows a Scatchard analysis of CaN binding to rhodamine X-CaM. These data gave a K_d of 1.4 nM using 1:1 stoichiometry.

Ca^{2+} binding to rhodamine X-CaM produced only small changes in its polarization, either in the presence or in the absence of Mg^{2+}. Thus, it was possible to determine the [Ca^{2+}] dependence of the interaction of rhodamine X-CaM with CaN by following the changes in rhodamine X-CaM polarization that occur with CaN binding. Ca^{2+} titrations of 5 nM rhodamine X-CaM and 10 nM CaN in the presence and absence

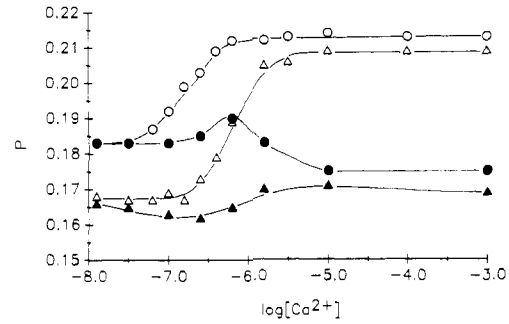

FIGURE 8: Calcium titrations of rhodamine X-CaM in the presence and absence of CaN using polarization (P). 5 nM rhodamine-CaM alone (●), or in the presence of 5 mM Mg^{2+} (▲) and 10 nM rhodamine X-CaM + 10 nM CaN alone (○), or in the presence of 5 mM Mg^{2+} (△). Buffer was 200 mM MOPS, 2 mM EGTA, and 90 mM KCl, pH 7.0. Buffer fluorescence was 3% of the total fluorescence under these conditions.

of Mg^{2+} (Figure 8) showed $K_{0.5}$ (concentration which produced half-maximal change) for Ca^{2+} to be very similar to that found with MIANS-CaM. However, the greater fluorescence of rhodamine X allowed the binding to be determined with equal precisions at lower concentrations of CaM. The Hill coefficients for the Ca^{2+} titrations of the rhodamine X-CaM–CaN complex were 1.9 (r = 0.996) and 2.0 (r = 0.999) in the absence and presence of Mg^{2+}, respectively, with 5 mM Mg^{2+} reducing the p$K_{0.5}$ for Ca^{2+} of the CaM–CaN complex from 6.77 to 6.20. At 3 mM Mg^{2+}, the p$K_{0.5}$ was 6.29 (data not shown). Thus, CaM interaction with its target proteins can be easily monitored by changes in the polarization of rhodamine X on CaM.

Discussion

Spinach CaM has been labeled with several sulfhydryl-selective probes, presumably at cysteine-26. Consistent with the label being at cysteine-26 is our observation that the highly homologous brain CaM, which has a threonine for cysteine at position 26, shows no reactivity with MIANS. Furthermore, the maleimide reagent is quite specific for the free sulfhydryl groups at neutral pH (Means & Feeney, 1971). In addition, MIANS is incorporated into spinach CaM with 1:1 stoichiometry, suggesting that maleimides can be used to label the cysteine of plant CaM in a highly specific manner. The reaction of N-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)maleimide was previously shown to block the reactivity of wheat germ CaM with 5,5′-dithiobis(2-nitrobenzoic acid) (Yoshida et al., 1983), again consistent with the sulfhydryl selectivity of maleimides. MIANS-CaM appears to retain biological activity since this modification produced no detectable change in its ability to activate either cGMP PDE or CaN. In addition, the binding of terbium to MIANS-CaM is virtually identical with unlabeled spinach or bovine brain CaM. Thus, the labeling of cysteine-26, which is in a calcium binding loop (Vanaman, 1980), with MIANS does not alter either the terbium binding or the biological activity of spinach CaM.

The interaction of MIANS-CaM with MLCK produces a 4.6-fold increase in its fluorescent yield with a K_d of 9 nM. This compared favorably with the reported K_d's of 1–4 nM determined by direct assays of enzyme activity (Nishikawa et al., 1985; Conti & Adelstein, 1982; Ngai & Walsh, 1984) and by fluorescence studies (Malencik & Anderson, 1986). Olwin et al. (1984) have shown that the presence of light chains and the nonhydrolyzable analogue of ATP, AppNHp, reduced the dissociation constant of CaM for skeletal MLCK by a factor of 5. Thus, a K_d of 9 nM compares favorably with what should be expected in the absence of substrates. MI-

ANS-CaM binds to caldesmon with an affinity that is 25 times lower than that for MLCK. At present, the physiological relevance of the interaction between caldesmon and CaM is not known, although caldesmon has been implicated in the regulation of actin–myosin interactions in smooth muscle (Ngai & Walsh, 1984). K_d determinations much below 10 nM are difficult with MIANS-CaM because of its low fluorescence relative to background at low nanomolar concentrations. The affinity of CaN for MIANS-CaM could not be accurately determined for this reason.

Labeling of spinach CaM with rhodamine X-maleimide allowed binding of CaM to CaN to be investigated in the low nanomolar range. Titrations of MIANS-CaM with melittin, prenylamine, and calmidazolium indicate that the binding of these compounds to CaM does not alter MIANS fluorescence. Thus, MIANS-CaM is clearly different from DANS-CaM, which shows similar fluorescence changes with the binding of proteins (Malencik et al., 1982; Kincaid et al., 1982), small peptides (Malencik & Anderson, 1982), and CaM antagonist drugs (Johnson & Wittenauer, 1983). DANS-CaM also undergoes large changes in fluorescence upon binding Ca^{2+} (Kincaid et al., 1982). MIANS-CaM undergoes only small changes in its fluorescence upon binding Ca^{2+}, especially in the presence of Mg^{2+}. Thus, the fluorescence of MIANS-CaM appears to be specifically enhanced by its interactions with CaM's target proteins. This is presumably because the fluorescent label on Cys-26 detects and reports a conformer of CaM which can be produced by CaM target proteins but not by CaM–drug or CaM–peptide complexes. Alternatively, the larger size of target proteins could bring them into direct contact with the fluorescent probe on Cys-26 of CaM.

Both MIANS-CaM and rhodamine X-CaM bind with high affinity to CaN, producing an increase in fluorescence in the case of MIANS-CaM or an increase in polarization in the case of rhodamine X-CaM. These changes allowed the Ca^{2+} dependence of the interaction of CaM with CaN to be monitored. In the presence of a 2-fold molar excess of CaN, half-maximal binding was observed at pCa 6.7 for MIANS-CaM and at pCa 6.8 for rhodamine X-CaM. In both cases, the changes were indicative of cooperative binding with Hill coefficients of 1.7 and 1.9, respectively. Mg^{2+} shifted the Ca^{2+} dependence to higher Ca^{2+} concentrations with a 2-fold reduction in apparent Ca^{2+} affinity at 3 mM Mg^{2+} and a 4-fold reduction at 5 mM Mg^{2+} without affecting the cooperativity in Ca^{2+} binding (Hill coefficients near 2 for both MIANS-CaM and rhodamine X-CaM). Our Hill coefficients of 1.7–1.9 for the Ca^{2+} dependence of CaM binding to CaN are lower than the Hill coefficients of Ca^{2+} activation of enzyme activity (Hill coefficients of 3.3–3.8) observed by Kincaid and Vaughn (1986) and Stewart et al. (1983). While the reason for the steeper cooperativity for activity relative to binding is uncertain, it is possible that CaM–CaN complexes exist in an inactive and not fully Ca^{2+}-saturated state. Also, complete occupation of the calcium binding sites on CaN may be necessary for activation but not for CaM binding. The greater cooperativity observed for Ca^{2+}-dependent activation of target proteins than for the Ca^{2+} dependence of calmodulin binding may also result from the presence of substrate in the activation studies. The presence of substrate is known to increase the affinity of calmodulin for its target proteins (Olwin et al., 1984), and this would be expected to enhance the cooperativity of Ca^{2+} binding (Cox et al., 1984).

In contrast to what is observed with CaN, the Ca^{2+} dependence of activation of skeletal muscle myosin light-chain kinase is only slightly more cooperative (Hill coefficient = 2.5; Crouch et al., 1981) than the calcium dependence of CaM binding (Hill coefficent = 1.8; Johnson et al., 1981). The differences observed in cooperativity of the Ca^{2+} dependence of activation of CaN and myosin light-chain kinase may reflect differences in CaM interactions with its various target proteins.

The fluorescence of the four labeled CaMs shown in Figure 6 covers the entire visible range. These fluorescent CaMs should prove particularly useful for the microscopic localization of CaM target proteins in cells. Rhodamine X-CaM exhibits high absorption at the long-wavelength lines of mercury and can be detected at subnanomolar levels, and should be ideal for use in the microscopic localization of CaM target proteins. These low concentrations of rhodamine X-CaM might not be detected on instruments with low senstivity in the red. MIANS-CaM undergoes fluorescence increases which appear to be specific for its binding to target proteins, and it should prove very useful for monitoring the stoichiometry, affinity, Ca^{2+} dependence, and the effects of CaM antagonists in altering CaM interactions with these proteins. Since MIANS-CaM retains its biological activity, we can directly relate its Ca^{2+}-dependent binding to its target proteins with their activation. For the above reasons, MIANS-CaM should also be very useful in monitoring CaM interactions with its target proteins at the single-cell level.

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Conversion of 15-Hydroxyeicosatetraenoic Acid to 11-Hydroxyhexadecatrienoic Acid by Endothelial Cells†

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ABSTRACT: Cultured endothelial cells take up 15-hydroxyeicosatetraenoic acid (15-HETE), a lipoxygenase product formed from arachidonic acid, and incorporate it into cellular phospholipids and glycerides. Uptake can occur from either the apical or basolateral surface. A substantial amount of the 15-HETE incorporated into phospholipids is present in the inositol phosphoglycerides. 15-HETE is converted into several metabolic products that accumulate in the extracellular fluid; this conversion does not require stimulation by agonists. The main product has been identified as 11-hydroxyhexadecatrienoic acid [16:3(11-OH)], a metabolite of 15-HETE that has not been described previously. Formation of 16:3(11-OH) decreases when 4-pentenoic acid is present, suggesting that it is produced by β-oxidation. The endothelial cells can take up 16:3(11-OH) only 25% as effectively as 15-HETE, and 16:3(11-OH) is almost entirely excluded from the inositol phosphoglycerides. These results suggest that the endothelial cells can incorporate 15-HETE when it is released into their environment. Through partial oxidation, the endothelium can process 15-HETE to a novel metabolite that is less effectively taken up and, in particular, is excluded from the inositol phosphoglycerides.

15(*S*)-Hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE)[1] is an arachidonic acid derivative formed by cells that contain 15-lipoxygenase activity (Ford-Hutchinson, 1985). 15-HETE inhibits several of the enzymes involved in eicosanoid synthesis, including phospholipase A_2 (Chang et al., 1985), 5-lipoxygenase (Borgeat & Samuelsson, 1979), 12-lipoxygenase (Vanderhoek et al., 1980), and cyclooxygenase (Vanderhoek et al., 1984). In this regard, 15-HETE reduces prostacyclin (PGI_2) formation when it is added to endothelial cultures (Hadjiagapiou & Spector, 1986), suggesting that localized concentrations of this lipoxygenase product might comprise the antithrombotic and vasodilator capacity of the endothelium.

Since the endothelium synthesizes small quantities of 15-HETE (Gorman et al., 1985; Mayer et al., 1986), the amount available to the cells probably is regulated in part through an autocrine mechanism. In addition, several cells that are either adjacent to, or can come in contact with, the endothelium such as smooth muscle (Larrue et al., 1983), eosinophilic leukocytes (Turk et al., 1982), and macrophages (Rabinovitch et al., 1981) release 15-HETE. A number of different cells can take up monohydroxyeicosatetraenoic acids from the extracellular fluid (Bonser et al., 1981; Pawlowski et al., 1982; Stenson et al., 1983), including endothelial cells (Kühn et al., 1985; Schafer et al., 1986; Richards et al., 1986), suggesting that 15-HETE released from adjacent cells also may influence the amount available to the endothelium. Therefore, we have examined the factors that influence the interaction between 15-HETE and endothelial cells to further evaluate the potential role of exogenously derived 15-HETE in endothelial function.

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[1] Abbreviations: 15-HETE, 15-hydroxyeicosatetraenoic acid; PGI_2, prostaglandin I_2 or prostacyclin; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; GLC, gas-liquid chromatography; ECL, equivalent chain length; HHT, 12-hydroxyheptadecatrienoic acid; NDGA, nordihydroguaiaretic acid; 8,15-diHETE, 8,15-dihydroxyeicosatetraenoic acid; 16:3(11-OH), 11-hydroxyhexadecatrienoic acid; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; MEM, minimal essential medium; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; CoA, coenzyme A.

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