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Calcium Binding to Complexes of Calmodulin and Calmodulin Binding Proteins

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Received May 21, 1985

ABSTRACT: The free energy of coupling for binding of Ca^{2+} and the calmodulin-sensitive phosphodiesterase to calmodulin was determined and compared to coupling energies for two other calmodulin binding proteins, troponin I and myosin light chain kinase. Free energies of coupling were determined by quantitating binding of Ca^{2+} to calmodulin complexed to calmodulin binding proteins with Quin 2 to monitor free Ca^{2+} concentrations. The geometric means of the dissociation constants ($\bar{K}_d$) for Ca^{2+} binding to calmodulin in the presence of equimolar rabbit skeletal muscle troponin I, rabbit skeletal muscle myosin light chain kinase, and bovine heart calmodulin sensitive phosphodiesterase were 2.1, 1.1, and 0.55 μM. The free-energy couplings for the binding of four Ca^{2+} and these proteins to calmodulin were −4.48, −6.00, and −7.64 kcal, respectively. The Ca^{2+}-independent K_d for binding of the phosphodiesterase to calmodulin was estimated at 80 mM, indicating that complexes beween calmodulin and this enzyme would not exist within the cell under low Ca^{2+} conditions. The large free-energy coupling values reflect the increase in Ca^{2+} affinity of calmodulin when it is complexed to calmodulin binding proteins and define the apparent positive cooperativity for Ca^{2+} binding expected for each system. These data suggest that in vitro differences in free-energy coupling for various calmodulin-regulated enzymes may lead to differing Ca^{2+} sensitivities of the enzymes.

Calmodulin (CaM)[1] mediates Ca^{2+} stimulation of a number of enzymes and is implicated in Ca^{2+} regulation of a variety of cellular functions [for reviews, see Cheung (1980), Rasmussen (1981), Klee & Vanaman (1982), and Manalon & Klee (1984)]. Recent work with intracellularly trapped fluorescent Ca^{2+} chelators (Tsien et al., 1982) indicates that free Ca^{2+} concentrations vary from 100–200 nM at rest to 500–1000 nM upon stimulation in rat myocytes (Williamson et al., 1983), PC-12 cells (Meldolesi et al., 1984), hepatocytes (Joseph et al., 1984), lymphocytes (Pozzan et al., 1982), and thymocytes and fibroblasts (Hesketh et al., 1985). However,

† This work was supported by National Institutes of Health Grants HL 23606 and GM 33708.

[1] Abbreviations: CaM, calmodulin; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; Quin 2, 2-[[2-[bis-(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis-(carboxymethyl)amino]quinoline; Tris, tris(hydroxymethyl)aminomethane.

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the dissociation constant for Ca^{2+} binding to CaM determined in vitro at physiological ionic strength and Mg^{2+} concentrations was 10–15 μM (Crouch & Klee, 1980; Dedman et al., 1977; Keller et al., 1982; Haiech et al., 1981) with variability dependent on Mg^{2+} concentration and ionic strength. Recently, we have reported large increases in affinity of CaM for Ca^{2+} when CaM is bound to CaM binding proteins (Keller et al., 1982; Olwin et al., 1984a). These reports have been confirmed by other laboratories with different CaM binding proteins (Burger et al., 1983; Maulet & Cox, 1984). Taken together, the data indicate that the affinity of CaM for Ca^{2+} is greatly increased in the presence of a CaM binding protein, allowing potential CaM regulation of Ca^{2+}-dependent events at free Ca^{2+} concentrations normally encountered with stimulated cells.

In this paper, we measured the average dissociation constant for Ca^{2+} binding to CaM in the presence of troponin I, myosin light chain kinase, and the CaM-sensitive phosphodiesterase by using Quin 2 to monitor Ca^{2+} binding. By comparison of the free-energy coupling values that are (Weber, 1975; Keller et al., 1983; Olwin et al., 1984b) for the binding of Ca^{2+} and these three CaM binding proteins to CaM under identical conditions, we concluded that CaM complexes with CaM binding proteins in vitro have different affinities for Ca^{2+}.

Materials and Methods

Materials

Quin 2 was purchased from Calbiochem, La Jolla, CA. All other reagents were of the highest grade available.

Methods

Calmodulin was prepared from modification of the procedures reported by Dedman et al. (1977) and Gopalakrishna & Anderson (1982) as described previously (Olwin et al., 1984a). Rabbit skeletal muscle myosin light chain kinase and phosphorylatable rabbit skeletal muscle myosin light chains were purified and assayed essentially as described by Blumenthal & Stull (1980) with modifications described by Olwin et al. (1984a).

Purification of Bovine Heart CaM-Sensitive Phosphodiesterase. The purification of bovine heart phosphodiesterase was a scaled up version of the procedure originally reported by LaPorte et al. (1979) and contained the following modifications. Four kilograms of fresh bovine heart was used for starting material. The pool of phosphodiesterase activity from the first DEAE-52-cellulose (Whatman) column (30 × 6 cm) was diluted 3-fold, made 0.1 mM in EGTA, applied to the second DEAE-52 column (30 × 3.6 cm), and developed with a linear NaCl gradient as described by LaPorte et al. (1979). Calmodulin affinity chromatography of the phosphodiesterase was on calmodulin–agarose resin prepared from Affi-Gel 15 (Bio-Rad laboratories) as described by the manufacturer with a calmodulin concentration of 0.3 mg of CaM/mL of agarose. The pool of phosphodiesterase from the second DEAE-52-cellulose column was combined with 10 mL of CaM–agarose resin, made 0.5 mM in $CaCl_2$ and 10 μg/mL in leupeptin, and rotated end over end for 1 h at 4 °C. CaM–agarose was washed successively with 3 or 4 column volumes of 10 mM MOPS, pH 7.2, 50 mM NaCl, 0.2 mM $CaCl_2$, 50 mM NaCl, and 2 mM DTT (buffer B), buffer B including 200 mM NaCl, buffer B containing 0.1% Lubrol PX, and finally by buffer B. Phosphodiesterase activity was eluted with 10 mM MOPS, pH 7.2, 1 mM $MgCl_2$, 2 mM EGTA, 50 mM NaCl, and 2 mM DTT. The protein peak was collected, concentrated on a small pad of DEAE-cellulose (0.2 mL), and eluted in the CaM column elution buffer including 150 mM NaCl in a total volume of 0.5 mL. Typical yields ranged from 0.5 to 0.9 mg of homogeneous CaM-sensitive phosphodiesterase from 4 kg of bovine heart.

Ca^{2+} Depletion. Removal of a Ca^{2+} from bovine heart CaM-sensitive phosphodiesterase and rabbit skeletal myosin light chain kinase was by parvalbumin–Sepharose chromatography as described by Keller et al. (1982). Total Ca^{2+} present in these protein samples was reduced to ≤ 0.1 μM as measured by atomic absorption spectroscopy on a Perkin-Elmer 305B spectrometer equipped with a graphite furnace.

Determination of Ca^{2+} Dissociation Constants for CaM–Protein Complexes. All buffers and protein stocks were depleted of Ca^{2+} and were used in subsequent experiments unless otherwise noted. Fluorescence emission spectra for Quin 2, as a function of increasing total Ca^{2+} concentration, were determined to obtain a $\bar{K}_d$ for Ca^{2+} binding to Quin 2 in our buffer system.

Fluorescence emission spectra for Quin 2 as a function of increasing Ca^{2+} concentrations were acquired and integrated on an SLM 4800 spectrofluorometer as described by the manufacturer. The excitation wavelength for all spectra was set at the Quin 2 maximum of 340 nm with resolution of the excitation and emission monochromators at 4 nm. The integrated spectra demonstrated a saturable increase in fluorescence intensity as a function of the total Ca^{2+} added (Figure 1) in a buffer composed of 10 mM MOPS, pH 7.2, 150 mM KCl, and 1 mM $MgCl_2$ (buffer A). All fluorescence emission spectra were integrated from 420 to 600 nm. The fluorescence intensity was saturated at 10 μM total molar Ca^{2+} and exhibited a maximum increase in fluorescence intensity of 6-fold. The values for the integrated spectral intensity were plotted as a function of the free Ca^{2+} concentration according to the method of Tsien et al. (1982). A K_d of 115 nM was assumed for these calculations (Tsien et al., 1982). The binding isotherm (Figure 1b) plotted by this method was consistent with a single class of Ca^{2+} binding sites and yielded a K_d of 117 ± 1 nM. This value is in close agreement to the 115 nM reported by Tsien et al. (1982) and demonstrates that the buffer system in our experiments does not affect the K_d of Quin 2 for Ca^{2+} and that parvalbumin–Sepharose chromatography of the buffer reduces the free Ca^{2+} concentrations to 10 nM or less. In subsequent experiments, the value of 117 nM was used for the K_d of Ca^{2+} binding to Quin 2. The high affinity of Quin 2 for Ca^{2+} was suitable for monitoring Ca^{2+} binding to CaM–enzyme complexes but would not be useful for monitoring binding of Ca^{2+} to noncomplexed CaM since the $\bar{K}_d$ for Ca^{2+} binding to CaM is 10–14 μM at similar ionic strength and Mg^{2+} concentrations (Dedman et al., 1977, Crouch & Klee, 1981; Keller et al., 1982; Haiech et al., 1982).

Binding of Ca^{2+} to CaM in the presence of a CaM binding protein was performed as described for Ca^{2+}-saturation curves with Quin 2. The free molar Ca^{2+} concentration was calculated from the increase in integrated values of the fluorescence emission spectra as described for Quin 2. The moles of Ca^{2+} bound per mole of CaM were determined from the fractional change in integrated fluorescence emission intensity with

$$\alpha = (I_S - I_B)/(I_B - I_F) \quad (1)$$

where α is the mole fraction of Ca^{2+} bound to Quin 2, I_S is the integrated fluorescence emission intensity value of the sample point at intermediate Ca^{2+} concentrations, I_B is the integrated fluorescence emission intensity value of Quin 2 saturated with Ca^{2+}, and I_F is the integrated fluorescence emission intensity value of free Quin 2 in the presence of excess EGTA. The I_F value was determined at the end of the experiment by adding EGTA to a final concentration of 1 mM

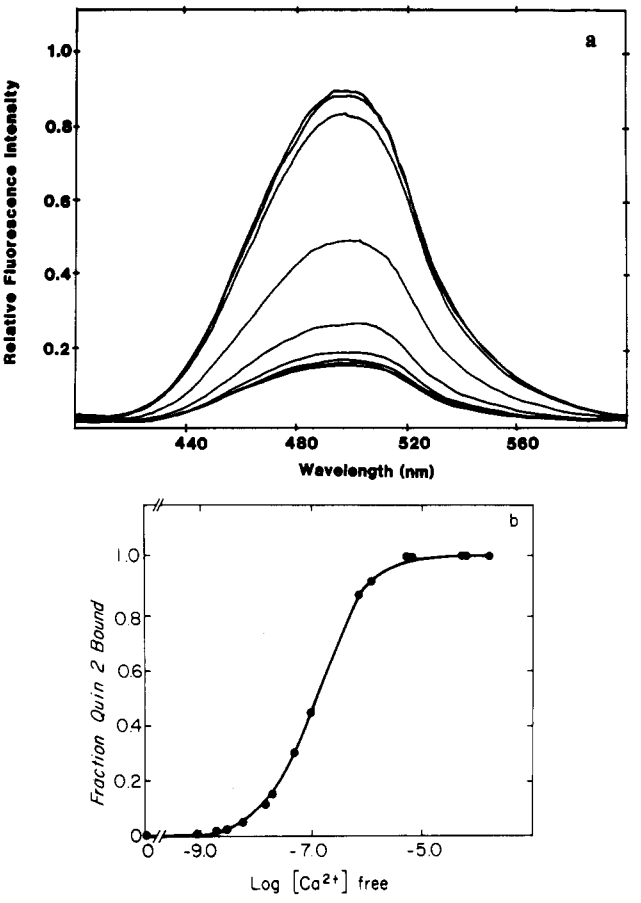


FIGURE 1: Quin 2 fluorescence emission spectra and saturation curve for increasing Ca^{2+} concentrations. The fluorescence emission spectra of Quin 2 (1.2 μmol) were plotted in (a) for increasing total molar $CaCl_2$ in 0.375 mL of buffer A including 2 mM DTT. The fluorescence emission spectra were integrated from 420 to 600 nM, and the fluorescence intensity increased a maximum of 6-fold on saturation of Quin 2 with $CaCl_2$. The total Ca^{2+} concentrations used were 10 nM, 40 nM, 140 nM, 440 nM, 1.4 μM, 4.4 μM, 14 μM, and 44 μM. The saturation curve (b) was calculated from the integrated values of the fluorescence emission spectra by assuming a K_D of Quin 2 for Ca^{2+} of 115 nM (Tsien et al., 1982). The solid line was fit to the data points by nonlinear least-squares regression analysis to the Adair equation for a single class of noninteracting binding sites (Keller et al., 1982) and yielded a K_D equal to 117 nM. The data points are the result of two independent experiments. The blank fluorescence intensity was $\leq$0.1% of the total. The final sample volume was 0.407 mL.

and unbuffered Tris until the pH was approximately 8.0. The moles of Ca^{2+} bound to Quin 2 were simply calculated by multiplying the value obtained for α by the dilution-corrected Quin 2 concentrations. The calculate moles of Ca^{2+} bound to CaM, the moles of free Ca^{2+} and the moles of Ca^{2+} bound to Quin 2 were subtracted from the total moles of Ca^{2+} added. Division of moles of Ca^{2+} bound to CaM by moles of CaM present in the experiment yielded $\bar{V}$. The values for $\bar{V}$ were then plotted vs. the free Ca^{2+} concentration, and curve was fit as previously described (Keller et al., 1982) to obtain $\bar{K}_d$, the geometric mean of the individual macroscopic K_d for the binding of Ca^{2+} to CaM–protein complexes.

Other Methods. Protein was assayed by the method of Lowry et al. (1951) with bovine serum albumin (Miles Laboratories, Pentex grade) or spectrophotometrically with $E_{1\%}$ = 1.8 at 277 nM for purified CaM (Watterson et al., 1976). Where molar concentrations are reported for CaM, myosin light chain kinase, and CaM-sensitive phosphodiesterase, molecular weight values of 16 723 (Vanaman et al., 1977), 90 000 (Blumenthal & Stull, 1980), and 57 000 per monomer of phosphodiesterase (LaPorte et al., 1979) were used, respectively.

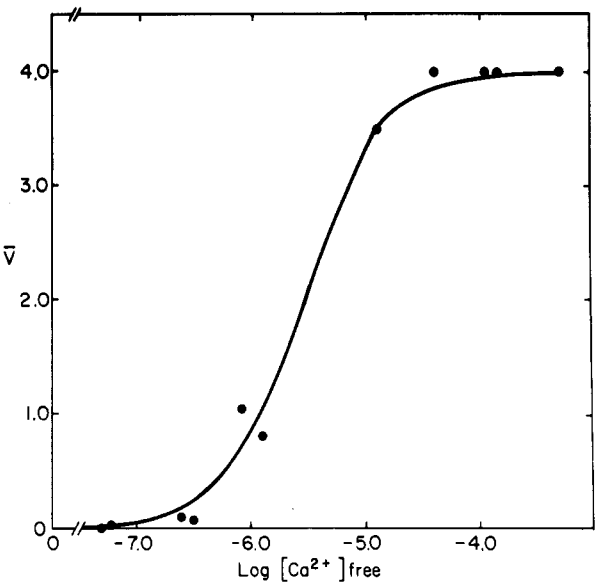


FIGURE 2: Saturation curve for Ca^{2+} binding to the CaM·TnI complex. The fluorescence emission spectra were integrated as described in Figure 1 and used to calculate the fraction of Ca^{2+} bound to the CaM·TnI complex ($\bar{V}$) and the free molar Ca^{2+} concentrations for the saturation curve plotted (see text for calculations). Titrations were with 2.5 nmol of Quin 2, 1.9 nmol of TnI, and 1.9 nmol of CaM in 0.375 mL of buffer A including 2 mM DTT. The final sample volume was 0.415 mL. The fluorescence intensity of Quin 2 in the absence of Ca^{2+} was determined at the conclusion of the titration by adding EGTA to a final concentration of 2.0 mM and raising the pH to $\geq$7.0 with unbuffered 2 M Tris-HCl. The data points represent two independent experiments, and the solid line represents the curve fit to the data points.

RESULTS

Previously Ca^{2+} binding of CaM–CaM binding protein complexes was performed by $^{45}Ca^{2+}$ equilibrium dialysis (Keller et al., 1982; Maulet & Cox, 1983; Olwin et al., 1984) or Hummel and Dryer chromatography (Burger et al., 1983) that required milligram quantities of CaM binding proteins. With the fluorescent Ca^{2+}-chelator Quin 2 as an indicator of free Ca^{2+} concentrations, only 50–100 μg of each CaM binding protein was required to determine the $\bar{K}_d$ for Ca^{2+} binding to CaM complexes of TnI, myosin light chain kinase, and bovine heart phosphodiesterase.

Ca^{2+} Binding to CaM·TnI Complexes. The binding of Ca^{2+} to the CaM·TnI complex at equimolar concentrations of CaM and TnI was determined with Quin 2 and plotted as moles of Ca^{2+} bound per mole of CaM ($\bar{V}$) vs. the free Ca^{2+} concentration (Figure 2). The solid curve is an iterative fit to the Adair equation (Keller et al., 1982) for the data points ilustrated and was best fit for a single class of four noninteracting binding sites yielding a $\bar{K}_d$ of 2.1 μM. The log interval of free Ca^{2+} concentration between 10 and 90% saturation of the protein complex by Ca^{2+} ($0.4 \leq \bar{J} \leq 3.6$) can be used as an indication of cooperativity or site heterogeneity (Weber, 1975). For a single class of noninteracting sites, the expected log interval is 1.908. The log interval for Ca^{2+} binding to the CaM·TnI complex between 10 and 90% saturation was 1.90, indicative of a single class of noninteracting sites. Approximately 30% of the total Quin 2 fluorescence intensity change was used to calculate $\bar{V}$ corresponding to 70–100% Quin 2 saturation and probably represents the upper limit of $\bar{K}_d$ determinations by this method. The $\bar{K}_d$ determined by this method is in good agreement with that determined by equi-

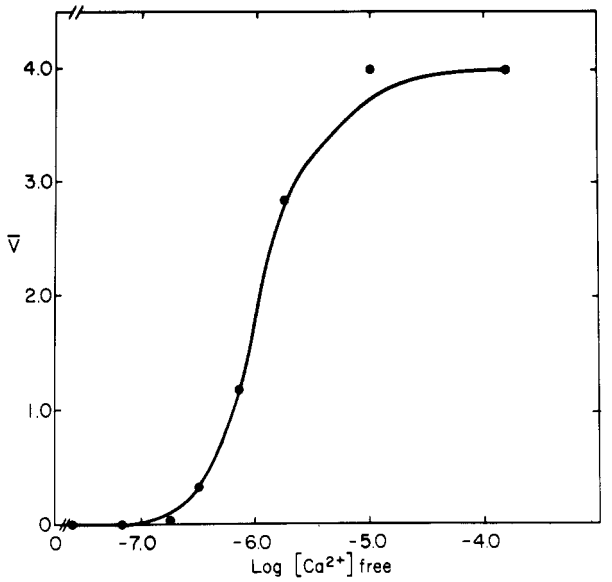


FIGURE 3: Saturation curve for Ca^{2+} binding to the CaM–myosin light chain kinase complex. The fluorescence spectra were acquired and integrated as described in the legend to Figure 2. Titrations were with 0.75 nmol of Quin 2, 1.4 nmol of CaM, and 1.4 nmol of myosin light chain kinase in 0.375 mL of buffer A including 2 mM DTT. The final sample volume was 0.421 mL. The solid line represents the curve fit of the data points.

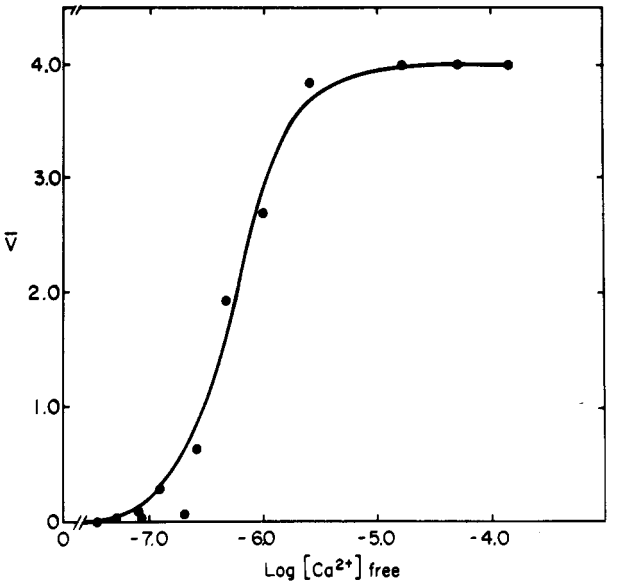


FIGURE 4: Saturation curve for Ca^{2+} binding to the CaM–PDE complex as a function of free molar Ca^{2+}. Fluorescence spectra were acquired and integrated as described in the legend to Figure 2. Titrations were performed with 0.64 nmol of Quin 2 in the presence of 1.12 nmol of CaM and 1.12 nmol of PDE in 0.375 mL of buffer A containing 2 mM DTT. The final sample volume was 0.421 mL. Data points are the results of two independent experiments. The solid line represents the curve fit to the data points.

librium dialysis (Keller et al., 1982; Olwin et al., 1984a).

Ca^{2+} Binding to CaM–Myosin Light Chain Kinase Complexes. Ca^{2+} binding to equimolar concentrations of CaM and myosin light chain kinase was quantitated by observing the increase in fluorescence emission intensity of Quin 2 as a function of the total Ca^{2+} added. A Ca^{2+} binding isotherm was generated by plotting $\bar{V}$ as a function of the free Ca^{2+} concentration and fitting the data points as described for troponin I. The curve fit yielded a $\bar{K}_d$ of 1.1 μM for binding of Ca^{2+} to equimolar concentrations of CaM and myosin light chain kinase (Figure 3). Unlike the binding of Ca^{2+} to the CaM·TnI complex, Ca^{2+} binding to CaM–myosin light chain kinase complexes displayed strong apparent positive cooperativity with a log interval of free Ca^{2+} concentrations between a $\bar{V}$ of 0.4 and $\bar{V}$ of 3.6 equal to 1.15. Observed positive cooperativity for Ca^{2+} binding was not a result of cooperative interactions between the four individual Ca^{2+} binding sites but arose from a nonrandom distribution of free CaM and CaM–myosin light chain kinase complexes as a function of the total Ca^{2+} concentration. Approximately 50% of the total Quin 2 fluorescence signal was used to generate the Ca^{2+} binding isotherm, corresponding to 50–100% saturation of Quin 2 by Ca^{2+}.

Ca^{2+} Binding to CaM–Bovine Heart Phosphodiesterase Complexes. Binding of Ca^{2+} to equimolar concentrations of CaM and bovine heart phosphodiesterase was monitored by Quin 2 fluorescence emission spectra for increasing Ca^{2+} concentrations. Moles of Ca^{2+} bound per mole of CaM ($\bar{V}$) was calculated and plotted as a function of the free Ca^{2+} concentration (Figure 4), and the curve was fit by a nonlinear least-squares regression analysis, yielding a $\bar{K}_d$ of 0.55 μM. In this experiment, 75% of the total Quin 2 fluorescent signal was used to generate a Ca^{2+} binding isotherm, corresponding to 25–100% saturation of Quin 2 by Ca^{2+}. Apparent strong positive cooperativity was demonstrated by a log interval of free Ca^{2+} concentration between 10 and 90% saturation of 1.20 (Figure 4).

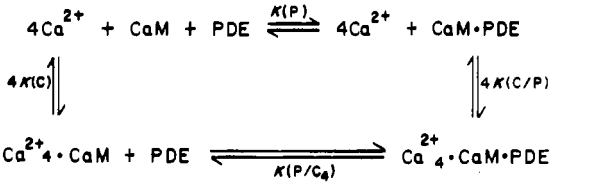
Free-Energy Coupling Calculations. The large increase in affinity of CaM for Ca^{2+} upon formation of complexes with

Table I: Dissociation Constants for Ca^{2+}–CaM–Protein Complexes

	CaM	CaM·TnI (1:1)	CaM–myosin kinase (1:1)	CaM–phospho-diesterase (1:1)
$\bar{K}_D$ (dialysis) (μM)[a]	14	1.7	0.86	nd[e]
$\bar{K}_D$ (Quin 2) (μM)[b]	nd	2.1	1.1	0.55
$4\Delta G_{cp}$ (dialysis) (kcal)[c]		−5.00	−6.60	nd
$4\Delta G_{cp}$ (Quin 2) (kcal)[d]		−4.48	−6.00	−7.64

[a] Average Ca^{2+} dissociation constants determined by microequilibrium dialysis (Keller et al., 1982; Olwin et al., 1984). [b] Ca^{2+} dissociation constants determined with Quin 2. [c] Free-energy coupling values for the interaction of 4 mol of Ca^{2+} and a calmodulin binding protein with CaM determined by equilibrium dialysis (Keller et al., 1982; Olwin et al., 1984a). [d] Free-energy coupling values for the interaction of 4 mol of Ca^{2+} and the respective CaM-binding protein with CaM determined by Quin 2. [e] Not determined.

Scheme I



CaM binding proteins (Table I) can be quantitatively expressed in terms of the free-energy coupling as outlined by Weber (1975). The free-energy coupling quantitatively describes the change in affinity of CaM for Ca^{2+} for the presence and absence of CaM binding proteins and the reciprocal change in affinity of CaM for CaM binding proteins in the presence and absence of Ca^{2+} (Keller et al., 1983; Olwin et al., 1984b). The free-energy coupling is expressed in kilocalories per mole of Ca^{2+} and is calculated from Scheme I with

$$RT \ln K(C/E) - RT \ln K(C) = \Delta G_{CE} \qquad (2)$$

where R is the gas constant in kilocalories, $K(C)$ is the K_d for binding 1 mol of Ca^{2+} to CaM, $K(C/E)$ is the K_d for binding

1 mol Ca^{2+} to the CaM–CaM binding protein complex, and ΔG_{CE} is the free-energy coupling value for the binding of 1 mol of Ca^{2+} and CaM binding protein to CaM. The free-energy coupling values vary considerably between the three CaM binding proteins, reflecting the energy that can be transmitted through CaM to a CaM binding protein from binding of Ca^{2+}.

An estimation of the Ca^{2+}-independent affinity of CaM for a CaM binding protein was calculated from Scheme I with

$$K(E) = K(E/C_4) - \exp[4\Delta G_{CE}/(RT)] \quad (3)$$

where T is the temperature in degrees Kelvin, $K(E)$ is the dissociation constant for CaM binding a CaM binding protein in the absence of Ca^{2+}, and $K(E/C_4)$ is the dissociation constant for the binding of a CaM binding protein to the CaM–Ca^{2+}_4 complex. For bovine heart phosphodiesterase, substitution of −7.64 kcal for $4\Delta G_{CE}$ and 3 nM for $K(E/C_4)$ (Olwin et al., 1983) yielded a $K(E)$ of 80 mM. A $K(E)$ of 5.7 μM was determined for Ca^{2+}-independent binding of CaM to TnI by substituting −4.84 kcal for $4\Delta G_{CE}$ and 3 nM for $K(E/C_4)$ (Olwin et al., 1983), suggesting that some CaM could be complexed to CaM binding proteins even at resting intracellular free Ca^{2+} concentrations.

Discussion

To compare the affinities of various CaM–CaM binding protein complexes for Ca^{2+}, we used Quin 2 to monitor free Ca^{2+} levels. While current techniques for determination of Ca^{2+} binding constants to CaM–protein complexes require milligram quantities of CaM binding proteins, the fluorescence technique presented here required 50–100 μg of CaM binding protein. This technique was required because of difficulties in purifying significant quantities of the CaM-sensitive phosphodiesterase. Measurement of Ca^{2+} binding using a Ca^{2+} indicator requires that the K_d of the Ca^{2+} binding is at or near the K_d of the Ca^{2+} indicator; otherwise, only a small part of the Ca^{2+} indicator signal is used to determine Ca^{2+} binding constants, resulting in large experimental errors. While Quin 2 is suitable for monitoring the K_d for Ca^{2+} binding to CaM–protein complexes, it is not possible to measure Ca^{2+} binding to isolated CaM, which has a K_d for Ca^{2+} of 10–15 μM (Dedman et al., 1977; Crouch & Klee, 1982; Keller et al., 1982; Haiech et al., 1982). Determination of the $\bar{K}_d$ for Ca^{2+} binding to various CaM–protein complexes agreed well with values determined by equilibrium dialysis for binding of Ca^{2+} to the CaM·TnI and CaM–myosin light chain kinase complexes (Keller et al., 1982; Olwin et al., 1984a), demonstrating the utility of the fluorescence technique. Comparison of the $\bar{K}_d$ for binding of 4 mol of Ca^{2+} to TnI, myosin light chain kinase, and bovine heart phosphodiesterase reveals significant differences in the affinities of these complexes for Ca^{2+}. The differences in affinity of Ca^{2+} binding to the CaM–protein complexes suggest that activation of these Ca^{2+}-dependent events occurs at different free Ca^{2+} concentrations in vitro. However, the Ca^{2+} dependency of CaM-sensitive events in vivo will be complex and reflects not only the concentration of the CaM binding protein but also the relative concentrations of other competing CaM binding proteins present, the concentrations of substrates for CaM-dependent enzymes that alter the energy coupling values (Olwin et al., 1984a), and covalent modifications of the CaM-dependent enzyme that change affinity for CaM (Conti & Adelstein, 1981).

A direct result of the increase in affinity of CaM complexes for Ca^{2+} over nonbound CaM is strong positive cooperativity observed for Ca^{2+} binding to CaM–protein complexes. However, the apparent cooperativity is dependent on the Ca^{2+} concentration, CaM binding protein concentration, and substrate interactions. The positive cooperativity will be evident when Ca^{2+} binding to a substoichiometric number of sites on CaM is sufficient to promote CaM–protein complex formation. Ca^{2+} binding to CaM in the presence of TnI did not exhibit apparent positive cooperativity because formation of Ca^{2+}-independent CaM·TnI complexes was promoted at the experimental concentrations of both proteins (5 μM). Strong apparent positive cooperativity was observed for Ca^{2+} titrations of CaM with myosin light chain kinase and bovine heart phosphodiesterase at respective concentrations of 3.7 and 3.0 μM. These observations demonstrate that the free-energy coupling value and the concentration of the CaM-binding protein can be used to determine the degree of positive cooperativity expressed by the system. The low concentrations of CaM binding proteins in tissue extracts relative to CaM (Klee & Vanaman, 1982) may promote positive cooperative Ca^{2+} activation in response to Ca^{2+} influxes in vivo.

The free-energy coupling values vary between different CaM binding proteins in vitro and may provide a mechanism for ordering activation of different CaM-dependent processes according to their Ca^{2+} sensitivity as coupling free energy is independent of ionic strength and Mg^{2+} concentrations (Keller et al., 1981; unpublished observations). Clearly, myosin light chain kinase and the heart CaM-sensitive phosphodiesterase can be fully activated at Ca^{2+} concentrations of 1 μM or less, demonstrating that CaM-mediated events can be activated during small intracellular Ca^{2+} transients. Strong apparent positively cooperative Ca^{2+}-binding and activation curves may occur in vivo, depending on the relative concentrations of CaM-binding proteins. Therefore, activation of CaM-sensitive systems may occur over a very narrow range of Ca^{2+} concentration ($\leq$1 order of magnitude) and may reflect an "all or none" response to intracellular increases in free Ca^{2+} concentrations. This observation is consistent with the general conclusion made by Rasmussen that there is a narrow range of Ca^{2+} concentration in the cell cytosol (0.2–1.2 μM) over which the ion functions as a second messenger (Rasmussen, 1981). Thus, the free-energy coupling value provides a convenient number for comparisons of the relative strength for Ca^{2+} and CaM interactions with a CaM binding protein in vitro. If the free-energy coupling values are low, complexes of CaM and CaM binding proteins may even exist at resting intracellular Ca^{2+} concentrations.

Registry No. Ca, 7440-70-2.

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Increased Agonist Affinity Is Induced in Tetranitromethane-Modified Muscarinic Receptors

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Received May 21, 1985

ABSTRACT: Tetranitromethane (TNM) modifies the muscarinic receptors from rat cerebral cortex. The modified receptor possesses an increased binding affinity (6–9-fold) toward several agonists such as acetylcholine, carbamoylcholine, arecoline, etc. The binding of antagonists (B_{max} and K_d) is only slightly altered. The effects of TNM treatment can be prevented by atropine, thus indicating that TNM modifies residue(s) at the binding site. We carried out a series of successive chemical modifications which indicated that the modified residue(s) is (are) most probably a tyrosyl and not a cysteinyl residue. This conclusion gains support from the pH profile of agonist binding, which suggests the involvement of a residue with an apparent pK comparable to that of the phenolic hydroxyl of a nitrotyrosyl residue. The binding properties of the modified receptor, when compared to those of the native one, clearly indicate that the response to TNM modification with respect to the binding of agonists such as acetylcholine and carbamoylcholine is different from that when oxotremorine and its analogue are employed. This is interpreted as being the result of different binding modes exhibited by the various agonists. Nitration of the receptors can be prevented by the presence of an antagonist but not by an agonist. We propose that this differential response is due to the formation of ligand–receptor complexes that differ with respect to the microenvironment of the modified tyrosyl residue.

Previous studies on ligand binding to muscarinic receptors have demonstrated that the binding of antagonists from the benzilate or tropate classes yields binding isotherms that fit a homogeneous population of binding sites [reviewed in Sokolovsky (1984)]. Studies on the binding of agonists, on the other hand, point to the existence of a heterogeneous population of binding sites that differ in their affinities toward agonists [for review, see Ehlert et al. (1982), Birdsall & Hulme (1983), and Sokolovsky (1984)]. It thus appears that agonist binding is the more sensitive to the state of the receptor.

0006-2960/85/0424-8086$01.50/0