Calmodulin is a potent target for new hypothalamic neuropeptides

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Recently, five glycopeptides with coronaro-constrictory properties were isolated from bovine hypothalamus [(1988) Neurochemistry (USSR) 7, 519–524]. Calmodulin has been recognized in our laboratory as a target protein for the neuropeptides isolated from hypothalamus. The results of indirect enzyme-linked immunosorbent assay have shown that the new hypothalamic neuropeptides antagonize with the monospecific anti-calmodulin antibody for calmodulin binding although they are not fragments of calmodulin. The inhibitory potency of the peptides is dependent on their concentration and the length of the polypeptide chain. Four out of five peptides are effective in nM concentration range. Ca^{2+} stimulates the binding of peptides to calmodulin; however, immunocomplex can be formed in the absence of Ca^{2+} as well. The effects of trifluoperazine and peptides on the calmodulin/antibody interaction are not additive, suggesting the cooperativity between the binding sites on calmodulin. Under physiological conditions the presence of the peptides could produce distinct conformers of calmodulin which may exhibit altered potency for stimulation/inhibition of target enzymes.

Anti-CaM activity; Neuropeptide; Enzyme-linked immunosorbent assay; Cooperative binding

1. INTRODUCTION

Galoyan and his coworkers [1] isolated five neuropeptides with molecular masses of 3, 3.5, 3.5, 3.5 and 7 kDa from bovine hypothalamus. Determination of amino acid compositions have shown their high Ser, Gly and Glu content. Both acidic and enzymatic hydrolyses resulted in the loss of activity of these peptides [2]. Recently, it has been shown that two of these peptides stimulate the calmodulin-sensitive activities of PDE and that all stimulate the MLCK activity in the absence of Ca^{2+} [2,3]. This finding may indicate that the effective peptides could perform the function of endogenous substitutes of Ca^{2+} [4].

CaM is involved in the regulation of numerous Ca^{2+}-mediated events [5,6] and it can mediate activation/inhibition of target enzymes; however, the precise mechanism of its action regulating metabolic processes has not been defined ([7] and references therein). Anti-CaM drugs prevent or modify the interactions of CaM with target enzymes. The binding sites for a classic CaM antagonist, TFP, in the N- and C-terminal domains of CaM are identified [8]. The C-terminal domain of CaM has been shown to be the important region for immunoreactivity [9].

In this work we have investigated the anti-CaM activity of the coronaro-constrictory peptides using a sensitive immunochemical approach which requires small quantity of the hypothalamic neuropeptides. This approach renders it possible to detect the binding of peptides to CaM, to analyse the mutual effect of the neuropeptides and TFP on the immunocomplex formation and to study the Ca^{2+}-sensitivity of the binding.

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Abbreviations: CaM, calmodulin; TFP, trifluoperazine; ELISA, enzyme-linked immunosorbent assay; MLCK, myosin light chain kinase; PDE, phosphodiesterase; IgG, immunoglobulin

2. MATERALS AND METHODS

CaM from human erythrocytes was purified to homogeneity using trifluoperazine-Sepharose-4B chromatography [10]. Rabbits were immunized with 1-fluoro-2,4-dinitrobenzene derivatized bovine CaM as described in [11]. The IgG fraction was isolated from the antiserum using the QAE-Sephadex A-50 (Pharmacia) chromatographic method [12]. Monospecific antibodies were further purified on a CaM-Sepharose-4B affinity column [14,15].

Standard ELISA protocols [14] for indirect assays were used with some modifications as follows. *Indirect assay:* coating of plate with human CaM was done for 18 h at 6°C. Antibodies were diluted in wells, and the plate was allowed to stand for 2 h at room temperature. The goat anti-rabbit globulin conjugated with horseradish peroxidase (Human, Budapest, Hungary) at 1000 times dilution was allowed to interact for 2 h at room temperature. *o*-Phenylenediamine, with peroxide added, was used as substrate solution. Plates were read at 492 nm after 1 h of substrate hydrolyzis time. The OD_{492nm} values are the average of least five measurements. Error of determination is ± 10%.

Peptides were isolated from bovine hypothalamus by gel-filtration on Sephadex G-10, ion-exchange chromatography on Dowex 50W × 8, and high-performance liquid chromatography on Nucleosil C_{18} semipreparative reverse-phase column (0.46 × 25 cm) [1].

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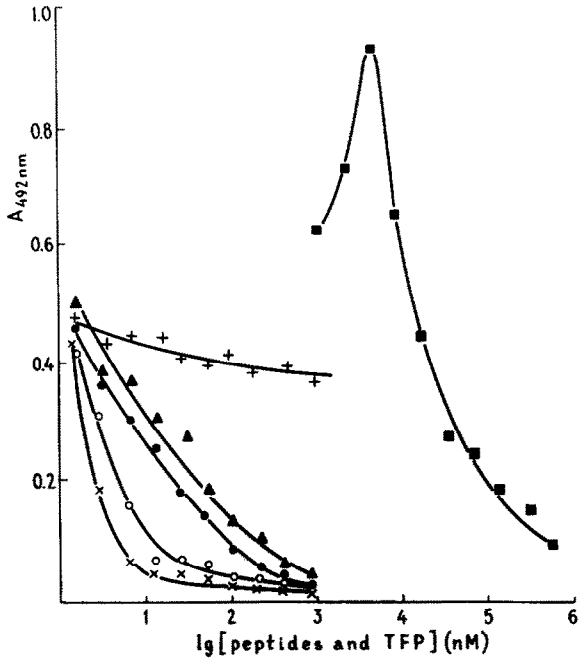

Fig. 1. Displacement curves of peptides and TFP at 2.5 μg/ml coating concentrations of CaM and 7.5 μg/ml antibody concentration. For other details see section 2. (×) P_1; (○) P_2; (●) P_3; (▲) P_4; (+) P_5; (■) TFP.

3. RESULTS AND DISCUSSION

3.1. *The binding of peptides and TFP to CaM*

Enzyme-linked immunoassay has been found to be especially sensitive for the detection of binding of enzymes or ligands like drugs to CaM [7]. We have demonstrated previously [13] that the sera did not form precipitine lines in double diffusion with CaM, and 'sandwich' ELISA [14] was nonreactive when anti-CaM IgG was applied. These observations, in accordance with the literary data, suggest that only major antigenic site is present on the CaM molecules [11].

Table I

Effect of neuropeptides and CaM on antibody/anti-antibody interactions

Additions (μM)	[$Ab_{immobilized}$] (μg/ml)	Dilution of anti-antibody	OD_{492nm}
no	5	1:2000	-
no	5	1:1000	-
no	5	1:500	-
no	10	1:1000	0.130
no	10	1:500	0.230
CaM (0.15)	10	1:500	0.165
P_2 (0.207)	10	1:500	0.230
P_2 (0.006)	10	1:500	0.230
P_4 (0.207)	10	1:500	0.230
P_4 (0.006)	10	1:500	0.220

For details see section 2.

We have used indirect ELISA to investigate whether coronaro-constrictory peptides can display anti-CaM activity. On the basis of the optimization experiments, 2.5 μg/ml CaM and 7.5 μg/ml antibody were found to ensure the very sensitive detection of relationships of antigen/antibody [13]. Fig. 1 shows the effect of neuropeptides (P_1–P_5) on the immunocomplex formation in a range of peptide concentrations. The dilution curve of a 'classic' CaM antagonist, TFP, was also measured under the same conditions. Binding of antibody to CaM was agressively inhibited by increasing the concentrations of P_1–P_4; however, P_5 exhibited much less effect. The concentrations inducing 50% inhibition (I_{50}) of the formation of immunocomplex are 2.5, 4.6, 15.8 and 31.6 nM, for P_1, P_2, P_3 and P_4, respectively. Therefore, it can be concluded that (i) P_1–P_4 are much more potent inhibitors of the immunocomplex formation than TFP; (ii) while the peptides exhibit simple displacement curves, the effect of TFP on the binding of antibody to CaM seems to be more complex [7]. The anti-CaM activity of the peptides can be interpreted in two different ways: (i) the peptides form a complex with CaM and inhibit the formation of immunocomplex; (ii) the peptides as real or apparent fragments of CaM bind directly to antibody and the binding sites are not accessible to the intact CaM. To distinguish between these two possibilities, the binding of peptides to antibody has been tested. To solid-phase immobilized antibody, anti-antibody was added at subsaturating concentrations in the absence and the presence of peptides (Table I). The presence of peptides did not perturb anything; however, CaM impedes the binding of anti-antibody to antibody. This observation together with the data presented in Fig. 1 suggests that the peptides are not the fragments of CaM. This idea is also supported by the comparison of amino acid compositions of peptides and segments of CaM [1].

It can be seen from the results that the inhibitory potency of the peptides decreased with increasing length of the polypeptide chain. P_5, having about the double molecular mass (M_w 7000) compared to the other four peptides, has much less effect on antibody binding to CaM. Although the sequences of the neuropeptides have not been determined, their amino acid compositions suggest that the smaller peptides cannot be deduced from the larger.

To investigate whether the binding of peptides to CaM interferes with that of TFP, they were added together to the ELISA mixture. At constant concentrations of the peptides which induce 50% inhibition of the formation of CaM-antibody complex, the concentrations of TFP were varied (P_5 was used in higher concentration: 100 nM). As shown in Fig. 3, while P_5 had practically no effect on the TFP dilution curve (cf. Fig. 1), the other peptides could eliminate partially or completely the activating effect of TFP on the antibody binding

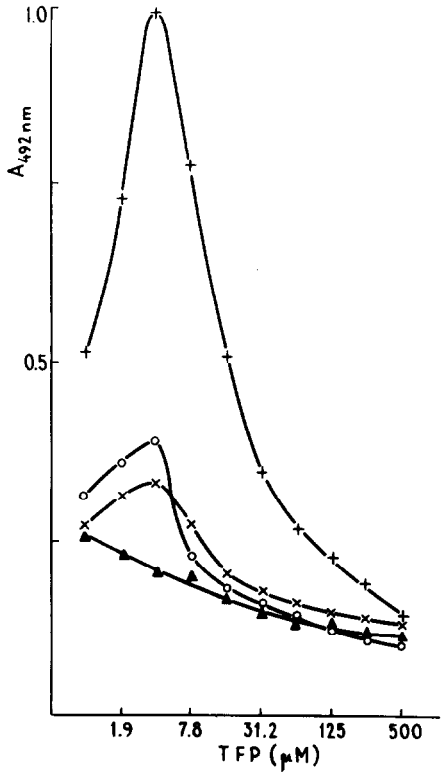

Fig. 2. Mutual effects of TFP and peptides on the binding of antibody to CaM. The concentrations of CaM and antibody were 2.5 μg/ml and 7.5 μg/ml, respectively. (A) The concentration of peptides was kept constant: 2.5, 4.6, 15.8 and 31.6 nM for P_1 (×), P_2 (○), P_4 (▲) and P_5 (+), respectively. For other details see the text.

to CaM. As a simple interpretation of these experiments, only one binding site for the peptides has been assumed. It is known from the literature that both N- and C-terminal domains of CaM have one TFP binding site each [15], and the monospecific-antibody interacts with the antigenic site in C-terminal domain [16]. While the binding of TFP to the N-terminal domain may stimulate the binding of antibody to CaM, the binding of TFP to the C-terminal domain may compete with antibody for CaM binding [7]. The binding of the peptides to CaM prevents the immunocomplex formation. Since the effect of the peptides on the antibody binding to CaM is more pronounced in the presence of low concentrations of TFP than in its absence or in the presence of high concentrations of TFP, it can be hypothesized that the peptides and TFP at low concentrations can bind simultaneously to CaM, and within this ternary complex CaM loses the ability to interact with antibody. Therefore, it seems likely that the binding sites for peptides and TFP are different and interact in a cooperative manner on the surface of CaM.

3.2. *Effect of Ca^{2+} on the binding of peptides to CaM*

In order to investigate the role of Ca^{2+} in the interaction between CaM and neuropeptides, we analysed the formation of the immunocomplex in the presence of peptides under various conditions. Control experiments showed that the formation of CaM-antibody complex is Ca^{2+}-dependent; however, the presence of Ca^{2+} was important merely during but not after the coating procedure (data not shown). This finding can be interpreted to mean that CaM molecules could, at least partly, preserve their active conformations induced by Ca^{2+} in solid-phase bound form.

We have carried out two sets of experiments with neuropeptides. A representative experiment carried out with P_2 is presented in Fig. 3. In one set Ca^{2+}-CaM complex was coated to the plate and the assay was further done in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. The binding of antibody to CaM was inhibited by peptide; however, the degree of inhibition does not depend on the presence of Ca^{2+} in solution. In the second set of experiments, CaM was coated to the plate in the absence of Ca^{2+}, then the mixture of the peptide and antibody was added to the solid-phase bound CaM in the presence of 1 mM Ca^{2+} or 1 mM EGTA. As shown in Fig. 3, the inhibitory effect of peptides manifests itself at higher peptide concentrations if the assay did not contain Ca^{2+} at all. Since the inhibition percentages are referred to the corresponding control measured in the absence of peptide; thus, it can be concluded that although neuropeptides can bind to Ca-free CaM, the peptides display higher affinity toward Ca^{2+}-CaM complex. Investigations with other peptides showed similar results (data not shown).

It is well-known that CaM antagonists like TFP bind

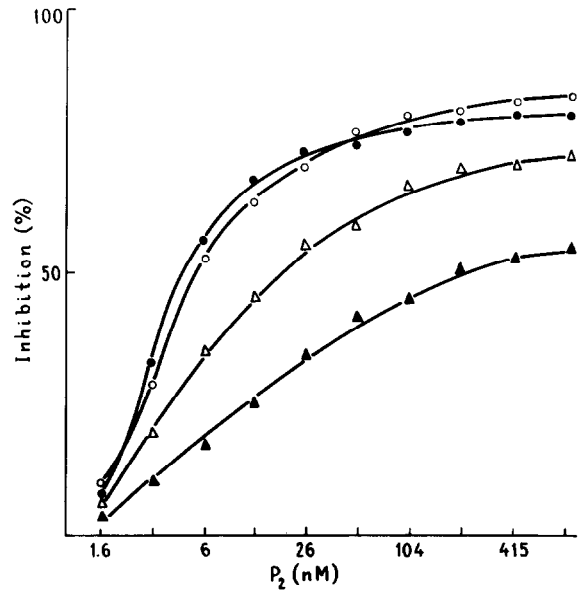

Fig. 3. Effect of Ca^{2+} on the binding of P_2 to CaM. The concentrations of CaM and antibody were 2.5 μg/ml and 7.5 μg/ml, respectively. CaM was coated to the microplate in the presence of 1 mM Ca^{2+}, and the assay contained 1 mM Ca^{2+} (●) or 1 mM EGTA (○). CaM was coated to the microplate in the absence of 1 mM Ca^{2+}, and the assay contained 1 mM Ca^{2+} (△) or 1 mM EGTA (▲). For other details see section 2.

to CaM in a Ca^{2+}-dependent manner [6,7] and that this binding can be enhanced by interaction of CaM antagonists at distinct sites via an allosteric mechanism. Newton [17] has observed increased rates of incorporation of affinity label when TFP was added at low concentration. We have previously shown that CaM can interact simultaneously with target enzyme and fendiline [18]. In this study evidence is presented that the neuropeptides inhibit not only the binding of antibody to CaM, but also eliminate the activating effect of TFP. Therefore, a combined binding of peptides and TFP to CaM has been assumed which induces the formation of a distinct ternary structure for CaM. In addition, it can be hypothesized that the concentrations of both neuropeptides and Ca^{2+} could be responsible for the regulation of the ratio of the active and inactive conformers of CaM. Thus, the neuropeptides from the hypothalamus as ellosteric regulators of CaM could alter its sensitivity to drugs and influence selectivity for CaM binding to its target proteins.

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