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Binding of Simple Peptides, Hormones, and Neurotransmitters by Calmodulin†

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ABSTRACT: We have prepared a fluorescent conjugate of porcine calmodulin with 5-(dimethylamino)-1-naphthalenesulfonyl chloride that is highly sensitive to both calcium binding and protein binding. We have used the fluorescence of this conjugate in addition to the intrinsic peptide fluorescence to show that adrenocorticotropic hormone (ACTH), β-endorphin, glucagon, and substance P undergo calcium-dependent binding by calmodulin, with competition for common binding sites. The dissociation constants determined in the presence of 0.85 mM $CaCl_2$ and 0.2 N KCl, pH 7.3 at 25 °C, range from 1.5 μM to 3.4 μM. The α-melanocyte-stimulating hormone, bombesin, and somatostatin also bind, with dissociation constants between 60 μM and 90 μM. Angiotensins I and III, bradykinin, neurotensin, physaelemin, substance P octapeptide, insulin, and Leu- and Met-enkephalin show little or no binding. Sequence comparisons show that the peptides that bind calmodulin well contain regions structurally similar to the recognition sequence for the cAMP-dependent protein kinase and to the sequences surrounding phosphorylated serine residues in several calmodulin binding proteins. This result suggests that modification of calmodulin binding sites in calmodulin-dependent proteins is one of the functions of protein kinase. Calcium has a dual role in peptide binding by calmodulin. The occupation of calcium binding sites having a $pK \sim 4$ results in a 2-fold increase in peptide binding affinity.

The intricate workings of the nervous system and endocrine system, evolved by animals for intercellular coordination, converge on two known major intracellular messengers. Cell surface receptors for hormones such as adrenocorticotropic hormone (ACTH), glucagon, and epinephrine transfer information across the cell membrane with cAMP[1] acting as the second messenger [cf. reviews by Greengard (1978) and by Krebs & Beavo (1979)]. The nervous system stimulates membrane depolarization and the release of *calcium* recognized by calmodulin and related intracellular proteins [cf. reviews by Kretsinger (1979), Cheung (1980), and Means (1981)]. The two systems are closely interconnected, as shown by the following examples. First, the enzymes catalyzing cAMP synthesis and degradation, adenylate cyclase and cyclic nucleotide phosphodiesterase, are both activated by the calcium–calmodulin complex (Cheung, 1971, 1981; Cheung et al., 1975; Brostrom et al., 1975; Kakiuchi et al., 1970). cAMP and calcium nearly always act together in mediating cellular response. Rasmussen (1980) has applied the term synarchic in reference to this dualism. Examples of synarchic control are found in phosphorylase kinase [cf. review by Fischer et al. (1975)] and in smooth muscle myosin light chain kinase (Conti & Adelstein, 1981), strictly calmodulin-dependent enzymes with activities stimulated and inhibited, respectively, after phosphorylation by the cAMP-dependent protein kinase. Finally, the nervous and endocrine systems seem to use some of the same peptide messengers. Peripheral hormones including somatostatin, angiotensin II, insulin, glucagon, and members of the gastrin/cholecystokinin group occur in the brain, where they may function as neurotransmitters [cf. reviews by Hokfelt et al. (1980), Synder & Innis (1979), and Snyder (1980)].

Weiss et al. (1980) found that ACTH and β-endorphin inhibit the purified cyclic nucleotide phosphodiesterase and showed that the peptides compete with the enzyme for calmodulin. We have pursued this observation with fluorescence measurements on the binding of 17 different peptides by calmodulin. When possible, we have used the intrinsic peptide fluorescence to detect interaction. We have also prepared and used a fluorescent conjugate of porcine calmodulin with 5-(dimethylamino)-1-naphthalenesulfonyl chloride that is exceptionally responsive to both calcium and protein binding. Our goal of obtaining information on the protein binding specificity of calmodulin was fulfilled. The peptides that calmodulin binds well contain regions structurally homologous to the marker sequence for the cAMP-dependent protein kinase and to the sequences surrounding phosphorylated serine residues in several calmodulin binding proteins.

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[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; cAMP, adenosine 3′,5′-phosphate; CaM, calmodulin; TnI, troponin I; MLCK, smooth muscle myosin light chain kinase; α-MSH, α-melanocyte-stimulating hormone; SP, substance P; dansyl, 5-(dimethylamino)-1-naphthalenesulfonyl; ρ, rotational relaxation time; τ, lifetime of the excited state; Q, quantum yield; p_0, limiting polarization; $\bar{A}$, anisotropy; K, dissociation constant; F_∞, fluorescence of totally bound ligand; F_0, fluorescence of unbound ligand; F, observed fluorescence; ϕ, fractional degree of saturation; $NaDodSO_4$, sodium dodecyl sulfate.

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Materials and Methods

All experiments were carried out in the presence of 0.2 N KCl, 50 mM Mops, and indicated concentrations of EDTA or $CaCl_2$, pH 7.3 at 25 °C. Glass-distilled water was used throughout. Angiotensins I and III, bradykinin, bombesin, mixed bovine and porcine glucagon, human β-endorphin (Met), Leu- and Met-enkephalin, porcine ACTH (93 IU/mg), neurotensin, physaelemin, substance P, substance P octapeptide, somatostatin, and bovine insulin (24 IU/mg) were purchased from Sigma Chemical Co. Porcine α-melanocyte-stimulating hormone was a gift from Professor Aaron B. Lerner several years ago. All peptides were dissolved or suspended at 1 mg/mL in the buffer used for the experiments. The insulin and glucagon samples were titrated with 0.1 N HCl for solubilization. The glucagon sample was used immediately after opening. All concentrations are based on weight except for ACTH, which exhibits varying degrees of glycosylation. The concentrations of ACTH were determined by measurements of the absorbance at 280 nm and of the tryptophan fluorescence relative to that of free tryptophan in solutions containing 4.8 N guanidine hydrochloride. The results, based on 2 mol of tyrosine and 1 mol of tryptophan per mol of ACTH, showed that the preparation is 58–63% protein by weight. Poly(L-arginine), molecular weight range 60 000, poly(L-lysine), molecular weight range 4000, and histone-free salmine were also supplied by Sigma.

Troponin I was prepared by the method of Kerrick et al. (1980), and smooth muscle myosin light chain kinase from both chicken and turkey gizzards was prepared by the method of Adelstein & Klee (1981). Porcine brain calmodulin, prepared according to Schreiber et al. (1981), was subjected to a final purification step by using affinity chromatography on a fluphenazine–Sepharose matrix (Charbonneau & Cormier, 1979). This additional purification removes tryptophan-containing impurities. The purified calmodulin binds and activates turkey gizzard myosin light chain kinase as detailed by Malencik et al. (1982).

5-(Dimethylamino)-1-naphthalenesulfonyl chloride was synthesized according to the method given by Weber (1952). The preparation of dansylcalmodulin was similar to the procedure used by Anderson & Weber (1966) with beef heart lactate dehydrogenase. Typically, 0.125 mL of acetone containing 20 mg/mL dansyl chloride was added slowly at 0 °C to 5 mL of solution containing 10 mg/mL calmodulin, 1% $NaHCO_3$, and 2 mM $CaCl_2$. After a 2-h incubation period, unreacted dansyl chloride was removed by centrifugation, and the sample was dialyzed at 4 °C for several days against changes of 0.2 N KCl–50 mM Mops (pH 7.3 at 25 °C) containing a 1% suspension of acid/base-washed Dowex 2. $NaDodSO_4$ gel electrophoresis showed that no unbound dye remained after dialysis. Bradford determination of calmodulin concentration (Bradford, 1976) together with absorption measurements at 340 nm, where $\epsilon = 3.3 \times 10^3$ cm^{-1} M^{-1} (Hartley & Massey, 1956), showed that the dansylcalmodulin used in these experiments contains an average of 1.7 mol of dye/mol of calmodulin. Labeling in the presence of 1 mM EDTA and no added calcium produced a slightly different product, with lower quantum yield and less bound dye.

Fluorescence spectra were recorded with the Hitachi Perkin-Elmer MPF2A fluorometer and corrected for grating transmission and detector response. Quantum yields were determined by integration of the corrected spectra and comparison to a matching quinine sulfate standard, having a quantum yield of 0.70 (Secrist et al., 1972). The titrations were carried out at fixed excitation and emission wavelengths of 340 and 440 nm for dansylcalmodulin and of 295 and 340 nm for the intrinsic tryptophan fluorescence. The corresponding bandwidths were 6 and 5 nm and 3 and 5 nm. Concentrated solutions of titrant were added to the cuvette solution with a 20-μL Pipetman, keeping the dilution correction factors below 5%.

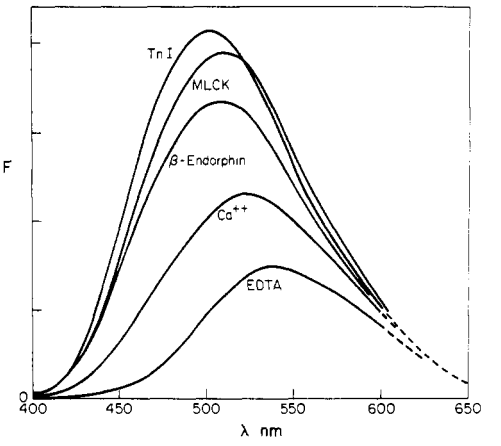

FIGURE 1: Fluorescence emission spectra of conjugate of calmodulin with 5-(dimethylamino)-1-naphthalenesulfonyl chloride. The spectra of 1.0 μM solutions of dansylcalmodulin were recorded in the presence of 1.2 mM EDTA, 0.85 mM $CaCl_2$, and 1.2 μM troponin I, 18 μM β-endorphin, or 2.0 μM myosin light-chain kinase *plus* 0.85 mM $CaCl_2$. The solutions also contained 0.2 N KCl and 50 mM Mops, pH 7.3 at 25 °C; excitation 340 nm.

Polarization and anisotropy measurements were made with the SLM 4000 fluorescence polarization spectrophotometer. The exciting wavelength was 340 nm, and the emitted light was observed through Schott KV 418 emission filters. The values of $\rho/(3\tau)$ were calculated from Perrin plots for which viscosities had been adjusted by the isothermal addition of sucrose (Weber, 1952). A circulating water bath maintained a constant temperature of 25.0 ± 0.1 °C.

Results

Binding of Hormones and Neuropeptides by Dansylcalmodulin. Dansylcalmodulin has exceptional fluorescence properties. The fluorescence spectrum and quantum yield are responsive to both calcium and protein binding, as shown in Figure 1. The addition of known calmodulin binding proteins, such as troponin I or smooth muscle myosin light chain kinase, produces distinctive changes in the fluorescence of the conjugate. The quantum yields of dansylcalmodulin are high—being 0.25 in the presence of calcium alone and 0.4–0.45 in the protein complexes. The ratio of the rotational relaxation time to the lifetime of the excited state (ρ/τ) determined from Perrin plots is 1.68 for dansylcalmodulin and 3.36 for the calmodulin–troponin I complex, an ideal range for fluorescence polarization or anisotropy measurements. The increase in ρ/τ obtained with troponin I is close to that expected from the molecular weight change. The limiting polarizations are moderately high, indicating relative rigidity of the dansyl moiety. Table I summarizes the fluorescence properties described here. We have reported stopped-flow studies of calcium binding by dansylcalmodulin (Malencik et al., 1981).

Our survey of 16 hormones and neuropeptides shows that 4—ACTH, β-endorphin, glucagon, and substance P—bind dansylcalmodulin well, producing fluorescence changes similar to those found for troponin I or myosin light chain kinase. Figure 2 contains representative titrations, showing the progressive increase in the fluorescence of dansylcalmodulin occurring on the addition of β-endorphin. The fluorescence was followed at 440 nm in order to take advantage of the wave-

Table I: Fluorescence Properties of Dansylcalmodulin[a]

additions	λ_{max} (nm)	Q	$\rho/(3\tau)$[b]	P_0[b]
1.2 mM EDTA	539	0.14	0.66	0.298
0.85 mM Ca^{2+}	522	0.25	0.56	0.316
1.1 mol of TnI + 0.85 mM Ca^{2+}	502	0.45	1.12	0.381
2.0 mol of MLCK + 0.85 mM Ca^{2+}	510	0.41		
9.0 mol of ACTH + 0.85 mM Ca^{2+}	501	0.34		
18 mol of β-endorphin + 0.85 mM Ca^{2+}	505	0.35		
20 mol of substance P + 0.85 mM Ca^{2+}	501	0.42		
poly(L-Arg)[c] + 0.85 mM Ca^{2+}	497	0.35		
poly(L-Arg)[c] + 1.2 mM EDTA	532	0.27		

[a] All values were determined in 0.2 N KCl–50 mM Mops, pH 7.3 at 25 °C. The dansylcalmodulin concentration was 1.0 μM. Excitation: 340 nm. [b] Measured with a Schott KV 418 emission filter. [c] At 0.36 mg/mg of calmodulin.

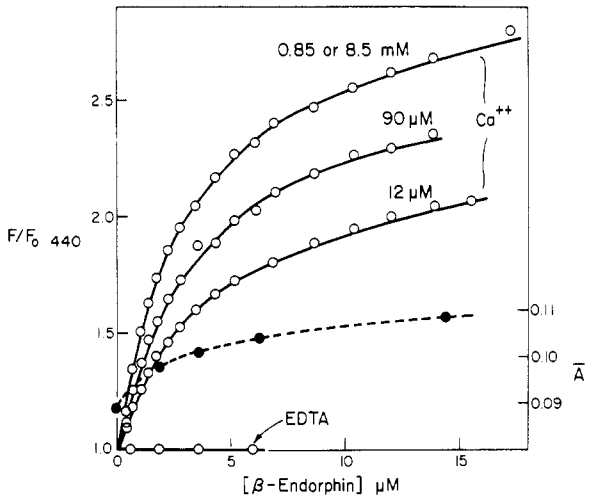

FIGURE 2: Fluorescence titrations of dansylcalmodulin with β-endorphin obtained at various concentrations of $CaCl_2$: 0 (with 1.2 mM EDTA), 12 μM, 0.09 mM, 0.85 mM, and 8.5 mM. The relative fluorescence (F/F_0) was measured at 440 nm, with 340-nm excitation. The continuous smooth curves were calculated for 1:1 binding by using the dissociation constants and enhancement factors given in Table I. (The dashed line shows the increase in anisotropy obtained in the presence of 0.85 mM $CaCl_2$, with a Schott KV 418 emission filter.) The solutions contained 1.0 μM dansylcalmodulin, 0.2 N KCl, and 50 mM Mops, pH 7.3 at 25 °C.

length change that accompanies binding.[2] The values of F_∞, the fluorescence of totally bound dansylcalmodulin, were determined through extrapolation of the changes in fluorescence to infinite β-endorphin concentration. The data were then fitted to the simple equilibrium

$$\text{CaM·}\beta\text{-endorphin} \underset{K}{\rightleftharpoons} \text{CaM} + \beta\text{-endorphin}$$

for which the average degree of saturation of dansylcalmodulin with β-endorphin (ϕ) is related to the fluorescence enhancement.

$$\phi = (F/F_0 - 1)/(F_\infty/F_0 - 1)$$

Double-reciprocal plots of the changes in fluorescence, $(F/F_0 - 1)^{-1}$, vs. the calculated concentrations of free β-endorphin, $([\beta\text{-endorphin}]_{total} - \phi[\text{CaM}]_{total})^{-1}$, are linear—allowing calculation of the dissociation constants and verification of the values of F_∞/F_0. The smooth curves in Figure 2 were calculated for the binding of 1 mol of β-endorphin by using the values of F_∞/F_0 and K obtained in this manner.

[2] This wavelength gives 32–46% of the maximum *observable* signal.

Table II: Peptide Binding by Dansylcalmodulin: Summary of Dissociation Constants and Fluorescence Enhancement Factors[a]

peptide	[CaM] (μM)	[Ca^{2+}] (mM)	K[b] (μM)	F_∞/F_0[b,c]
ACTH	1.0	0.85	1.5	2.66
ACTH	1.0	0.089	2.1	2.35
ACTH	10.0	0.85	1.7	2.54
β-endorphin	1.0	0.85	2.0	2.80
β-endorphin	1.0	0.089	2.7	2.59
β-endorphin	1.0	0.012	3.2	2.30
β-endorphin	0.2	0.85	1.9	2.01[d]
substance P	10.0	0.85		3.38
substance P	1.0	0.85	1.9	3.60
substance P	0.5	0.85	1.7	4.03
substance P	0.2	0.85	2.2	2.18[d]
substance P	0.1	0.85	2.0	2.31[d]
glucagon	1.0	0.85	3.4	3.3
α-MSH	10.0	0.85	59	1.90
somatostatin	1.0	0.85	65	2.33
bombesin	1.0	0.85	91	1.98

[a] Conditions: 0.2 N KCl–50 mM Mops, pH 7.3 (25 °C). [b] The reproducibility of these values was ±4–5% for the first four peptides. [c] F_∞/F_0 was measured at 440 nm. [d] Measured at 480 nm.

Table II shows that K and F_∞/F_0 both vary moderately with the calcium concentrations studied. These variations act in concert to produce the substantial differences among the titrations. The following analysis was carried out to estimate the linkage between β-endorphin binding and calcium binding to sites having dissociation constants *within the calcium concentration range studied.*[3]

$$\text{CaM·Ca}^{2+} \underset{K_1}{\rightleftharpoons} \textit{CaM} + \text{Ca}^{2+}$$

$$\text{CaM·Ca}^{2+}\text{·}\beta\text{-endorphin} \underset{K_3}{\rightleftharpoons} \textit{CaM·}\beta\text{-endorphin} + \text{Ca}^{2+}$$

$$\textit{CaM·}\beta\text{-endorphin} \underset{K_2}{\rightleftharpoons} \textit{CaM} + \beta\text{-endorphin}$$

$$\text{CaM·Ca}^{2+}\text{·}\beta\text{-endorphin} \underset{K_4}{\rightleftharpoons} \text{CaM·Ca}^{2+} + \beta\text{-endorphin}$$

The tabulated dissociation constants (K) for β-endorphin are related to the above microscopic equilibria and to the calcium concentration.

$$K = \left[\left(\frac{[\text{Ca}^{2+}]}{K_1} + 1\right)\Big/\left(\frac{[\text{Ca}^{2+}]}{K_3} + 1\right)\right]K_2$$

Application of this relationship to the values of K found at 8.5, 0.85, and 0.09 mM $CaCl_2$ gives $K_1 \sim 0.18$ mM, $K_3 \sim 0.09$ mM, $K_2 \sim 3.8$ μM, and $K_4 \sim 1.9$ μM. In other words, there are weak interactions (ΔG = ca. −0.4 kcal/mol) involving calcium binding sites having pKs near 4. When these sites are saturated, the dissociation constant for the calmodulin–β-endorphin complex is 1.9 μM. At nonsaturating calcium concentrations, the dissociation constant seems to approach 3.8 μM.

Calcium has a dual role in the binding of the peptides, however. No detectable interaction between β-endorphin and dansylcalmodulin occurs in solutions containing 1.2 mM EDTA and no added calcium (Figure 2), showing that β-endorphin binding to calmodulin is absolutely calcium dependent—as is the binding of most other proteins. The absolute calcium dependence involves the higher affinity calcium sites described under Discussion.

[3] The component denoted by italics may still contain calcium bound to higher affinity sites.

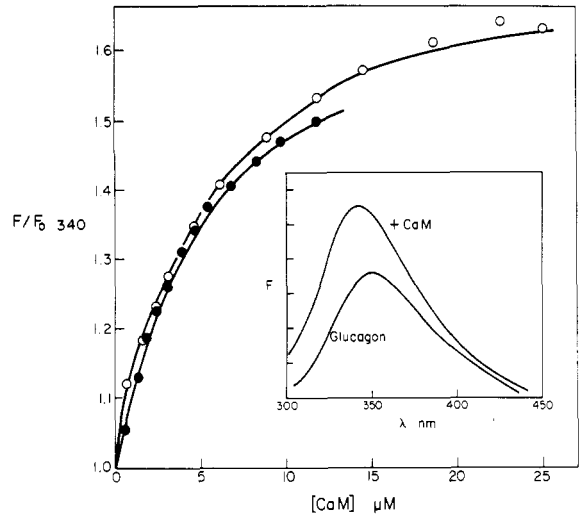

FIGURE 3: Fluorescence titrations of ACTH and glucagon with unlabeled calmodulin. The wavelengths of excitation and emission were 295 and 340 nm, respectively. The corresponding bandwidths were 3 and 5 nm. Glucagon, 2.5 μM (○); ACTH, 2.9 μM (●). The solutions contained 0.85 mM $CaCl_2$ plus constituents listed under Figure 1. The inset shows the change in the fluorescence spectrum of glucagon obtained on the addition of 22 μM unlabeled calmodulin.

Titrations of dansylcalmodulin with ACTH, substance P, and glucagon give dissociation constants and enhancement factors similar to those found for β-endorphin (Table II). The binding of these peptides also shows absolute dependence on calcium, with no interaction occurring in the presence of EDTA. The weak linkage to calcium binding sites having p*K*s near 4 was confirmed in the ACTH binding experiments. The enhancement factor obtained on the binding of substance P declines at higher concentrations of dansylcalmodulin. This may reflect binding of additional molecules of substance P accompanied by a decline in the fluorescence yield. The polarization changes occurring on the binding of substance P by dansylcalmodulin are small, however, suggesting that the number of any additional molecules bound is also small—probably no more than one. Nonetheless, double-reciprocal plots obtained with calmodulin concentrations between 0.1 and 1.0 μM are linear and give the same value of *K*. The binding of the α-melanocyte-stimulating hormone, bombesin, and somatostatin by calmodulin corresponds to dissociation constants of 60–90 μM.

Binding Studies Using the Intrinsic Fluorescence of ACTH and Glucagon. Measurements of the intrinsic peptide fluorescence confirm the results obtained with dansylcalmodulin and facilitate some interesting competition experiments. The following tabulation shows that, of the proteins concerned, only ACTH and glucagon contain tryptophan.

	CaM[a]	ACTH[b]	glucagon[b]	β-endorphin[b]	SP[b]
Tyr	2	2	2	1	0
Trp	0	1	1	0	0

[a] Cheung, 1980. [b] Bloom, 1981.

Since the fluorescence of tyrosine is largely excluded from the intrinsic protein fluorescence on excitation at 295 nm (Teale, 1960; Weber, 1961), ACTH and glucagon can be studied under conditions where unlabeled calmodulin, β-endorphin, and substance P do not fluoresce.

The fluorescence emission spectra and quantum yields of both ACTH and glucagon change when calmodulin binds (inset to Figure 3), showing increased shielding of the tryptophan residues from the polar solvent molecules. Titrations of ACTH and glucagon with calmodulin (Figure 3), following

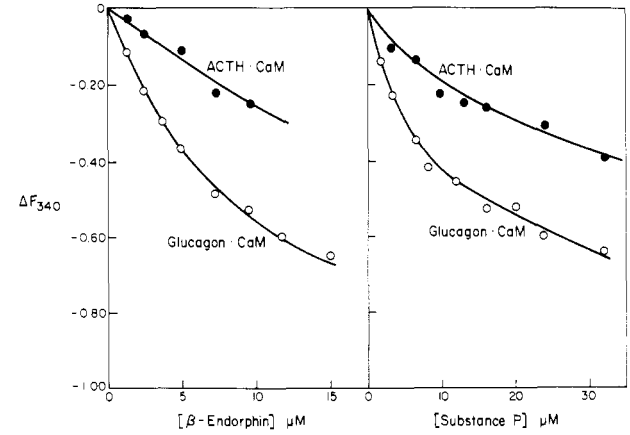

FIGURE 4: Dissociation of glucagon–calmodulin and ACTH–calmodulin complexes by β-endorphin and substance P. Δ*F* is the relative change in the fluorescence intensity measured at 340 nm. The solutions contained 1.6 μM ACTH and 8.0 μM unlabeled calmodulin or 2.5 μM glucagon and 8.0 μM calmodulin. Other conditions are described under Figure 2.

the fluorescence changes at 340 nm,[4] yield dissociation constants of 2.5 μM and 3.0 μM, respectively. These values are similar to the ones obtained with dansylcalmodulin. The errors are inevitably larger with the intrinsic fluorescence because of the smaller enhancement factors. Similar titrations of ACTH with troponin C, which is ancestrally related to calmodulin (Barker et al., 1977), showed no interaction at concentrations up to 50 μM.

The various peptides may occupy different subsites on the calmodulin molecules since calmodulin binding proteins are generally 10–100 times larger than the peptides. To detect competition for common binding sites, we titrated the calmodulin complexes of ACTH and glucagon with substance P and β-endorphin. The resulting changes in tryptophan fluorescence, plotted as fractions of the difference between the initial intensity and the intensity of ACTH or glucagon alone, show that displacement occurs in all four cases (Figure 4). As predicted from the dissociation constants in Table II, glucagon is displaced more easily than ACTH. Although substance P and β-endorphin bind equally well to dansylcalmodulin, β-endorphin is more effective in competition. This may relate to our observation on dansylcalmodulin suggesting weak binding of substance P to additional sites. The binding of substance P, lacking both tyrosine and tryptophan, has no effect on the tyrosine fluorescence of calmodulin.

Binding of Poly(L-*arginine*), *Poly*(L-*lysine*), *and Salmine by Dansylcalmodulin.* The following experiments were prompted by the basic nature of the peptides that calmodulin binds well. Figure 5 shows that the addition of poly(L-arginine) to dansylcalmodulin causes a large fluorescence enhancement similar to that obtained with the peptides, troponin I, and myosin light chain kinase. Unlike the previous ligands, the binding of poly(L-arginine) is not absolutely calcium dependent. The fluorescence enhancement obtained in the presence of EDTA confirms the responsiveness of dansylcalmodulin to protein binding in the absence of calcium. The fluorescence enhancements reach a maximum on the addition of 0.36 mg of poly(L-arginine)/mg of calmodulin. Higher ratios result in precipitation. Salmine, high in arginine, also binds dansylcalmodulin well and is not absolutely calcium dependent. Titrations similar to those in Figure 4 show that

[4] This wavelength choice avoids the corrections for Raman scattering needed at shorter wavelengths and further reduces traces of tyrosine fluorescence.

Table III: Structural Homologies in Calmodulin Binding Proteins

substance P				Arg	Pro	Lys	Pro	Gln	Gln	Phe Phe	Gly Leu Met
glucagon (16-26)				Ser	Arg	Arg	Ala	Gln	Asp	Phe Val	Gln Trp Leu-
ACTH (16-26)				Lys	Arg	Arg	Pro	Val	Lys	Val Tyr	Pro Asn Gly-
β-endorphin (29-19)				Lys	Lys	His	Ala	Asn	Lys	Ile Ile	Ala Asn Lys-
synthetic substrate of cAMP-dependent protein kinase (9-18)				Lys	Arg	Lys	Glu	Ile	Ser[a]	Val Ala	Gly Leu
phosphorylase kinase (β subunit)				Thr	Lys	Arg	Ser	Gly	Ser[a]	Val Tyr	Glu Pro- Leu
histone H2B (33-42)				Arg	Lys	Glu	Ser	Tyr	Ser[a]	Val Tyr	Val Tyr-
skeletal muscle TnI				Arg	Gln	His	Leu	Lys	Ser[a]	Val Met	Glu Leu-
cardiac TnI (17-25)				Val	Arg	Arg	Ser[a]	Asp	Arg	Ala Tyr	Ala-
myelin basic protein (8-16)				Gln	Arg	His	Gly	Ser[a]	Lys	Tyr Leu	Ala-

[a] Designates phosphorylatable serine residues. The hormone sequences were copied from the article by Bloom (1981) and the phosphorylated sequences from the review by Carlson et al. (1979).

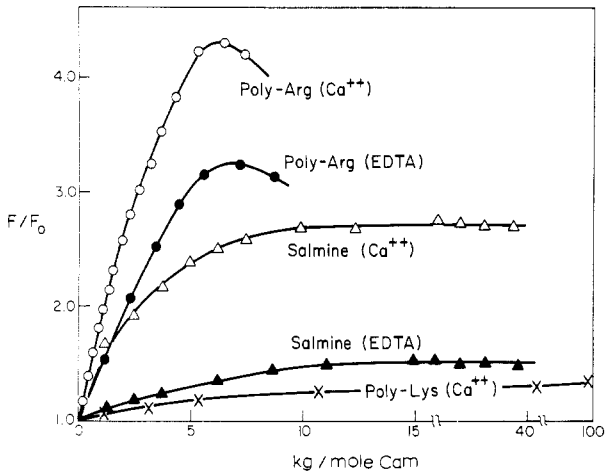

FIGURE 5: Binding of poly(L-arginine), poly(L-lysine), and salmine by dansylcalmodulin. 2.3 μM dansylcalmodulin was titrated with poly(L-arginine) in the presence of 0.85 mM $CaCl_2$ (○) or 1.2 mM EDTA (●) and with poly(L-lysine) in 0.85 mM $CaCl_2$ (×). 1.0 μM dansylcalmodulin was titrated with salmine in the presence of 0.85 mM $CaCl_2$ (△) and 1.2 mM EDTA (▲). F/F_0 was measured at 440 nm when Ca^{2+} was present and at 480 nm when EDTA was present. Other conditions are given under Figure 1.

salmine displaces the bound ACTH, with 50% change in intrinsic fluorescence occurring at ~0.25 mg/mL salmine. Poly(L-lysine) binds dansylcalmodulin weakly.

Discussion

We have shown that ACTH, β-endorphin, glucagon, and substance P undergo calcium-dependent binding to calmodulin with dissociation constants in the micromolar range. This affinity is high, considering the sizes of the peptides, but does not strongly support a physiologically significant role for the isolated interactions. The binding of peptide hormones such as glucagon by cell surface receptors (Goldfine et al., 1972) and the binding of proteins such as the cyclic nucleotide phosphodiesterase (Wang et al., 1980) or smooth muscle myosin light chain kinase (Conti & Adelstein, 1981) by calmodulin occur in the nanomolar concentration range. An exception to these high affinities exists in phosphorylase kinase, for which calmodulin binding to a second site takes place in the micromolar range (Cohen, 1980). The interaction between the peptides and calmodulin probably reflects similarities or homologies between the peptides and parts of the recognition sequences of calmodulin binding proteins. The competition of the peptides for common binding sites on calmodulin suggests the existence of structural homologies among them.

Substance P, with 11 amino acid residues, is the smallest peptide that calmodulin binds well and probably has a major portion of its structure committed to the interaction. Sequence comparisons show similarities between the first eight amino acid residues of substance P and the residues in positions 16–23 of both ACTH and glucagon. The aligned sequences in Table III show a variable section of three amino acid residues flanked on the N-terminal side by a strongly basic tripeptide sequence and on the C-terminal side by a pair of bulky hydrophobic residues. β-Endorphin contains a His-Lys-Lys sequence two positions away from the C terminal. Reversal of the β-endorphin sequence gives an antiparallel alignment in which the basic and hydrophobic segments coincide with those of the other peptides. Chain direction should have little effect on binding so long as interactions do not involve the polypeptide backbone and the reversed sequence contains no proline. (β-Endorphin has none.)

We recognized a striking similarity between the above pattern and the marker sequence for the cAMP-dependent protein kinase. Serine residues most readily phosphorylated by protein kinase are flanked by clusters of basic residues including arginine on the N-terminal side and by hydrophobic residues on the C-terminal side [cf. review by Carlson et al. (1979)]. Table III shows the similarities between one of the most effective synthetic cAMP-dependent protein kinase substrates and the four peptides. The suggestion that calmodulin and the cAMP-dependent protein kinase have similar binding specificities is consistent with the dual regulation of enzymes like phosphorylase kinase and smooth muscle myosin light chain kinase. Other proteins such as the histones (Grand & Perry, 1979; Malencik et al., 1982), troponin I (Amphlett et al., 1976; Dedman et al., 1977a,b), and the myelin basic protein (Grand & Perry, 1979) also bind calmodulin in addition to undergoing phosphorylation. The table contains sequences surrounding several of the phosphorylated serine residues in these proteins. The pattern envisioned from the peptide binding studies to calmodulin is repeated. Since a marker sequence accessible to the cAMP-dependent protein kinase is probably also accessible to calmodulin, the coincidence suggests that modification of at least certain calmodulin binding sites is one of the functions of the cAMP-dependent protein kinase. Known changes in calmodulin binding following phosphorylation occur in smooth muscle myosin light chain kinase, with an ~20-fold (Conti & Adelstein, 1981) to ~500-fold (Malencik et al., 1982) decrease in affinity, and in the myelin basic protein, troponin I, and histone H2A with 2.5–5-fold decreases in affinity (Malencik et al., 1982). The presence of calmodulin blocks one of the two phosphorylatable sites in smooth muscle myosin light chain kinase, suggesting competition between calmodulin and the cAMP-dependent protein kinase (Conti & Aldestein, 1981; Malencik et al., 1982). Although other subsites must take part in calmodulin binding, a marker sequence with a microscopic dissociation constant of 2 μM could be the major source of free energy of stabilization and hence a natural target for modification. The unmodified marker sequence would contribute −7.7 kcal to the

Table IV: Summary of Peptides Showing Slight or No Interaction with Dansylcalmodulin[a]

peptide	max concn used (μM)	max fluorescence change obsd at 440 nm (%)
angiotensin I	110	+55
substance P octapeptide	38[b]	+29.5
insulin	49	+13.8
physaelemin	68	+11.0
neurotensin	54	+8.0
angiotensin III	52	0
bradykinin	100	0
Leu-enkephalin	180	0
Met-enkephalin	180	0
Arg-Arg-Arg	1700	+10

[a] Conditions: 1.0 μM dansylcalmodulin, 0.85 mM $CaCl_2$, 0.2 N KCl, and 50 mM Mops, pH 7.3 (25 °C). [b] Incompletely dissolved. The true concentration in solution must be smaller.

total free energy charge of −12.2 kcal needed for an overall dissociation constant of 1 nM.

Similarities between the first four peptides and the α-melanocyte-stimulating hormone, bombesin, and somatostatin are less pronounced. All are basic. Somatostatin contains a Phe-Phe-Trp sequence that may bind calmodulin. Substance P octapeptide, lacking the first three residues of substance P, gives detectable fluorescence enhancement of dansylcalmodulin (Table IV). Physaelemin, homologous to substance P but containing an N-terminal p-Glu-Ala-Asp sequence, binds weakly as does neurotensin. Even though they are strongly basic, bradykinin and angiotensin III do not bind. The binding of β-endorphin seems not to involve the N-terminal opioid pentapeptide since Leu-enkephalin and Met-enkephalin neither bind dansylcalmodulin nor displace bound β-endorphin. Proopiomelanocortin, the precursor molecule containing the sequences of ACTH and β-endorphin [cf. review by Herbert (1981)], probably binds calmodulin well. The small enhancement seen with insulin may reflect a trace of glucagon.

The binding of strongly basic proteins, such as poly(L-arginine) and salmine, seems to involve the specific protein binding site plus electrostatic interaction with the many acidic side chains of calmodulin. Binding of these proteins by dansylcalmodulin in the presence of calcium produces the fluorescence changes characteristic of other calmodulin binding proteins. The binding persists in the presence of EDTA, with fluorescence spectra different from those obtained under other conditions. Binding to remote sites is consistent with experiments on parvalbumin, a protein descended from calmodulin that is considered to have lost the specific protein binding site (Blum et al., 1977). Parvalbumin binds poly(L-arginine), salmine, and histone H2A well—both in the presence and in the absence of calcium—but does not bind troponin I or the myelin basic protein (D. A. Malencik and S. R. Anderson, unpublished observations). Other proteins high in arginine would be expected to bind calmodulin strongly. An interesting example is phosphorylase phosphatase inhibitor I, which is phosphorylatable by cAMP-dependent protein kinase at a threonine residue adjacent to a sequence of four consecutive arginine residues (Huang & Glinsman, 1976; Cohen et al., 1977; Nimmo & Cohen, 1978). This affinity for strongly basic proteins is generally shared by the cAMP-dependent protein kinase (Krebs & Beavo, 1979).

Our results confer a sense of unity on the major elements of cellular regulation. Structural features recognized by cell surface receptors occur in the recognition sequences for both calmodulin and the cAMP-dependent protein kinase. These similarities reflect a relationship that may be functional or evolutionary. X-ray diffraction studies showed that the Phe-Val sequence that we believe is important in the binding of glucagon by calmodulin is directly involved, along with leucine at position 26, in the self-association of glucagon. Sasaki et al. (1975) suggested interaction of these hydrophobic groups with the glucagon receptor.

A companion publication by us deals with the dual effects of phosphorylation and calmodulin binding on the structural and functional properties of turkey gizzard myosin light chain kinase (Malencik et al., 1982). The results include experiments showing that ACTH, glucagon, β-endorphin, substance P, troponin I, the myelin basic protein, and histone H2A inhibit myosin light chain kinase through competition for calmodulin.

Recent experiments with model peptides support the hypothesis that calmodulin and the cAMP-dependent protein kinase may recognize the same sequences in target proteins (S. R. Anderson and D. A. Malencik, unpublished results). We demonstrated calcium-dependent binding to calmodulin for two synthetic protein kinase substrates, Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ser_2-NH_2 and Leu-Arg_2-Ala-Ser-Leu-Gly. Phosphorylation of the decapeptide, the more strongly bound of the two, causes the dissociation constant for the peptide–calmodulin complex to increase from 2.4 mM to 7.0 mM. The enzymes from which the two peptides are derived, glycogen synthase and pyruvate kinase, are not calmodulin dependent. We are presently working on the isolation and characterization of low molecular weight calmodulin binding properties from calcineurin, myosin light chain kinase, troponin I, and the myelin basic protein. We plan to determine their sequences and the effects of phosphorylation on their binding to calmodulin.

Calmodulin has four calcium binding sites of varying affinities. Although reported dissociation constants differ somewhat, there is agreement that calcium binding to sites having p*K*s between 5 and 6 accounts for most of the conformational changes in calmodulin and is essential for specific protein binding (Teo & Wang, 1973; Lin et al., 1974; Watterson et al., 1976; Dedman et al., 1977a,b; Klee, 1977; Wolff et al., 1977; Crouch & Klee, 1980; Seamon, 1980). We find that calcium has a dual role in peptide binding by calmodulin, with a 2-fold increase in affinity occurring on the occupation of calcium binding sites having a $pK \sim 4$. This suggests subtle final conformational changes in calmodulin associated with saturation of the weak calcium binding sites. Crouch & Klee (1980) have related observations on the cyclic nucleotide phosphodiesterase, which undergoes an abrupt activation at calcium concentrations near 10^{-4} M when the calmodulin concentration is limiting.

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