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Binding of Hormones and Neuropeptides by Calmodulin†

Dean A. Malencik and Sonia R. Anderson*

ABSTRACT: Calmodulin exhibits high-affinity, calcium-dependent binding of 1 mol/mol of the vasoactive intestinal peptide (VIP), secretin, and either the 42- or 43-residue gastric inhibitory peptide (GIP) with dissociation constants of 0.05–0.14 μM. The affinity of VIP for calmodulin approaches its affinity for the cell-surface VIP receptors. These peptides compete with both smooth muscle myosin light chain kinase and glucagon in calmodulin binding. Calculation of amino acid frequencies for eight calmodulin binding peptides (VIP, GIP, secretin, ACTH, β-endorphin, substance P, glucagon, and dynorphin [Malencik, D. A., & Anderson, S. R. (1982) *Biochemistry 21*, 3480]) shows a below-average incidence of glutamyl residues, above-average incidence of glutaminyl residues, and average incidence of both aspartyl and asparaginyl residues. Predictions of structure from sequence suggest that the bound peptides contain strongly basic turns and coils in close association with regions having above-average β-sheet potential. The temperature dependence of glucagon binding by calmodulin shows that the association is enthalpy driven.

As the major intracellular receptor for calcium, calmodulin is involved in the regulation of diverse cellular functions. The binding of calcium stabilizes one or more specific conformations of the calmodulin molecule recognized by calmodulin-dependent enzymes such as cyclic nucleotide phosphodiesterase, adenylate cyclase, and myosin light chain kinase [cf. reviews by Means (1981) and Cheung (1980, 1982)]. The complementary protein binding sites of both calmodulin and the affected enzymes are yet to be characterized in terms of primary and higher levels of structure. In fact, no calmodulin-dependent enzyme has been completely sequenced up to the present time. In order to obtain information on the protein binding specificity of calmodulin, we have previously studied the calcium-dependent binding of 19 different model peptides ranging in length from 7 to 39 amino acid residues (Malencik & Anderson, 1982; Malencik et al., 1982b). Our survey revealed four peptides that compete for common binding sites on calmodulin with dissociation constants in the micromolar range: adrenocorticotropic hormone (ACTH),[1] β-endorphin, substance P, and glucagon. The report by Weiss et al. (1980), that ACTH and β-endorphin inhibit the purified cyclic nucleotide phosphodiesterase, had suggested to us that calmodulin binds other peptide hormones and neurotransmitters. Regardless of the physiological relevance of these interactions, they may provide clues regarding a recognition sequence for calmodulin. We found that the peptides that calmodulin binds well contain common structural features, notably a strongly basic tripeptide sequence three positions away from a pair of bulky hydrophobic residues. Since this pattern is similar to the recognition sequence for the cAMP-dependent protein kinase, we suggested that calmodulin and protein kinase act on common sequences in proteins subject to dual control by calcium and cAMP. This hypothesis was supported by experiments showing that phosphorylation by the protein kinase affects calmodulin binding by smooth muscle myosin light chain kinase (Conti & Adelstein, 1981; Malencik et al., 1982a), skeletal muscle troponin I, histone H2A, myelin basic protein (Malencik et al., 1982a), and synthetic protein kinase substrates (Malencik et al., 1982b).

This report extends our previous observations with emphasis on other peptides of the glucagon family—secretin, the vasoactive intestinal peptide (VIP), and the gastric inhibitory peptide (GIP) (Table IV). The affinities of these peptides for calmodulin are 10–70 times greater than those of the peptides previously studied and are within an order of magnitude of that for the cell-surface neurohormone receptor in the case of VIP. The close relationships among the peptides permit observations on the effects of amino acid substitutions.

Materials and Methods

Porcine brain calmodulin was prepared according to Schreiber et al. (1981) and subjected to a final purification step by affinity chromatography on a fluphenazine–Sepharose matrix (Charbonneau & Cormier, 1979). This additional

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[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; 9AC, 9-anthroylcholine; cAMP, adenosine cyclic 3′,5′-phosphate; CaM, calmodulin; MLCK, myosin light chain kinase; TnI, troponin I; ACTH, adrenocorticotropic hormone; VIP, vasoactive intestinal peptide; GIP, gastric inhibitory peptide; dansyl, 5-(dimethylamino)-1-naphthalenesulfonyl; *K*, dissociation constant; *F*, observed fluorescence; F_0, fluorescence of unbound ligand; F_∞, fluorescence of totally bound ligand.

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Table I: Peptide Binding by Calmodulin: Summary of Dissociation Constants[a]

peptide	K_d[b] (μM)	K_d[c] (μM)	F_∞/F_0[c] (440 nm)	$K_{MLCK\cdot CaM}/K_d$[e]
VIP				0.026
GIP (43 residues)	~0.08			~0.014
secretin[d]		0.14	2.04[d]	0.010
ACTH[f]	2.5	1.5	2.66	
β-endorphin[f]		2.0	2.80	~0.0008
substance P[f]		1.9	3.60	
glucagon[f]	3.3	3.4	3.3	~0.001
dynorphin		4.5	2.43	
des-Gln^6-substance P		9.7	3.20	
phosphorylase kinase peptide[g]		94	2.5	
phosphorylated phosphorylase kinase peptide[g]		550[h]	1.8	
Lys-bradykinin		700	3.5	
Met-Lys-bradykinin		500	3.0	

[a] Conditions were generally as follows: 0.2 N KCl–50 mM Mops–1 mM $CaCl_2$, pH 7.3, 24 °C. [b] K_d estimated from changes in intrinsic peptide fluorescence (±10–20%). [c] K_d and fluorescence enhancement factors obtained with dansylcalmodulin (usually ±5%). [d] 0.15 N KCl; fluorescence measured at 480 nm. [e] Ratio of dissociation constants determined in competition experiments (Figure 1). [f] Data from Malencik & Anderson (1982). [g] KCl was omitted. [h] ±20%.

purification removes tryptophan-containing impurities. The purified calmodulin was labeled with 5-(dimethylamino)-1-naphthalenesulfonyl chloride as described by Malencik & Anderson (1982). Turkey gizzard myosin light chain kinase was prepared according to the procedure of Adelstein & Klee (1981). Troponin I was prepared from rabbit muscle by the method of Kerrick et al. (1980).

Dynorphin, VIP, and des-Gln^6-substance P were obtained from Vega Biochemicals; secretin was from Research Plus; lysyl-bradykinin, methionyl-lysyl-bradykinin, and mixed bovine and porcine glucagon were from Sigma Chemical Co. The synthetic 42- and 43-residue gastric inhibitory peptides were supplied by Peninsula Laboratories. Prof. John C. Brown donated a sample of the natural GIP (42 residues). The phosphorylase kinase peptide was custom synthesized by Peninsula, phosphorylated in a reaction catalyzed by the cAMP-dependent protein kinase, and characterized according to Malencik & Anderson (1983).

3-(*N*-Morpholino)propanesulfonic acid (Mops), dithiothreitol, and ATP were obtained from Sigma Chemical Co. 9-Anthroylcholine bromide was obtained from Molecular Probes, Inc. All other chemicals were reagent grade, and glass-distilled water was used throughout.

Fluorescence measurements were obtained with the Hitachi Perkin-Elmer MPF2A fluorometer. The details of the titrations of dansylcalmodulin and of the tryptophan-containing peptides were described by Malencik & Anderson (1982). The conditions of the competition experiments, using 9-anthroylcholine and myosin light chain kinase, were given by Malencik et al. (1982a).

Results

We continue to apply three of the methods that we previously used to detect peptide binding by calmodulin. The first is based on the enhancement of the intrinsic fluorescence of tryptophan-containing peptides accompanying calmodulin binding (Malencik & Anderson, 1982). This method requires comparatively high peptide concentrations since the exciting wavelength must be removed from the absorption maxima of both tryptophan and tyrosine in order to avoid excitation of the intrinsic tyrosine fluorescence of calmodulin.[2] The second approach uses the large changes in the fluorescence of dansylcalmodulin that accompany the binding of both peptides and proteins to it (Malencik et al., 1981; Malencik & Anderson, 1982). This method is highly sensitive but requires covalent modification of calmodulin. The third technique is based on the characteristic binding of the fluorescent probe 9-anthroylcholine (9AC) by smooth muscle myosin light chain kinase (Malencik et al., 1982a). Myosin light chain kinase binds 1 mol of 9AC with fluorescence enhancement. Interaction of the enzyme with calmodulin increases its affinity for 9AC, with the dissociation constant decreasing from 20 to 6.4 μM. The resulting changes in 9AC fluorescence are sensitive to calmodulin binding by the enzyme and are useful in evaluation of the competition with high-affinity calmodulin binding proteins such as troponin I (TnI), histone H2A, and the myelin basic protein.

Figure 1 shows the competition resulting when glucagon, secretin, GIP (42 or 43 residues),[3] VIP, and TnI are added to solutions containing the smooth muscle myosin light chain kinase–calmodulin complex (0.5 μM) and 9-anthroylcholine (5.0 μM). The fluorescence intensities shown on the ordinate range from the value obtained with the enzyme alone (normalized to 100) to that obtained with the enzyme–calmodulin complex (250). Since the enzyme and calmodulin are present at equal concentrations in excess of the dissociation constant, ~2 nM (Malencik et al., 1982a), the concentration of unbound calmodulin is negligible. The displacement of the enzyme corresponds to the following net equilibrium, where P represents the competing protein or peptide:

$$\text{MLCK·CaM} + \text{P} \rightleftharpoons \text{MLCK} + \text{P·CM}$$

Only two of the components in this equilibrium bind 9AC efficiently. None of the peptides affects the binding of 9AC by the enzyme alone. We previously showed that the ratio of the dissociation constants for the two competing equilibria, $K_{MLCK\cdot CaM}$ and $K_{P\cdot CaM}$, can be calculated from the fractional change in fluorescence (ΔF):[4]

$$\frac{K_{MLCK\cdot CaM}}{K_{P\cdot CaM}} = \frac{\Delta F^2}{([P]/[MLCK]_{total})(1 - |\Delta F|)}$$

[2] Calmodulin contains no tryptophan.

[3] The synthetic 42- and 43-residue GIP's differ by one Gln residue at positions 29 and 30, reflecting an error in the sequencing of the natural 42-residue GIP (Jornvall et al., 1981). The two peptides bind calmodulin about equally well.

[4] This equation applies when most of the competing protein or peptide is unbound; i.e., $[P] \gg [P\cdot CaM]$. If a significant fraction of P is bound and $[P]_{total} > [CaM]_{total}$, the equation used is

$$\frac{K_{MLCK\cdot CaM}}{K_{P\cdot CaM}} = \frac{\Delta F^2[MLCK]_{total}}{(1 - \Delta F)[[P]_{total} - [CaM]_{total} + (1 - \Delta F)[MLCK]_{total}]}$$

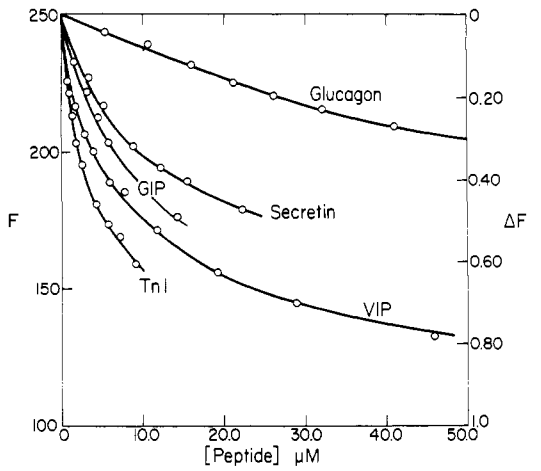


FIGURE 1: Dissociation of smooth muscle myosin light chain kinase–calmodulin complex by calmodulin binding peptides (troponin I, VIP, GIP, secretin, and glucagon). ΔF is the fraction of the difference between the fluorescence intensities of 0.5 μM myosin light chain kinase plus 0.5 μM calmodulin and of 0.5 μM myosin light chain kinase alone. The solutions contained 5.0 μM 9-anthroylcholine, 0.15 N KCl, 50 mM Mops, 1 mM dithiothreitol, and 1 mM $CaCl_2$, pH 7.3, 25 °C. Excitation 360 nm; emission 460 nm.

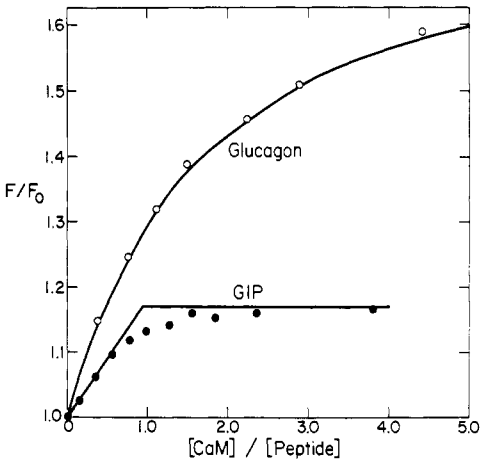


FIGURE 2: Fluorescence titrations of 43-residue GIP (1.1 μM) and glucagon (3.5 μM) with calmodulin. The wavelengths of excitation and emission were 295 and 340 nm, respectively. The corresponding bandwidths were 3 and 5 nm. F_0 is the fluorescence of the peptide in the absence of calmodulin. The molar ratio of calmodulin/peptide is shown on the abscissa. The following conditions were used: 0.2 N KCl, 50 mM Mops, and 1 mM $CaCl_2$, pH 7.3, 24 °C.

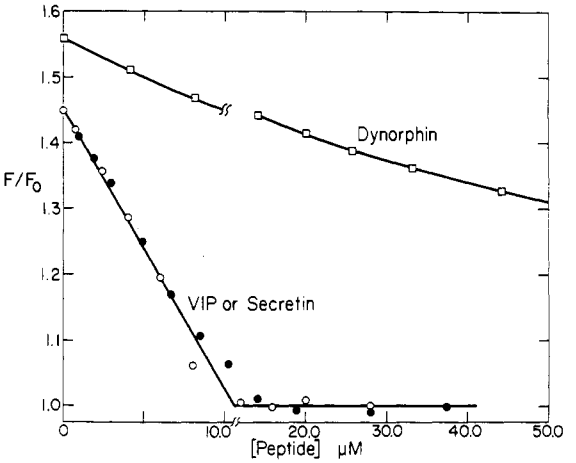


FIGURE 3: Dissociation of glucagon–calmodulin complex by VIP, secretin, and dynorphin. F_0 is the fluorescence of glucagon in the absence of calmodulin. The solutions contained 10.0 μM calmodulin and either 4.3 μM glucagon for the titrations with VIP (○) and secretin (●) or 3.5 μM glucagon for the titration with dynorphin (□). The abscissa gives the concentration of added peptide (exclusive of glucagon). Other conditions are described under Figure 2.

The ratios of dissociation constants calculated from Figure 1 are 0.048 for TnI, 0.026 for VIP, ~0.014 for GIP, 0.010 for secretin, and ~0.001 for glucagon (Table I). Surprisingly, VIP, with just 28 amino acid residues, binds calmodulin nearly as well as the 21 000-dalton TnI. TnI, the inhibitory subunit of troponin, is often used as a model in calmodulin binding studies (Grand et al., 1979). LaPorte et al. (1981) obtained a dissociation constant of 60 nM for the calmodulin–TnI complex under somewhat different conditions.

These ratios are reasonable representations of the relative affinities of the enzyme and the peptides for calmodulin. The preferential binding of 9AC by the enzyme–calmodulin complex implies that the value for $K_{MLCK\cdot CaM}$ is 71% of the value expected in the absence of 9AC.[5] The comparatively weak binding of 9AC by calmodulin, with K_{av} = ~440 μM (LaPorte et al., 1980), should have little effect. Experiments in a later section show that the dissociation constant for the secretin–calmodulin complex is 1.4×10^{-7} M. By combining this value with the results of the competition experiments, we calculate values of 1.4 nM and 2.0 nM for $K_{MLCK\cdot CaM}$ in the presence and absence of 9AC.

The fluorescence emission spectra and quantum yields of the tryptophan-containing peptides—ACTH, glucagon, and GIP—change when calmodulin binds. A stoichiometric titration of 1.1 μM GIP (43 residues) with calmodulin, following the fluorescence changes at 340 nm, shows 17% enhancement and a dissociation constant of ~80 nM (Figure 2). The larger changes obtained with glucagon or ACTH, up to 75% maximum enhancement at 340 nm (Figure 2; Malencik & Anderson, 1982), facilitate competition experiments with peptides containing no tryptophan— such as secretin, VIP, and dynorphin. The titration of a 10 μM solution of calmodulin containing 4.3 μM glucagon with either VIP or secretin shows a linear decrease in glucagon fluorescence with an endpoint of 1 mol of peptide/calmodulin (Figure 3). Virtually all of the VIP or secretin is bound up to saturation, showing that the dissociation constant for the glucagon–calmodulin complex (3.3 μM) is much larger than the dissociation constants for the other two peptides. The results are consistent with competitive displacement, i.e.

$$\text{glucagon·CaM} \rightleftharpoons \text{glucagon} + \text{CaM}$$

$$\text{CaM} + \text{VIP} \rightleftharpoons \text{VIP·CaM}$$

The addition of dynorphin results in a gradual displacement of glucagon consistent with the larger dissociation constant (4.5 μM) shown later. The well-defined stoichiometries and the competition found both here and in our previous work

Table II: Effect of Temperature on Glucagon Binding by Calmodulin[a]

T (°C)	K (μM)	F_∞/F_0[b]
11.3	0.96	1.67
23.2	3.1	1.74
29.9	6.6	1.81
38.1	13.5	1.94

[a] 5.0 mM Mops–0.2 N KCl–1.0 mM $CaCl_2$, pH 7.3; 3.5 μM glucagon. [b] Excitation 295 nm; emission 340 nm.

[5] $K_{app} = [([9AC]/K_1 + 1)/([9AC]/K_2 + 1)]K$, where K_1 is the dissociation constant for the enzyme–9AC complex (20 μM), K_2 is the constant for the (calmodulin–enzyme)–9AC complex (6.5 μM), and K is the dissociation constant for the enzyme–calmodulin complex.

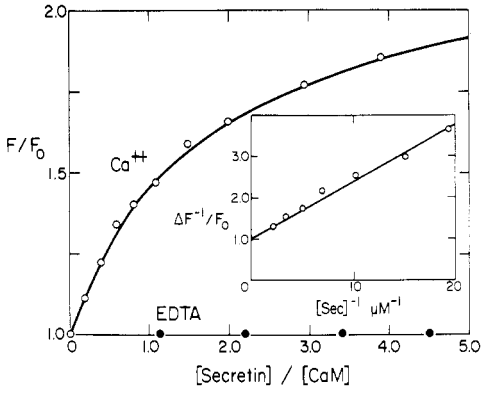


FIGURE 4: Fluorescence titration of dansylcalmodulin with secretin. The relative fluorescence (F/F_0) was measured at 480 nm, with 340-nm excitation. The double-reciprocal plot of the changes in fluorescence vs. the concentration of unbound secretin corresponds to 1:1 binding with K_d = 1.4 ± 0.2 μM and a fluorescence-enhancement factor of 2.0 (inset). The solution contained 0.2 μM dansylcalmodulin and 1 mM $CaCl_2$ (○) or 1.0 μM dansylcalmodulin and 1.0 mM EDTA (●) in 0.15 N KCl–50 mM Mops, pH 7.3, 25 °C.

demonstrate that the peptides compete for a common binding site on calmodulin.

We determined the temperature dependence of calmodulin binding by glucagon in titrations following the changes in the intrinsic tryptophan fluorescence (Table II). The van't Hoff plot of the results is linear, giving $\Delta H°_{assoc} = -17.0$ kcal. Since the values of $\Delta G°$ range from −6.9 to −7.8 kcal, the binding of glucagon by calmodulin is enthalpy driven with the net entropy change being negative.

We used dansylcalmodulin to further characterize the binding of the preceding peptides as well as of des-Gln[6]-substance P (Arg-Pro-Lys-Pro-Gln-Phe-Phe-Gly-Leu-Met-NH_2), dynorphin, lysyl-bradykinin, methionyl-lysyl-bradykinin, and a synthetic substrate[6] for the cAMP-dependent protein kinase derived from the phosphorylation site in the β-subunit of phosphorylase kinase (Yeaman et al., 1977). The binding of specific peptides and proteins by dansylcalmodulin causes the quantum yield to increase and the fluorescence emission maximum to shift from 522 to 500–510 nm (Malencik & Anderson, 1982). Figure 4 shows the fluorescence increase measured at 480 nm when 0.2 μM danyslcalmodulin is titrated with secretin. The fractional degree of saturation (ϕ) of dansylcalmodulin with the peptide is related to the fluorescence enhancement:

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

The double-reciprocal plot of the changes in fluorescence vs. the calculated concentration of free secretin is linear (inset to Figure 4)—allowing calculation of the dissociation constant [1.4 (±0.2) × 10^{-7} M] and verification of the value of F_∞/F_0 (2.0). Table I summarizes the dissociation constants obtained with 13 of the model peptides. Phosphorylation of the protein kinase substrate,[6] which occurs primarily at a single site probably corresponding to position 7 (Yeaman et al., 1977; Malencik & Anderson, 1983), causes the dissociation constant to increase 6-fold. None of these peptides interacts appreciably with dansylcalmodulin in the absence of calcium, confirming the strong calcium dependence previously reported by us (Malencik & Anderson, 1982).

Altogether, we have located eight distinct small peptides that bind calmodulin well: VIP, GIP, secretin, ACTH, β-endorphin, substance P, glucagon, and dynorphin. They contain a total of 221 amino acid residues. We calculated the average frequencies of occurence for the 19 component amino acids and compared them to the frequencies tabulated by King & Jukes (1969) for 53 randomly selected polypeptides containing a total of 5492 residues. Table III shows that *arginine* and *glutamine* are found with relatively high frequencies in the calmodulin binding peptides while *glutamic* acid is found with strikingly low frequency. Lysine, phenylalanine, and tyrosine occur with above-average frequencies while valine, proline, and alanine seem to occur less often than average.

[6] Arg-Thr-Lys-Arg-Ser-Gly-Ser-Val-Tyr-Glu-Pro-Leu-Lys-Ile.

Table III: Amino Acid Frequencies in Calmodulin Binding Peptides[a]

residue	no. of occurrences	obsd frequency[c]	av frequency[b,c]
Asp	13	5.9	5.9
Asn	9	4.1	4.2
Glu	6	2.7	5.8
Gln	16	7.2	3.7
Lys	21	9.5	7.2
Arg	16	7.2	4.2
His	6	2.7	2.9
Pro	8	3.6	5.0
Ser	18	8.1	8.1
Thr	12	5.4	6.2
Ala	12	5.4	7.4
Gly	14	6.3	7.4
Trp	4	1.8	1.3
Phe	14	6.3	4.0
Tyr	10	4.5	3.3
Val	9	4.1	6.8
Leu	19	8.6	7.6
Ile	8.0	3.6	3.8
Met	6.0	2.7	1.8
Cys	0	0	3.3

[a] VIP, GIP, secretin, ACTH, β-endorphin, substance P, glucagon, and dynorphin. [b] Tabulated by King & Jukes (1969) for 53 randomly selected polypeptides. [c] Number of occurrences per 100 residues.

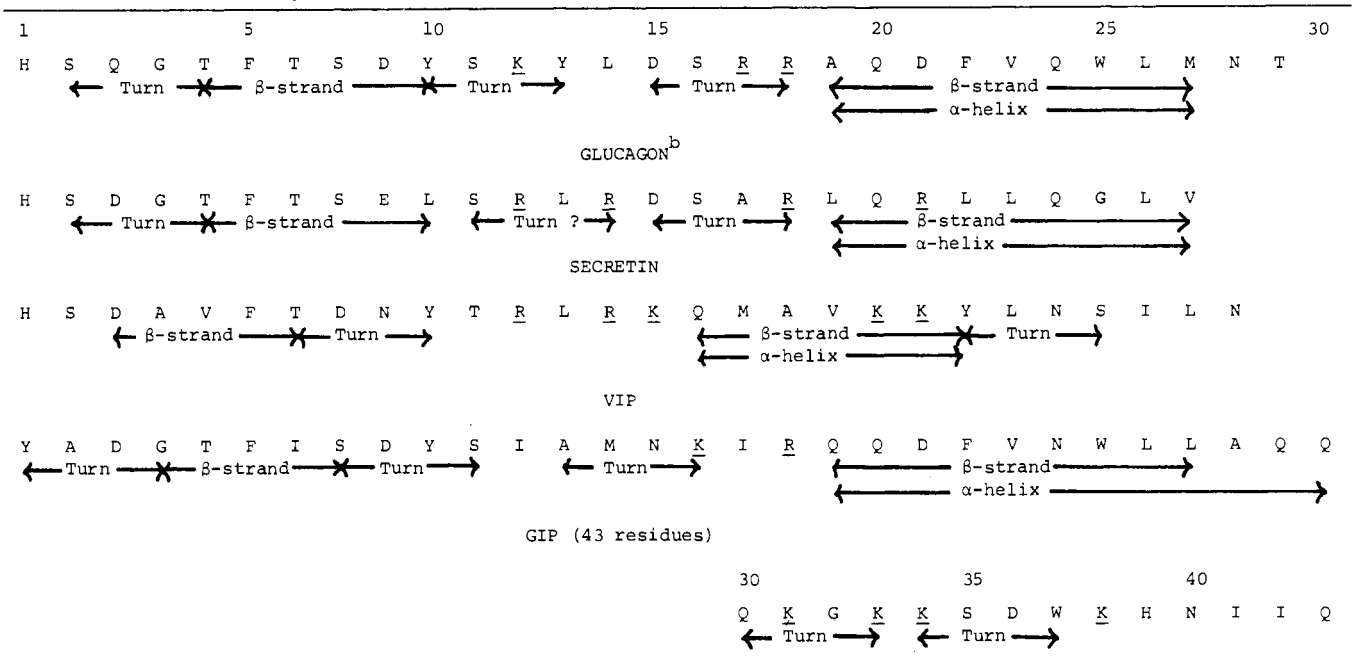
Discussion

Our work on the binding of ACTH, β-endorphin, substance P, and glucagon had suggested a model consisting of a strongly basic tripeptide sequence three positions away from a pair of hydrophobic residues as part of a recognition sequence for calmodulin (Malencik & Anderson, 1982). This model is demonstrated in positions 1–8 of substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) and 16–23 of glucagon (Table IV). VIP, GIP, and secretin are comparable in size to the previous peptides but bind calmodulin 10–70 times more strongly. The occurrence of a second cluster of basic amino acid residues is the major distinguishing feature of the present peptides. Secretin, for example, shows 50% sequence homology with glucagon; significant amino acid differences probably include those at positions 14 (Leu → Arg) and 21 (Asp → Arg) (Table IV).

The distribution of glutamyl residues in the peptides indicates that the low frequency of occurrence is probably relevant to calmodulin binding. Of the six Glu residues tabulated, four occur in ACTH: one in the N-terminal region and three in the strongly acidic C-terminal region, which is glycosylated (Herbert et al., 1980) and contains no basic residues. The effects of the latter three Glu residues on calmodulin binding may be atypical. Secretin and β-endorphin each contain a single Glu. The α-melanocyte-stimulating hormone[7] (Ac-

[7] The α-melanocyte-stimulating hormone is derived from the N-terminal sequence of ACTH.

Table IV: Predicted Secondary Structures of Calmodulin Binding Peptides Belonging to Glucagon Family[a]



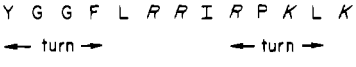
[a] Sequences are from the paper by Bloom (1981). [b] Predicted by Chou & Fasman (1975).

Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) (Malencik & Anderson, 1982), and the phosphorylase kinase peptide[6] each contain Glu and bind calmodulin poorly. Immediate electrostatic effects do not explain the low incidence of Glu since aspartyl residues occur at the normal rate. The strong influence of Glu on secondary structure may be involved. Glu is the amino acid residue found most often in α-helices, least often in β-sheets, and next to the least often in β-turns (Chou & Fasman, 1974). The high incidence of glutaminyl residues may reflect an active role in calmodulin binding or simply replacement of the unfavorable glutamyl residues. While Gln is prominent in substance P and the 43-residue GIP, VIP contains only one Gln. Des-Gln^6-substance P, with a single Gln, binds calmodulin less well than substance P (Table I).

The involvement of clusters of basic amino acid residues in calmodulin binding accounts for the above-average frequencies obtained with the lysyl and arginyl residues. The experiments with lysyl-bradykinin (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and methionyl-lysyl-bradykinin support our hypothesis that the basic clusters are important in binding since bradykinin does not interact with calmodulin. Although poly-L-arginine binds calmodulin more strongly than poly-L-lysine, Arg and Lys are apparently equally important in specific peptide binding. The calcium dependence is lost when the proportion of basic residues is large. Salmine, high in arginine, and poly-L-arginine bind calmodulin in both the presence and absence of calcium (Malencik & Anderson, 1982). The absence of cysteine is probably expected in such small peptides.

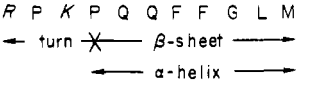
The environment of the protein binding site in calmodulin probably stabilizes specific secondary structure in the peptides, which are largely unfolded in dilute aqueous solutions [cf. review by Blundell & Wood (1982)]. Calmodulin may possibly bind more than one type of secondary structure, reflecting its broad range of biochemical function. In some cases, at the expense of binding energy, the conformation of the bound peptide may differ from the most stable conformation. Keeping these possibilities in mind, we applied the rules of Chou & Fasman (1974) for the prediction of secondary structure from sequence to the eight peptides.

Dynorphin and substance P demonstrate structural features also found in the larger peptides. The ability of calmodulin to bind strongly basic, nonrepetitive "coils" is evident with dynorphin. Except for the β-turns, including the one found in the opioid N-terminal sequence,[8] the structure of dynorphin is probably irregular. An α-helix is impossible for the C-terminal section, due to Pro, while the β-strand is unfavorable:[9]



dynorphin

Substance P is a more representative calmodulin binding peptide. The rules of Chou & Fasman (1974) indicate a well-defined structure containing a rigid N-terminal β-turn connected to a β-strand or, less likely, an α-helix. The average β-sheet, $\langle P_\beta \rangle$, and α-helix, $\langle P_\alpha \rangle$, potentials for the C-terminal heptapeptide are 1.25 and 1.09, respectively. The Phe-Phe sequence that we treated as a part of the recognition sequence appears in the core of the C-terminal structure:



substance P

The predicted secondary structures of glucagon [also described by Chou & Fasman (1975)], secretin, VIP, and GIP each contain sections of β-strand in the N-terminal region and of β-strand or α-helix in the C-terminal region (Table IV). The average β-sheet and α-helix potentials of the latter region are similar, favoring β-sheets. In glucagon, $\langle P_\alpha \rangle$ for positions 19–27 is 1.19 while $\langle P_\beta \rangle$ is 1.25. The ability of glucagon to exist in more than one conformation is well established.

[8] Calmodulin does not bind Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu).
[9] Sequence from Goldstein et al. (1979).

Glucagon is largely helical in the crystalline state (Sasaki et al., 1975) but forms fibrils of antiparallel β-sheet in acidic solutions (Gratzer et al., 1967, 1698; Beaven et al., 1969; Epand, 1971). We believe that the low incidence of glutamyl residues, taken together with the predicted β-strands, indicates β-sheet formation in the peptide–calmodulin complexes.[10] Interaction with a complementary β-strand in calmodulin could stabilize the β-strand conformation in the peptides and perhaps also in calmodulin-dependent enzymes. The two residues found most often in β-sheets are methionine and valine (Chou & Fasman, 1974). Although valine occurs less frequently than average in the peptides, most of the proposed β-strands contain valine. Since valine also stabilizes α-helices, the frequency of glutamate is more useful in distinguishing between α-helices and β-sheets. The relatively large number of β-turns contributes to the low incidence of both glutamyl and valyl residues. Chou & Fasman (1974) reported that valine is the amino acid least likely to occur in turns.

The arginyl and lysyl residues are generally concentrated in β-turns and regions of irregular structure, as they are in substance P and dynorphin. The proposed C-terminal β-strand is associated with basic residues flanking its N-terminal end. The C-terminal β-strands of secretin and VIP also contain internal basic residues. The larger GIP molecule reveals a more complex structure, where an α-helix may adjoin the C-terminal strand. The predicted N-terminal β-strands may contribute little to binding, at least in some cases, since 11-residue substance P (which resembles the C-terminal β-strand) actually binds calmodulin more strongly than glucagon does (Table I; Malencik & Anderson, 1982).

ACTH (39 residues) and β-endorphin (31 residues) are unrelated to the glucagon family of peptides. Nonetheless, the prediction rules indicate that the basic sequence in ACTH is also associated with a possible β-strand, i.e.

K K R R P V K V Y P
← β-strand →

positions 15–24

β-Endorphin shows a different pattern, where the basic sequence occurs in a long, irregular C-terminal region. Possibly the predicted β-strand actually extends through the strongly stabilizing Ile-Ile sequence:

P L V T L F K N A I I K N A H K K G Q
← β-strand → ← turn →

positions 13–31

Phosphorylation of the phosphorylase kinase peptide, of a synthetic decapeptide derived from glycogen synthase (Malencik et al., 1982b), or of TnI, histone H2A, and the myelin basic protein (Malencik et al., 1982a) results in a moderate 3–6-fold decrease in calmodulin binding affinity. The effects of phosphorylation on binding support our hypothesis that calmodulin and the cAMP-dependent protein kinase can act on common polypeptide sequences. The magnitude of the changes probably reflects the short-range consequences of altered primary structure. Much larger effects on calmodulin binding, 20-fold (Conti & Adelstein, 1981) to more than 100-fold (Malencik et al., 1982a), following phosphorylation of smooth muscle myosin light chain kinase probably result from long-range conformational changes.

The phosphorylase kinase peptide was selected for study since it is derived from a calmodulin-dependent enzyme [cf. review by Fischer et al. (1975) and Yeaman et al. (1977)], its sequence[6] resembles those of the peptides that calmodulin binds well, and phosphorylation of the corresponding site in phosphorylase kinase is blocked when excess calmodulin is present (Cox & Edstrom, 1981). Although the peptide binds calmodulin, the affinity is much less than originally expected. Either the peptide is not part of a calmodulin binding site in phosphorylase kinase, or other amino acid residues, absent in the peptide, provide stabilization. Perhaps, the Glu residue in the synthetic peptide is actually Gln in the enzyme.

Simple peptides can approach enzymes such as myosin light chain kinase in efficiency of calmodulin binding. The free-energy changes accompanying binding are −9.9 kcal/mol with VIP (28 residues) and −11.8 kcal/mol with smooth muscle myosin light chain kinase (~1100 residues). The calmodulin binding sites in calmodulin-dependent enzymes probably contain the major structural features of the peptides: strongly basic sequences occurring in reverse turns and nonrepetitive coils together with hydrophobic sequences having high β-sheet potential. The binding sites may include phosphorylatable serine or threonine residues since the cAMP-dependent protein kinase acts on turns or coils (Kaiser et al., 1981) containing sequences such as Arg-Arg-X-Ser and Lys-Arg-X-X-Ser (Huang et al., 1979; Huang & Krebs, 1979; Carlson et al., 1979).

The sequence of calmodulin (Watterson et al., 1980) shows several regions with high β-sheet potential. Their sequence positions and corresponding values of $\langle P_\beta \rangle$ are as follows: 32–36, 1.31; 48–52, 1.30; 68–72, 1.41; 108–112, 1.28; 141–146, 1.45.[11] Positions 68–72 (Phe-Leu-Thr-Met-Met), occurring between calcium binding sites II and III in the model proposed by Kretsinger (1979), and positions 141–146 (Phe-Val-Gln-Met-Met-Thr) near the C-terminal end (148) stand out as possible sites of interaction with the peptides. Selective oxidation of methionyl residues 71, 72, and 76 abolishes the interaction between calmodulin and the cyclic nucleotide phosphodiesterase, showing that residues in and near the β-sheet nucleus at positions 68–72 are vital to calmodulin function. In addition, these particular methionine residues become more accessible to solvent and, perhaps also, to peptides or proteins, when calmodulin binds calcium (Walsh & Stevens, 1978). A C-terminal fragment of calmodulin, containing residues 72–148, fails to activate the phosphodiesterase (Schreiber et al., 1981). The peptide binding site probably includes complementary acidic residues such as those in positions 78–84 (Asp-Thr-Asp-Ser-Glu-Glu-Glu) or 118–123 (Asp-Glu-Glu-Val-Asp-Glu). That the binding of glucagon by calmodulin is enthalpy driven is consistent with the suggested role of hydrogen bonding in complex formation. At least three regions of rabbit skeletal muscle troponin C, which is ancestrally related to calmodulin (Barker et al., 1977), are involved in interaction with TnI (Grabarek et al., 1981). Regions near calcium binding sites II and III show calcium-dependent binding of TnI while a region near calcium binding site IV binds TnI in both the presence and absence of calcium. The high-affinity calmodulin binding peptides such as VIP should be useful in X-ray diffraction studies to locate and define the protein binding site of calmodulin.

Our observations suggest similarities between the peptide binding sites of calmodulin and some of the cell-surface neurohormone receptors. Ottesen et al. (1982) studied the binding of several peptides by the VIP receptors on crude smooth

[10] The 220-nm circular dichroism band becomes fractionally more negative when calmodulin binds VIP.

[11] Positions 25–29 give $\langle P_\beta \rangle = 1.2$ but are believed to be directly involved in calcium binding at site I.

Table V: Comparison of Peptide Binding by Calmodulin and the VIP Receptors

peptide	pK_{CaM}	$pK_{receptor}$[a]
VIP	7.3	8.4
secretin	6.8	6.6
glucagon	5.8	<5

[a] Ottesen et al., 1982.

muscle membranes from porcine uterus. Their results, expressed in terms of the peptide concentration required to give 50% displacement of 70 pM bound [^{125}I]iodo-VIP, are compared to the dissociation constants for the peptide–calmodulin complexes in Table V. VIP, secretin, and glucagon show the same relative orders of affinity for calmodulin and the VIP receptors. However, the differences between the peptides are larger with the VIP receptors—showing a higher degree of specificity than that found with calmodulin.

Since smooth muscle myosin light chain kinase is subject to dual control by calcium and cAMP (Conti & Adelstein, 1981), VIP may possibly be a first messenger in the regulation of its activity. Bitar & Makhlouf (1982) showed that VIP causes a rapid, dose-dependent relaxation of gastric smooth muscle cells and an increase in intracellular cAMP coincident with maximum relaxation.

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Registry No. VIP, 37221-79-7; secretin, 1393-25-5; GIP, 59392-49-3; synthetic 43-GIP, 84910-57-6; MLCK, 51845-53-5; glucagon, 9007-92-5; ACTH, 9002-60-2; β-endorphin, 60617-12-1; substance P, 33507-63-0; dynorphin, 74913-18-1.

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