STIMULATION OF ENZYME ACTIVITIES BY FRAGMENTS OF CALMODULIN

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1. Introduction

Calmodulin is a calcium-binding protein which activates a number of Ca^{2+}-dependent enzymes, and is a major cytoplasmic calcium receptor of eukaryotic cells [1–4]. It is unique in its ability to interact with so many different proteins, and this raises the question of which regions in the molecule are responsible for its biological properties. Calmodulin possesses 4 binding sites for Ca^{2+}, each associated with a particular region of the primary structure [4]. One approach which can be used to investigate this problem is therefore to isolate peptide fragments containing particular Ca^{2+} binding sites [5,6], and to test whether they can activate calmodulin-dependent enzymes. In this paper the ability of such fragments to activate phosphorylase kinase and cyclic nucleotide phosphodiesterase has been compared, as well as their ability to substitute for troponin-C in neutralizing the inhibition of actomyosin-ATPase by troponin-I. These studies demonstrate that more than one region of the calmodulin molecule is capable of interacting with its target proteins, and that different calmodulin-dependent proteins do not interact with calmodulin in an identical manner.

2. Materials and methods

2.1. *Preparation of proteins and fragments of calmodulin and troponin-C*

Calmodulin and cyclic nucleotide phosphodiesterase were isolated from bovine brain as in [7], and phosphorylase kinase from rabbit skeletal muscle as in [8]. The subunits of troponin [9], actin [10], myosin [11] and tropomyosin [12] were isolated from rabbit skeletal muscle by standard procedures.

Fragments of calmodulin were obtained by controlled tryptic digestion and isolated by polyacrylamide gel electrophoresis in the presence of urea as in [5,6]. Different tryptic fragments were obtained depending on whether Ca^{2+} (TR-C peptides) or EDTA (TR-E peptides) was present during the digestions. These fragments contained different Ca^{2+}-binding regions and their compositions are illustrated in fig.1. Protein concentrations were determined by amino acid analysis.

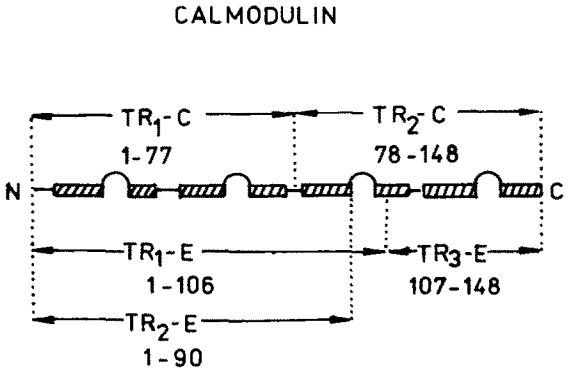

Fig.1. Compositions of the different fragments of calmodulin. TR-C fragments are produced by limited tryptic digestion in the presence of Ca^{2+}, and TR-E fragments by digestion in the presence of EDTA. The 4 Ca^{2+}-binding domains are represented by the hatched areas.

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2.2. Measurements of enzyme activities

(a) Actomyosin ATPase was measured at 25°C by the phosphate released from ATP as in [13]. The incubation mixture (1.0 ml) contained 0.2 mg tropomyosin/ml, 0.06 mg troponin-I/ml, 10 mM Tris–HCl (pH 7.5), 1.0 mM dithiothreitol, 1.0 mM $MgCl_2$, 30 mM KCl and 1.0 mM ATP. The assays also contained either troponin-C, calmodulin, or tryptic fragments derived from these proteins, and either 1.0 mM EGTA or 0.1 mM $CaCl_2$. The reactions were initiated with ATP and terminated after 5 min by the addition of sodium dodecyl sulphate to 1%.

(b) Phosphorylase kinase was assayed at pH 6.8 and 30°C as in [14] using *N'*-(2-hydroxymethyl)-ethylene diamine-*N*,*N'*,*N'*-triacetate to buffer the free Ca^{2+} concentration at 0.05 mM. The assays were carried out in the presence and absence of calmodulin or calmodulin fragments.

(c) The activity of cyclic nucleotide phosphodiesterase was determined in the presence of 0.1 mM $CaCl_2$ by a two stage assay, the phosphate liberated in the second stage being measured as in [15].

3. Results

3.1. Neutralization of the inhibition of actomyosin ATPase by troponin-I using calmodulin fragments

Calmodulin can replace troponin-C in neutralizing the inhibition of actomyosin ATPase by troponin-I [16]. As reported briefly [17], troponin-C fragments can substitute for intact troponin-C in this reaction and the most effective fragments are TR_2-C and TR_1-E. Fragments of calmodulin behave similarly to the homologous peptides of troponin-C, although the effect of the TR_1-E fragment is somewhat weaker than that of the TR_2-C fragment (fig.2). These and the results in [5,6] concerning the interaction of calmodulin fragments with troponin-I under the condition of urea–polyacrylamide gel electrophoresis, implicate residues 78–90 (which are common to both the TR_1-E and TR_2-C fragments) as an important region in the binding of calmodulin to troponin-I.

3.2. Activation of phosphorylase kinase by fragments of calmodulin

Phosphorylase kinase is a Ca^{2+}-dependent enzyme whose dependence on Ca^{2+} is conferred by a tightly

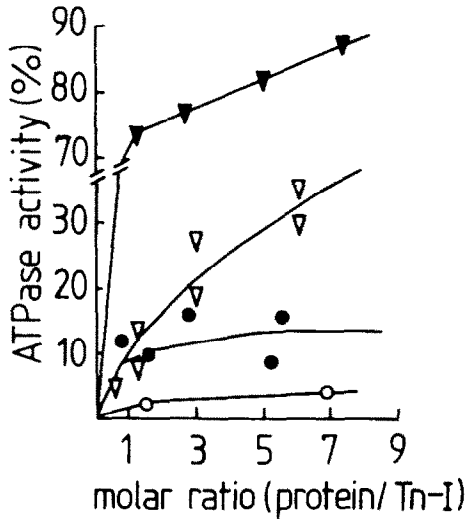

Fig.2. Neutralization of troponin-I (Tn-I) inhibition of actomyosin ATPase by calmodulin and its tryptic fragments. Assays were carried out in the presence of Ca^{2+} as in section 2. The activity of actomyosin ATPase in the presence of Tn-I was taken as 0% and in the absence as 100%: (▼) calmodulin; (▽) TR_2-C; (●) TR_1-E; (○) TR_1-C.

bound molecule of calmodulin, termed the δ-subunit. The enzyme is, however, activated a further 5-fold at pH 6.8 and saturating concentrations of Ca^{2+} by interaction with a second molecule of calmodulin (termed the δ'-subunit) [14]. Half-maximal activation by the δ'-subunit occurs at 1.0×10^{-8} M calmodulin. The calmodulin fragments TR_2-C, TR_1-E and TR_1-C were found to be capable of activating phosphorylase kinase, whereas fragment TR_3-E was completely inactive (fig.3). Half-maximal activation occurred at 9×10^{-7} M for TR_2-C and 2×10^{-5} M for both TR_1-C and TR_1-E. These fragments were therefore 100- and 2000-fold less effective than intact calmodulin, respectively.

3.3. Activation of cyclic nucleotide phosphodiesterase by fragments of calmodulin

Half-maximal activation of cyclic nucleotide phosphodiesterase by intact calmodulin required a concentration of 10^{-9} M.

It was reported in [6] that calmodulin fragment TR_1-E was 220-fold less effective than the intact calmodulin molecule in the activation of cyclic nucleotide phosphodiesterase. The TR_2-C fragment was 850-times weaker, while other fragments (e.g., TR_1-C and TR_2-E) were at least several thousand-fold weaker

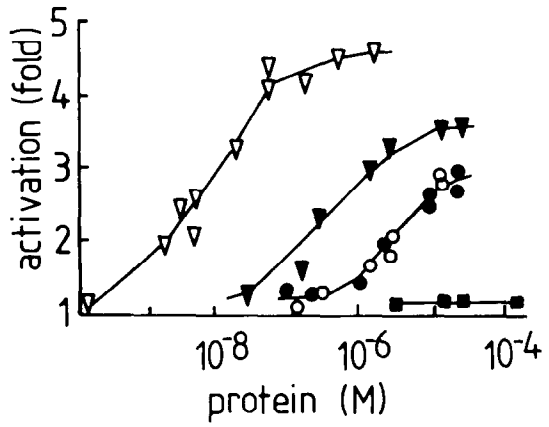

Fig.3. Activation of phosphorylase kinase by calmodulin and its fragments. The assays were performed at pH 6.8 and 0.05 mM $CaCl_2$, using phosphorylase kinase purified up to the 30 000 rev./min supernatant step [14]: (▽) intact calmodulin; (▼) TR_2-C; (●) TR_1-E; (○) TR_1-C; (■) TR_3-E.

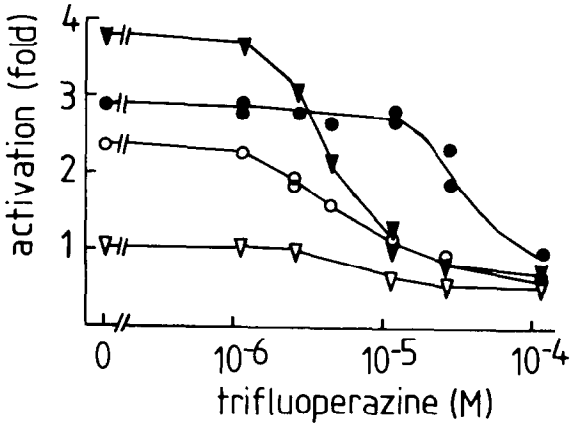

Fig.4. Effect of trifluoperazine on the activation of phosphorylase kinase by calmodulin (δ′-subunit) and its fragments. Assays were performed at pH 6.8 and 0.05 mM $CaCl_2$ using homogeneous preparations of phosphorylase kinase: (▼) intact calmodulin, 1.9×10^{-7} M; (●) TR_2-C, 1.1×10^{-5} M; (○) TR_1-C, 1.3×10^{-5} M; (▽) no additions. Trifluoperazine was a gift from Smith, Kline and French.

[6]. Similar results were reported in the case of myosin light-chain kinase [19].

More extensive studies have indicated, however, that the ability of the TR_1-E fragment to activate these enzymes varies from preparation to preparation, and may be caused by contamination with intact calmodulin (see section 4).

In this work the same highly purified fragments of TR_1-E and TR_2-C that were used to activate phosphorylase kinase (section 3.2, fig.2) were tested for their ability to activate cyclic nucleotide phosphodiesterase. These experiments demonstrated that fragment TR_1-E is a weaker activator than was reported previously. Its ability to activate phosphodiesterase is similar to the TR_2-C fragment, half-maximal activation occurring at $\sim 8 \times 10^{-7}$ M.

3.4. *The effect of trifluoperazine on activation of phosphorylase kinase by calmodulin and its fragments*

It had been shown that activation of phosphorylase kinase by calmodulin (the δ′-subunit) is inhibited by μM levels of the phenathiazine drug trifluoperazine [18]. As shown in fig.4 activation of phosphorylase kinase by the TR_1-C and TR_2-C fragments is also inhibited in the presence of trifluoperazine. However, activation by the TR_2-C fragment is inhibited at much higher concentrations than activation by the TR_1-C fragment. For instance at 1.25×10^{-5} M trifluoperazine activation by fragment TR_2-C was unaffected, whereas activation by fragment TR_1-C was inhibited almost completely.

These results were extended by measuring the ability of different concentrations of calmodulin and its fragments to activate phosphorylase kinase in the presence of 1.25×10^{-5} M trifluoperazine (fig.5). Under these conditions half-maximal activation of phospho-

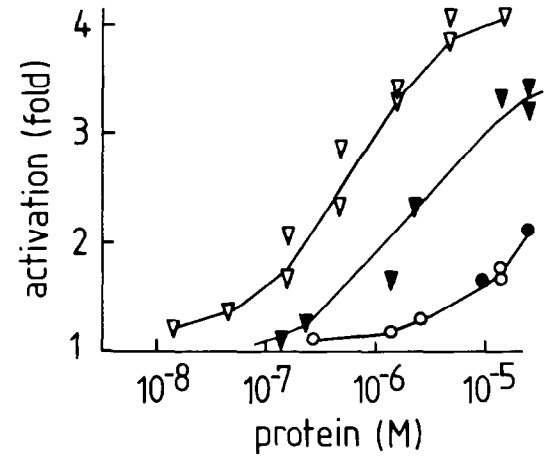

Fig.5. Activation of phosphorylase kinase by calmodulin and its fragments in the presence of 12.5 μM trifluoperazine. Experimental conditions are given in fig.4 and the symbols are as in fig.3.

rylase kinase was achieved at 6×10^{-7} M calmodulin (60-fold higher than in the absence of trifluoperazine) and at 6×10^{-6} M TR_2-C fragment (only 7-fold higher than in the absence of trifluoperazine). In order to obtain half-maximal activation of the phosphorylase kinase in the presence of 1.25×10^{-5} M trifluoperazine, it was necessary to add only 10-times more of fragment TR_2-C than calmodulin (fig.5), whereas in the absence of trifluoperazine 100-times more of the fragment was needed (fig.3).

4. Discussion

These experiments clearly demonstrate that fragments of calmodulin are capable of activating a number of calmodulin-dependent enzymes. The fragment TR_2-C containing Ca^{2+}-binding domains 3 and 4 is a more effective activator of phosphorylase kinase (only 100-times less effective than native calmodulin) than TR_1-C containing Ca^{2+}-binding domains 1 and 2 (2000-times less effective than calmodulin). Moreover fragment TR_2-C, but not TR_1-C, forms a Ca^{2+}-dependent complex with troponin-I [5,6], and is a much better activator of actomyosin ATPase than fragment TR_1-C (fig.2). Since the fragment TR_3-E containing Ca^{2+} binding domain 4 is completely unable to activate phosphorylase kinase and actomyosin ATPase, these results suggest that the region comprising residues 78–106 may be particularly important in the interaction of calmodulin with phosphorylase kinase or troponin-I. This region does not contain the trimethyllysine present at residue 115 in all calmodulins [4], except that from *Dictyostelium* [20].

On the other hand, fragment TR_2-C only activated cyclic nucleotide phosphodiesterase weakly, being 850-times less effective than calmodulin [6] and fragment TR_1-C was virtually inactive. The ability of fragment TR_1-E (containing Ca^{2+}-binding domains 1 and 2 and an incomplete domain 3) to activate cyclic nucleotide phosphodiesterase varied from preparation to preparation, and in the most highly purified preparations was similar to that of the TR_2-C fragment. The results in [6,19,21,22] which suggested that the TR_1-E fragment was a relatively good activator of myosin light chain kinase and phosphodiesterase may therefore be explained by trace contamination with intact calmodulin. Fragment TR_1-E migrates only slightly faster than native calmodulin under the conditions of urea–polyacrylamide gel electrophoresis used for the separation of the fragments [5,6].

This work has demonstrated that identical preparations of calmodulin fragments differ in their ability to activate particular enzymes. For instance, fragment TR_2-C was much more effective in the activation of phosphorylase kinase than in the activation of phosphodiesterase. The results also demonstrate that more than one region of calmodulin is capable of interacting with its target proteins, and this concept has also been suggested by a further experiment involving phosphorylase kinase. Crosslinking studies using [^{14}C]calmodulin have shown that the second molecule of calmodulin (the δ'-subunit) is complexed with both the α- and β-subunits of phosphorylase kinase [23], indicating that at least two regions of calmodulin can interact with this enzyme. The existence of two or more protein binding regions on calmodulin has important biological implications, since one molecule of calmodulin might be capable of activating two different enzymes simultaneously. It also raises the possibility that several calmodulin-dependent proteins could exist as a complex in vivo in the presence of Ca^{2+}.

The phenathiazine drug, trifluoperazine, binds to calmodulin in the presence of Ca^{2+} and thereby inhibits calmodulin-dependent enzymes. This interaction may underlie some of the therapeutic actions of this drug [24]. This work has demonstrated that activation of phosphorylase kinase by the TR_2-C fragment is much less susceptible to inhibition by trifluoperazine than is activation by the TR_1-C fragment. These results may indicate that the N-terminal domains 1 and 2 of calmodulin have a higher affinity for trifluoperazine than the C-terminal domains 3 and 4.

The affinity of trifluoperazine for the different fragments of calmodulin is now under investigation in our laboratory.

Acknowledgements

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