Interactions of basic polypeptides and proteins with calmodulin

Toshifumi ITANO, Ritsuko ITANO and John T. PENNISTON

Section of Biochemistry, Department of Cell Biology, Mayo Foundation, Rochester, MN 55901, U.S.A.

(Received 12 February 1980)

Low concentrations ($<10\mu\text{g/ml}$) of a number of highly basic polypeptides inhibit the calmodulin-stimulated cyclic nucleotide phosphodiesterase. Inhibitory compounds include synthetic polypeptides [polylysine (D and L) and polyarginine] and basic proteins (protamine, histones H1, H2A, H2B, H3 and H4 and myelin basic protein). Polylysine of mol.wt. about 2000 or higher was inhibitory, but pentyllysine did not inhibit. Other basic proteins and compounds did not inhibit, including bradykinin, spermine and putrescine. In mixtures of calmodulin and basic protein, complexes were formed whether Ca^{2+} was present or not. This was true for polylysine, myelin basic protein and histone H2B. These interactions suggest that the inhibition of the phosphodiesterase is due to interaction of these basic proteins with calmodulin. The wide variety of basic polypeptides and proteins that affect the calmodulin stimulation of phosphodiesterase indicates that these interactions are not specific.

Calmodulin is a ubiquitous and multifunctional Ca^{2+} -binding protein that is believed to be a major translator of the intracellular Ca^{2+} message. It regulates a large number of intracellular enzymes including cyclic nucleotide phosphodiesterase, adenylate cyclase, protein kinases, Ca^{2+} -pumping ATPase of plasma membranes, microtubules, phospholipase A_2 and NAD^+ kinase. The role of calmodulin in regulation has been reviewed recently Wolff & Brostrom, 1979; Wang & Waisman, 1979; Cheung, 1980).

In addition to the effect of calmodulin on known enzymes, it has been shown to bind strongly to other proteins of unknown function. Two such proteins from bovine brain have been purified; one of these proteins is a heat-labile one (Klee & Krinks, 1978; Wallace *et al.*, 1978; Sharma *et al.*, 1979), and the other is heat-stable (Sharma *et al.*, 1978). Both of these inhibitor proteins require Ca^{2+} for binding of calmodulin. A number of other calmodulin-binding proteins have been detected, but not characterized (LaPorte & Storm, 1978; Grand & Perry, 1979). Although only the molecular weight of most of these latter proteins has been established, one of them has been identified as myelin basic protein (Grand & Perry, 1979).

In view of the considerable interest in calmodulin-binding proteins we present here evidence concerning a non-specific interaction between highly

basic proteins and calmodulin. Knowledge of the nature of these non-specific interactions is vital in helping to assess the possible physiological roles of various calmodulin-binding proteins.

Materials and methods

Materials

Oligolysines were obtained from Vega, histone H4 was from Boehringer and other proteins and chemicals were from Sigma.

Turbidity

Turbidity was measured on solutions that were $50\mu\text{g/ml}$ in both calmodulin and basic polypeptide. The initial medium was 30 mM-sodium Pipes, pH 6.5, 0.5 mM-EGTA. Where Ca^{2+} addition is indicated, the medium was made 1 mM in CaCl_2 . Absorbance measurements were made at 360 nm in a 1 cm path-length cuvette, with a model 25 Beckman spectrophotometer.

Phosphodiesterase assay

Phosphodiesterase activity was measured by coupling the phosphodiesterase reaction with 5'-nucleotidase (Wang & Desai, 1977). P_i determination was done by the extraction of phosphomolybdate into organic solvent (Martin & Doty, 1949). The enzyme activity was measured at 37°C , pH 7.5, in a reaction mixture containing 40 mM-Tris/HCl, 40 mM-imidazole, 3 mM-magnesium acetate, 0.1 mM- CaCl_2 , 1.2 mM-cyclic AMP, 0.2 unit of

Abbreviation used: Pipes, 1,4-piperazinediethanesulphonic acid.

5'-nucleotidase and 20 μg of calmodulin-requiring phosphodiesterase in 0.5 ml. All inhibition assays were done with 0.10 μg of calmodulin in the assay mixture, except where otherwise indicated. The blank reading without calmodulin, which was 10–20% of the reading with calmodulin, was subtracted to give the calmodulin-stimulated activity. This blank reading was not affected by the presence of basic protein in the absence of calmodulin.

Inhibition by histone H1 and by poly-L-lysine was tested in the same medium that had been made 0.1 M in KCl. The inhibition pattern in this nearly iso-osmotic medium was the same as that in the standard assay medium.

Binding of calmodulin to polylysyl-agarose

^{125}I -labelled calmodulin was prepared by a lactoperoxidase iodination, which retained full biological activity (E. Graf, A. G. Filoteo & J. T. Penniston, unpublished work); poly-(L-lysine)-agarose 4B containing 0.5–1.0 mg of polylysine/ml of packed gel was obtained from Sigma. The medium used was 20 mM-sodium Pipes, pH 6.6, containing 0.5 mM-EGTA and 10 g of bovine serum albumin/litre; where Ca^{2+} is indicated, the medium was 1 mM- CaCl_2 . Packed agarose (10 μl) and 100 μl of ^{125}I -labelled calmodulin containing 0.62 μg of calmodulin were suspended in the medium to a final volume of 0.5 ml. This suspension was filtered, and then washed with 2 ml portions of the same medium, with or without Ca^{2+} , as required.

Results

The effects of a variety of basic proteins and polypeptides on the calmodulin-stimulated cyclic nucleotide phosphodiesterase reaction are shown in Figs. 1–3. Most of the highly basic proteins tested showed a strong inhibition of the reaction at low concentrations. As Figs. 1 and 2 show, all of the histones tested inhibited. Fig. 1 also shows that the inhibition due to histone H1 was not affected by prior heating of the histone.

The inhibition by histones could be overcome by the addition of polyanions, either DNA or calmodulin. In the presence of 0.5 μg of histone H1, a 37% inhibition was observed; this was decreased to 0% inhibition by addition of 5 μg of DNA to the mixture of histone, calmodulin and phosphodiesterase. The competitive effect of extra calmodulin in overcoming the inhibition by a histone is shown in Fig. 3. Fig. 4 shows tests of other basic proteins; protamine and myelin basic protein showed inhibition, but phosphorylase *b* did not.

Synthetic polypeptides also inhibited the enzyme reaction. Fig. 4 shows the effect of polyarginine, and Fig. 5 that of polylysines. Both were inhibitory at concentrations comparable with the concentrations

Fig. 1. Inhibition of phosphodiesterase by native (●) and heated (○) histone H1. Calmodulin was present at 0.1 μg per assay.

Fig. 2. Inhibition of phosphodiesterase by other histones: ●, Histone H2A; ○, histone H3; ▲, histone H4; ×, histone H2B. Calmodulin was present at 0.1 μg per assay.

Fig. 3. Competitive effects of histone H2B and calmodulin: ●, No histone H2B; ×, 0.3 μg of histone H2B per assay; ▲, 0.5 μg of histone H2B per assay.

Fig. 4. Inhibition of phosphodiesterase by other basic proteins and polypeptides (a) ▲, protamine; ●, myelin basic protein. (b) ▲, phosphorylase b; ●, poly-L-arginine. Calmodulin was present at 0.1 µg per assay.

at which basic proteins inhibited. Fig. 5 also shows the effects of chain length and of stereoisomer type on the inhibition by polylysine. The high-molecular-weight polylysines inhibited whether the D- or L-isomers were utilized, but short-chain oligolysines did not inhibit. This failure of the lower-molecular-weight basic materials to inhibit was reflected in the lack of effect of other small basic molecules on the reaction. Bradykinin, spermine and putrescine failed to inhibit the calmodulin-stimulated cyclic nucleotide phosphodiesterase (results not shown).

The existence of an interaction between basic proteins or polypeptides and calmodulin was detectable by the appearance of turbidity in mixtures of the two. Table 1 summarizes turbidity data obtained for the mixtures of calmodulin with three basic molecules. All solutions were uncoloured both before and

Fig. 5. Inhibition of phosphodiesterase by oligo- and poly-lysines O, L-Lys₃; X, L-Lys₅; ●, D-Lys₅₀₀; ▲, L-Lys₈₋₃₀. Calmodulin was present at 0.1 µg per assay.

Table 1. Complex formation as measured by turbidity The results presented were obtained by measurement of turbidity 1 min after addition of the polycation to the solution of calmodulin in buffer. CaCl₂ was then added, and the turbidity was measured again after 1 min.

	Turbidity (cm ⁻¹)	
	No Ca ²⁺	+ Ca ²⁺
Myelin basic protein	1.13	0.51
Poly-L-lysine	1.84	1.38
Histone H2B	1.04	0.97

after mixture, and turbidity of the solutions was apparent to the eye after mixing. It was apparent that interaction of calmodulin with the basic molecule occurred even in the absence of Ca²⁺. Addition of Ca²⁺ usually caused a decrease in the total turbidity, but never eliminated the interaction. The experiments shown were done by addition of Ca²⁺ to the medium after the complexes were formed. However, turbidity also appeared *de novo* when Ca²⁺ was in the medium before complex formation.

The effect of Ca²⁺ on the formation of complexes between calmodulin and polylysine was further checked by use of polylysine bound to agarose. Table 2 shows that the binding of calmodulin to this immobilized polylysine was not affected by Ca²⁺; the

Table 2. *Binding of ^{125}I -labelled calmodulin to poly-(L-lysine)-agarose*
The results show the percentage of calmodulin bound after each procedure.

	Added in EGTA medium		Added in Ca^{2+} medium
First wash	64	First wash	62
Two EGTA washes	58	Two Ca^{2+} washes	57
Two Ca^{2+} washes	52	Two EGTA washes	53

elution pattern of calmodulin was the same whether it was added and eluted in a Ca^{2+} -containing or an EGTA-containing medium. This indicated that the affinity of calmodulin for polylysine was not strongly affected by the presence of Ca^{2+} .

Discussion

The results presented suggest that the main requirement for non-specific inhibition of the calmodulin-stimulated cyclic nucleotide phosphodiesterase reaction by a polymer is that the polymer be a long-chain polycation with a high density of positive charge. This is understandable in terms of the theory of polyelectrolytes. Polyions are known to bind their counter ions much more strongly than do the monomeric or oligomeric analogues of the polyion (Rice & Nagasawa, 1961). Thus, it is expected that long-chain polycations such as polylysine, polyarginine and the basic proteins would interact when short-chain oligocations such as pentalysine do not do so.

In view of the highly acidic nature of calmodulin (Jarrett & Penniston, 1978), it seems likely that the basic polycations have their effect by direct interaction with calmodulin rather than with the phosphodiesterase enzyme. This suggestion is supported by the binding of calmodulin to polylysine-agarose and by the turbidity of mixtures of basic polycations with calmodulin shown in Table 1. The occurrence of turbidity indicates the formation of complexes between basic protein and calmodulin. Changes in the turbidity on addition of Ca^{2+} indicate some change in the nature of the aggregates with the change in the conformation of calmodulin expected when Ca^{2+} is added. However, the aggregates themselves persisted whether Ca^{2+} was present or not.

The data presented here suggest that calmodulin is capable of quite strong interactions with low concentrations of polycations. The wide variety of polycations that inhibit the phosphodiesterase reaction indicates that this interaction is non-specific in nature and depends mainly on the high charge density present on both macromolecules. The non-specificity of the interaction is made particularly evident by the effectiveness of the non-physiological polycation, poly-D-lysine.

The existence of such strong non-specific interactions is of particular interest in view of the recent reports on calmodulin-binding proteins of unknown function. Considerable caution must be used in interpreting the appearance of complexes between calmodulin and basic proteins, since non-physiological complexes may form during the treatment of cell homogenates.

We have called these interactions non-specific because of the wide range of strongly basic proteins involved. The absence of a Ca^{2+} requirement for interaction does not in itself show that this interaction is non-specific: a molecule of calmodulin appears to be specifically incorporated into each phosphorylase kinase complex without any Ca^{2+} requirement (Shenolikar *et al.*, 1979). This interaction of calmodulin with the other subunits of phosphorylase kinase may be of a different kind from its interaction with basic proteins; phosphorylase kinase is acidic, having an isoelectric point of 5.77 (Hayakawa *et al.*, 1973).

The heat-stable inhibitor protein described by Sharma *et al.* (1978) resembles the basic proteins and polypeptides studied here. Like it, they display heat stability and are basic. But their protein requires Ca^{2+} for its binding to calmodulin, giving it a greater claim to biological specificity.

Grand & Perry (1979) have isolated myelin basic protein from bovine brain, and have shown that it interacts with calmodulin. However, they did not show the existence of a myelin basic protein-calmodulin complex in their initial homogenate. Since myelin basic protein is one of the polycations considered here, it is necessary to proceed with caution in interpretation of this interaction. They also reported that the interaction of myelin basic protein with calmodulin was dependent on Ca^{2+} . The data in Tables 1 and 2 show that complexes of myelin basic protein with calmodulin can occur either in the presence or the absence of Ca^{2+} .

References

- Cheung, W. Y. (1980) *Science* **207**, 19-27
- Grand, R. J. A. & Perry, S. V. (1979) *Biochem. J.* **183**, 285-295
- Hayakawa, T., Perkins, J. P. & Krebs, E. G. (1973) *Biochemistry* **12**, 567-573

- Jarrett, H. W. & Penniston, J. T. (1978) *J. Biol. Chem.* **253**, 4676–4682
- Klee, C. B. & Krinks, M. H. (1978) *Biochemistry* **17**, 120–126
- LaPorte, D. C. & Storm, D. R. (1978) *J. Biol. Chem.* **253**, 3374–3377
- Martin, J. B. & Doty, O. M. (1949) *Anal. Chem.* **21**, 965–967
- Rice, S. A. & Nagasawa, M. (1961) *Polyelectrolyte Solutions*, p. 427, Academic Press, New York
- Sharma, R. K., Desai, R., Thompson, T. R. & Wang, J. H. (1978) *Can. J. Biochem.* **56**, 598–604
- Sharma, R. K., Desai, R., Waisman, D. M. & Wang, J. H. (1979) *J. Biol. Chem.* **254**, 4276–4282
- Shenolikar, S., Cohen, P. T. W., Cohen, P., Nairn, A. C. & Perry, S. V. (1979) *Eur. J. Biochem.* **100**, 329–337
- Wallace, R. W., Lynch, T. J., Tallant, E. A. & Cheung, W. Y. (1978) *J. Biol. Chem.* **254**, 377–382
- Wang, J. H. & Desai, R. (1977) *J. Biol. Chem.* **252**, 4175–4184
- Wang, J. H. & Waisman, D. M. (1979) *Curr. Top. Cell. Regul.* **15**, 47–107
- Wolff, D. J. & Brostrom, C. O. (1979) *Adv. Cyclic Nucleotide Res.* **11**, 27–88