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Interaction of β-Endorphin and Other Opioid Peptides with Calmodulin

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SUMMARY

A highly purified preparation of calmodulin activated a calmodulin-deficient phosphodiesterase by more than 10-fold. This activation of phosphodiesterase by calmodulin was completely inhibited by two opioid peptides, β-endorphin and dynorphin, at concentrations that had no appreciable effect on the basal phosphodiesterase activity. By contrast, similar concentrations of other structurally related peptides, including α-endorphin, (des-Tyr^1)-γ-endorphin, Leu-enkephalin, and Met-enkephalin, failed to block calmodulin's activation of phosphodiesterase. The inhibition by β-endorphin of calmodulin's action was not reversed by calcium or by the opiate antagonist naloxone but was overcome by increasing the concentration of calmodulin. Equilibrium dialysis studies showed that ^{125}I-labeled β-endorphin bound directly to calmodulin in a saturable, calcium-dependent manner with a dissociation constant of approximately 4.6 μM. There was substantially less binding of β-endorphin to troponin-C and little or no calcium-dependent binding of β-endorphin to bovine serum albumin, lactalbumin, or histone. This interaction of β-endorphin with calmodulin was similar in several respects to the interaction of certain antipsychotic drugs to calmodulin and may explain certain of the peptide's biochemical effects.

INTRODUCTION

Calmodulin is a calcium-binding protein that modulates the action of numerous calcium-dependent processes (1). Studies of its distribution and function suggest that it plays an important role in the central nervous system. It is found in high concentrations in brain (2), particularly in corpus striatum and frontal cortex (3). Subcellularly, calmodulin is present in both cytoplasmic and particulate fractions of cells (3, 4) and is associated with specific membranous components of neurons, including synaptic (5) and vesicular membranes (6) and postsynaptic densities (7).

Functionally, calmodulin has been shown to activate specific forms of phosphodiesterase (8), adenylate cyclase (9), and (Ca^{2+} + Mg^{2+})-ATPase (5) in brain. It also is involved in calcium-dependent phosphorylation of synaptic membranes (10), in axonal transport (11), and in the release of neurotransmitters (6).

Since calmodulin can influence a wide variety of neuronal functions, agents that alter its activity might have profound pharmacological effects on the nervous system. The calmodulin-inhibitory compounds that have been described so far include calcium chelators (12), a miscellaneous group of lipophilic compounds (13, 14), certain psychotropic agents (1), and a few endogenous proteins (15, 16).

A study of the influence of various psychotropic drugs on the activity of calmodulin showed that the most potent of these drugs are those that have in common the ability to reduce schizophrenic symptomatology (17, 18). These antipsychotic drugs prevent several actions of calmodulin such as its activation of phosphodiesterase (19, 20), (Ca^{2+} + Mg^{2+})-ATPase (21), protein kinase (13), adenylate cyclase (9), and phospholipase A_2 (22) as well as many other calmodulin-dependent processes (18).

The mechanism by which antipsychotic drugs block these calmodulin-dependent enzymes and processes is through a direct interaction of these agents with calmodulin (17, 23). This binding is reversible, calcium-dependent, saturable, and relatively selective for calmodulin (23, 24).

The proteins isolated from brain that have been shown to inhibit the activity of calmodulin include a heat-labile, calcium-binding protein termed calcineurin (15), a heat-stable protein (16), and a particulate protein found in synaptic membranes (5).

Since certain neuropeptides have been reported to exhibit behavioral effects (25), it was of interest to determine whether they might also interact with calmodulin. The present report shows that the neuropeptides β-endorphin and dynorphin share certain biochemical characteristics with the antipsychotic drugs in that they block the calmodulin-induced activation of phosphodiesterase and that this blockade is apparently due to a direct calcium-dependent interaction of the peptides with calmodulin.

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MATERIALS AND METHODS

Calmodulin was prepared from bovine brain as described by Teo *et al.* (26). A calmodulin-sensitive form of phosphodiesterase was isolated by preparative gel electrophoresis (11) from areas of rat brain (caudate nucleus, olfactory tubercle, and frontal cortex) rich in this form of the enzyme (8). Phosphodiesterase activity was measured in the absence or presence of calmodulin (1 unit/sample) by a modification of the luciferin-luciferase technique (27). One unit of calmodulin is defined as the amount required to produce 50% of the maximal activation of phosphodiesterase. One unit of calmodulin was approximately equal to 0.6 pmole of calmodulin.

To determine the effects of the peptides on calmodulin activity, various concentrations of the peptides were added to preparations of an activable form of phosphodiesterase. Enzyme activity was determined in the absence of calmodulin (basal or unactivated) or in the presence of 1 unit of calmodulin (activated). The concentration of peptide that reduced either the basal or the calmodulin-induced activation of phosphodiesterase by 50% is defined as the IC_{50} value. IC_{50} values were determined graphically using a linear transformation of the data (28).

The binding of β-endorphin to calmodulin was determined by equilibrium dialysis by a modification of a technique described previously (23). Calmodulin was dialyzed to equilibrium at 4° against varying concentrations of ^{125}I-labeled β-endorphin in a buffer containing 5 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$, and either 0.1 mM $CaCl_2$ or 0.3 mM EGTA.[1] After the samples reached equilibrium, the radioactivity bound to calmodulin was determined with a γ spectrometer.

To determine whether chlorpromazine could interfere with the binding of β-endorphin to calmodulin, chlorpromazine was irreversibly bound to calmodulin by ultraviolet light photoaffinity labeling according to the procedure of Prozialeck *et al.* (29). The samples were then dialyzed overnight with two changes of buffer to remove the free chlorpromazine, and the binding of β-endorphin to calmodulin was measured as described above.

α-Endorphin, Met-enkephalin, myokinase, and pyruvate kinase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); β-endorphin and (des-Tyr^1)-γ-endorphin from Beckman Instruments, Inc., Biological and Fine Chemicals Division (Palo Alto, Calif.); Leu-enkephalin and dynorphin from United States Biochemical Corporation (Cleveland, Ohio); ^{125}I-labeled β-endorphin from Immunonuclear Corporation (Stillwater, Minn.); and lactalbumin, bovine serum albumin, and histone from Sigma Chemical Company (St. Louis, Mo.). Troponin-C was kindly supplied by Dr. J. Potter, University of Cincinnati. S-100 was kindly supplied by Dr. R. Kretsinger, University of Virginia.

RESULTS

Effect of neuropeptides on calmodulin-induced activation of phosphodiesterase. Figure 1 shows the effects of β-endorphin on an activable form of phosphodiesterase as measured in the absence or presence of calmodulin. As may be seen, concentrations of β-endorphin that totally inhibited the activation of phosphodiesterase induced by calmodulin had no significant effect on the basal phosphodiesterase activity. The concentration of β-endorphin that reduced the activation of phosphodiesterase by 50% (IC_{50} value) was approximately 3.2 μM.

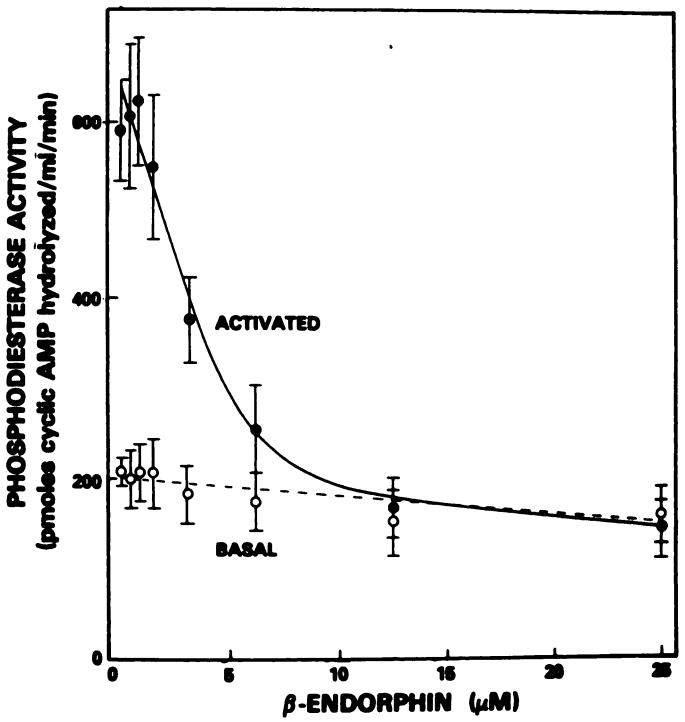

FIG. 1. *Effect of β-endorphin on calmodulin-induced activation of phosphodiesterase*

An activable phosphodiesterase was prepared from rat brain and assayed for phosphodiesterase activity in the absence and presence of calmodulin (1 unit) and varying concentrations of β-endorphin. Each point represents the mean of five determinations. Vertical brackets indicate the standard error.

Similar experiments were performed to determine the effects of other opioid peptides on the basal and calmodulin-stimulated phosphodiesterase activity. The IC_{50} values for these compounds are shown in Table 1. Of the compounds studied, only β-endorphin and dynorphin produced 50% inhibition of calmodulin-stimulated phosphodiesterase activity at concentrations up to 50 μM. None of the peptides, including β-endorphin and dynorphin, altered the basal phosphodiesterase activity at these concentrations.

The inhibitory effects of β-endorphin on calmodulin were not due to calcium chelation, since increasing the concentration of calcium had no effect on the action of β-endorphin. By contrast, the inhibitory actions of β-endorphin could be overcome by increasing amounts of calmodulin (Fig. 2); higher concentrations of calmodulin were required to antagonize the inhibitory effects of 25 μM β-endorphin than of 3 μM β-endorphin.

Other studies showed that the opiate antagonist naloxone, even at concentrations up to 500 μM, failed to

[1] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid.

TABLE 1

Effect of opioid peptides on calmodulin-induced stimulation of phosphodiesterase activity of rat brain

The activity of an activable form of phosphodiesterase was measured in the absence (unactivated) or presence (activated) of 1.0 unit of calmodulin and various concentrations of the compounds under study. These values represent the average of three experiments in which five concentrations of each drug were studied, with five replicates at each concentration. The IC_{50} values were determined graphically.

Peptide	IC_{50}	
	Activated	Unactivated
	μM	
β-Endorphin	3.4	>50
Dynorphin (1–13)	10	>50
α-endorphin	>50	>50
(des-Tyr¹)-γ-endorphin	>50	>50
Met-enkephalin	>50	>50
Leu-enkephalin	>50	>50

inhibit the activation of phosphodiesterase induced by calmodulin and failed to prevent the inhibition of the action of calmodulin produced by 3 μM β-endorphin (data not shown).

Binding of β-endorphin to calmodulin. To determine whether β-endorphin could bind directly to calmodulin, samples of calmodulin were dialyzed against varying concentrations of ^{125}I-labeled β-endorphin in the presence of either 0.3 mM EGTA or 0.1 mM $CaCl_2$. Because of the slow penetration of β-endorphin through the dialysis membrane, equilibrium was not attained until 6 days. At this time, the radioactivity associated with calmodulin was determined in a γ spectrometer. Figure 3A shows the binding of β-endorphin to calmodulin in the presence and absence of calcium. Binding in the presence of EGTA was relatively low and increased linearly with increasing concentrations of β-endorphin. By contrast, the binding of β-endorphin to calmodulin when measured in the presence of calcium was severalfold greater and was saturable. A Scatchard analysis (30) of the calcium-dependent binding of β-endorphin to calmodulin yielded an apparent dissociation constant (K_d) of 2.5 μM and a maximal binding (B_{max}) of approximately 0.96 mole of β-endorphin bound per mole of calmodulin (Fig. 3B). An average of two similar experiments resulted in a K_d value of 4.6 μM and a B_{max} of 1.3 moles of β-endorphin bound per mole of calmodulin.

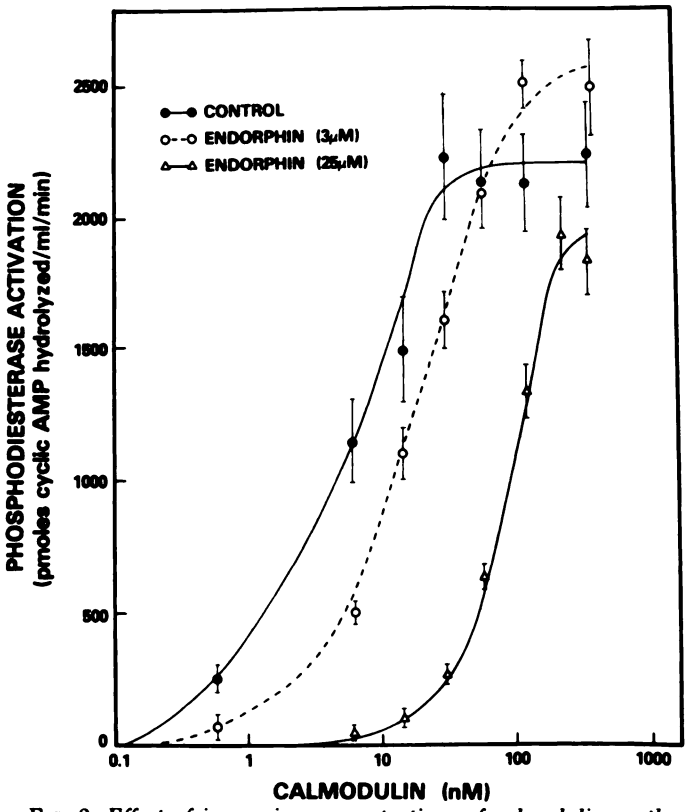

FIG. 2. *Effect of increasing concentrations of calmodulin on the β-endorphin-induced inhibition of phosphodiesterase activation*

The increase in phosphodiesterase activity induced by varying concentrations of calmodulin was assayed in the absence of β-endorphin (control) or in the presence of 3 μM and 25 μM β-endorphin. The basal phosphodiesterase activity, that is, that measured in the absence of calmodulin, was 400 pmoles of cyclic AMP hydrolyzed per milliliter per minute. Each point represents the mean of five determinations. Vertical brackets indicate the standard error of the mean.

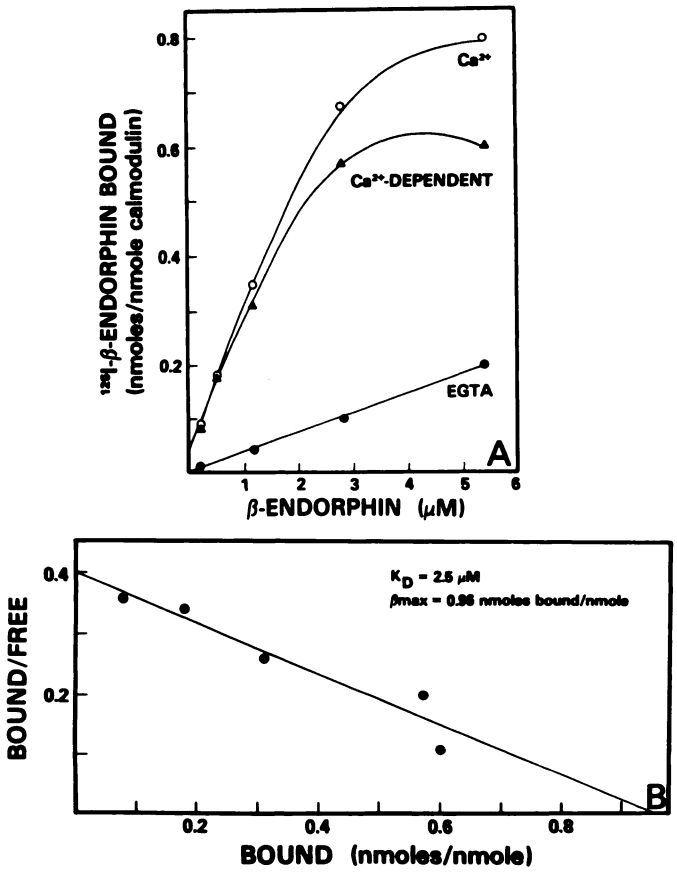

FIG. 3. *Binding of β-endorphin to calmodulin*

A. Calmodulin (7 μg) was dialyzed to equilibrium against varying concentrations of ^{125}I-labeled β-endorphin in the presence of 0.1 mM Ca^{2+} or 0.3 mM EGTA. Each point represents the average of two determinations. Data were plotted according to the Scatchard equation (29).

B. Calcium-dependent binding of β-endorphin to calmodulin. *Bound* = moles of β-endorphin bound per mole of calmodulin; *Free* = concentration of free β-endorphin at equilibrium. The K_d value for the binding of β-endorphin to calmodulin was 2.5 μM. The B_{max} was 0.96 mole of β-endorphin bound per mole of calmodulin.

Figure 4 shows that the calcium-dependent binding of β-endorphin to calmodulin increased linearly with increasing amounts of calmodulin.

To determine the relative specificity of the binding of β-endorphin to calmodulin, several other proteins were dialyzed to equilibrium against radiolabeled β-endorphin. In this experiment, 7 μg each of calmodulin, bovine serum albumin, lactalbumin, histone, or troponin-C or S-100 were dialyzed against 0.4 μM ^{125}I-labeled β-endorphin in the presence of either 0.3 mM EGTA or 0.1 mM $CaCl_2$, and the amount of β-endorphin bound to each protein was determined. In the presence of calcium, calmodulin and its close homologues troponin-C and S-100 bound a significant amount of β-endorphin (Table 2). Neither of the two other acidic proteins, bovine serum albumin or lactalbumin, nor the basic protein histone showed any significant calcium-dependent binding to β-endorphin. In the absence of calcium, all proteins studied bound β-endorphin to a small degree (0.1 nmol/mg of protein).

Effects of Met-enkephalin and chlorpromazine on the binding of β-endorphin to calmodulin. To determine whether the opiate peptide Met-enkephalin could inhibit the binding of ^{125}I-labeled β-endorphin to calmodulin, we dialyzed samples of calmodulin against 0.6 μM ^{125}I-labeled β-endorphin and varying concentrations of Met-enkephalin. At concentrations up to 130 μM, Met-enkephalin failed to reduce the calcium-dependent binding of β-endorphin to calmodulin.

Since antipsychotics and β-endorphin inhibit calmodulin-stimulated phosphodiesterase and since both bind directly to calmodulin, we determined whether the phenothiazine could prevent the binding of β-endorphin to calmodulin. Samples of calmodulin were irradiated in the presence of varying concentrations of chlorpromazine. These samples were then dialyzed overnight to remove the unbound drug, and the binding of ^{125}I-labeled β-endorphin to calmodulin was determined. As may be seen in Fig. 5, chlorpromazine produced a concentration-dependent inhibition of the calcium-dependent binding of β-endorphin to calmodulin (IC_{50} = 22 μM). In the absence of calcium, chlorpromazine failed to inhibit the binding of β-endorphin until higher concentrations of the phenothiazine were reached (IC_{50} = 100 μM).

TABLE 2

Calcium-dependent binding of ^{125}I-labeled β-endorphin to various proteins

Various proteins were dialyzed to equilibrium against 0.4 μM ^{125}I-labeled β-endorphin in the presence of either 0.1 mM calcium or 0.3 mM EGTA. Calcium-dependent binding is defined as the difference between β-endorphin bound in the presence and absence of calcium. Each value represents the mean of three separate determinations.

Protein	β-Endorphin bound
	nmoles/mg protein
Bovine brain calmodulin	12.4
S-100	10.4
Troponin-C	5.1
Bovine serum albumin	—
Lactalbumin	—
Histone	—

DISCUSSION

The present results show that certain opioid peptides, notably β-endorphin and dynorphin, inhibit the activation of phosphodiesterase induced by calmodulin. The mechanism by which β-endorphin blocked the action of

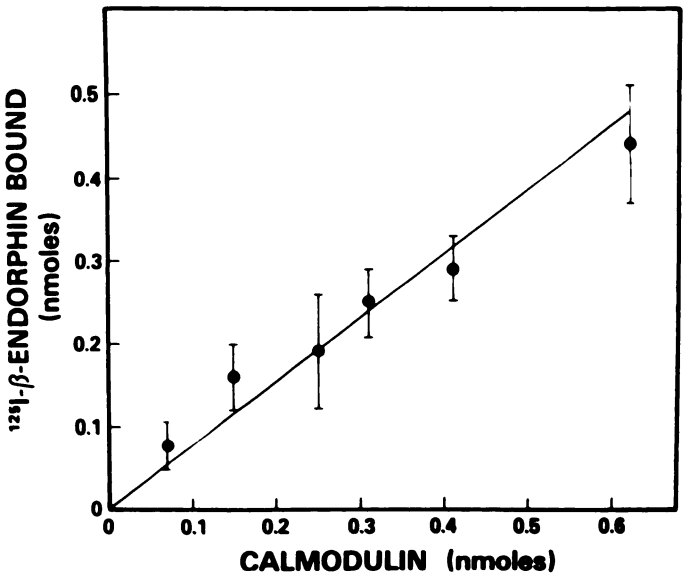

FIG. 4. *Binding of ^{125}I-labeled β-endorphin to calmodulin as a function of the concentration of calmodulin*

Various concentrations of calmodulin were dialyzed to equilibrium against 2.5 μM ^{125}I-labeled β-endorphin in the presence of either 0.1 mM Ca^{2+} or 0.3 mM EGTA. The data represent the mean ± standard error of four determinations of the calcium-dependent binding.

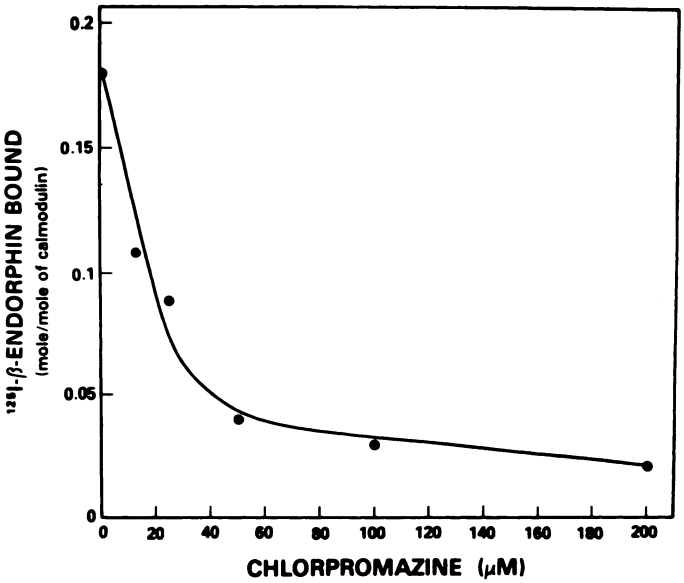

FIG. 5. *Inhibition by chlorpromazine of the binding of β-endorphin to calmodulin*

Calmodulin (60 μg/ml) and varying concentrations of chlorpromazine were irradiated with ultraviolet light for 30 min. After dialyzing the samples overnight, the binding of ^{125}I-labeled β-endorphin to calmodulin was determined by equilibrium dialysis in the presence of either 0.3 mM EGTA or 0.1 mM $CaCl_2$. The calcium-dependent binding of β-endorphin to calmodulin, shown on the ordinate, is the mean of three determinations.

calmodulin was through a direct calcium-dependent binding to calmodulin. This interaction was relatively selective, since the structurally related compounds α-endorphin, (des-Try[1])-γ-endorphin, and Leu- and Met-enkephalin failed to interfere with the action of calmodulin at concentrations at which β-endorphin totally inhibited the effects of calmodulin. Furthermore, there was no significant calcium-dependent binding of β-endorphin to bovine serum albumin, lactalbumin, or histone. However, β-endorphin did bind to S-100 and troponin-C, structural homologues of calmodulin.

Since calmodulin is an acidic protein and would be negatively charged at pH 7.0, one might expect that certain basic substances which would be positively charged at that pH could interfere with its actions. Indeed, several basic proteins and peptides have been reported to inhibit the calmodulin-induced activation of phosphodiesterase. These include, in addition to β-endorphin and dynorphin, ACTH 1-24 (18), histone (31, 32), myelin basic protein (31), and several synthetic polylysine and polyarginine peptides (32). However, for this interaction to occur, other physical and chemical properties besides the basic nature of a peptide must be important since several other basic proteins and peptides failed to inhibit calmodulin-stimulated phosphodiesterase activity. These include bradykinin, phosphorylase B, spermine, putrescene (32), substance P, arginine-vasopressin, neurotensin, and α-melanocyte-stimulating hormone (18).

Another factor that should be considered in explaining the interaction between β-endorphin and calmodulin is the hydrophobic nature of the peptides. In the presence of calcium, calmodulin undergoes a conformational change, exposing a hydrophobic site on the molecule (33), and β-endorphin has been reported to interact with a number of lipids (34).

Since both antipsychotic drugs and β-endorphin inhibit calmodulin by binding to it, and since both types of compounds are lipophilic and positively charged at physiological pH, these two characteristics apparently are important for inhibition of calmodulin. However, these are probably not the only properties a compound must possess for them to inhibit calmodulin; other physical, chemical, or structural requirements will surely come to light with additional experimentation.

The biological significance of inhibiting calmodulin by β-endorphin is still unclear. The concentrations of β-endorphin required to inhibit calmodulin are far greater than those found in most brain areas (35) but are of the same order of magnitude as those needed to produce analgesia, catatonia, and hypothermia, and the inhibition of neurotransmitter release (see ref. 35). However, few if any of these pharmacological actions are likely to be explained by the ability of β-endorphin to inhibit calmodulin, since naloxone blocks most of the pharmacological effects of β-endorphin but failed to inhibit the action of β-endorphin on calmodulin.

On the other hand, β-endorphin has been shown to produce certain behavioral effects that are not blocked by naloxone (36). Therefore, it is tempting to speculate that β-endorphin may have at least two sites of action: one on the opiate receptor that may account for its analgesic activity and which is blocked by naloxone, and another site, perhaps on calmodulin, that may explain other actions unrelated to analgesic activity.

In this regard, it should be noted that the action of β-endorphin on calmodulin is strikingly similar to the action of antipsychotic drugs on this calcium-binding protein. Both block the activation of phosphodiesterase by calmodulin at concentrations that have no effect on basal activity (17, 18). Furthermore, as in the case of antipsychotic drugs, the inhibition of calmodulin-stimulated phosphodiesterase by β-endorphin is not overcome by excess Ca^{2+} but can be overcome by increasing concentrations of calmodulin. Indeed, the mechanisms by which neuroleptic drugs and β-endorphin block phosphodiesterase activity appear similar. Both bind directly to calmodulin, and this binding is increased in the presence of calcium. Moreover, the calcium-dependent binding of β-endorphin and antipsychotics showed similar selectivity, since both bound to troponin-C to a lesser degree than they bound to calmodulin and neither showed any significant calcium-dependent binding to several other proteins. Finally, our results showing that chlorpromazine can prevent the binding of β-endorphin to calmodulin suggest the possibility that these two compounds might be acting at a similar site on the calcium-binding protein.

In conclusion, our results show that two neuropeptides, β-endorphin and dynorphin, inhibit the calmodulin-induced stimulation of phosphodiesterase without altering the basal phosphodiesterase activity. β-Endorphin produces this effect by binding directly to calmodulin in a calcium-dependent manner. This interaction may explain not only the inhibition of calmodulin-stimulated phosphodiesterase activity but also other effects of β-endorphin such as its inhibition of calmodulin-dependent protein phosphorylation (10, 37). The evidence that both antipsychotic drugs and β-endorphin inhibit calmodulin-dependent processes supports the suggestion that β-endorphin or a related compound may play a role in certain forms of mental disorders.

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