EFFECT OF Ca^{2+} ON THE STABILITY OF THE PROTEIN ACTIVATOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

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1. Introduction

Mammalian cyclic nucleotide phosphodiesterase exists in multiple molecular forms [1]. One of these forms depends on a protein activator for full activity [2–4]. Recently, the protein activator of cyclic nucleotide phosphodiesterase has been purified to homogeneity [5–7]. Studies from several laboratories have established that the enzyme activation has the following properties: a) the activation of the enzyme by its protein activator requires the presence of Ca^{2+} [6–9], b) the enzyme and the protein activator associate to form a protein complex in the presence of Ca^{2+} [10–12] and c) the protein activator binds Ca^{2+} with a high affinity [6–8]. Based on these observations, it has been postulated that the free protein exists in an inactive conformation which may be converted to an active state upon binding of Ca^{2+}. Direct evidence in support of a Ca^{2+} induced conformational change in the protein activator has not been reported.

In the present study, it is shown that Ca^{2+} exerts a significant effect on the stability of the protein activator. Especially, the resistance of the protein activator to tryptic or chymotryptic attack is greatly enhanced by Ca^{2+}. Concentrations of Ca^{2+} effective for these protections are in the same range as those required to show Ca^{2+} binding to the protein activator. The results lend support to the suggested role of Ca^{2+} in the enzyme activation. A preliminary account has been presented [12].

2. Materials and methods

The protein activator and cyclic nucleotide phosphodiesterase were prepared from bovine heart according to previously established procedures [7]. The activity of cyclic nucleotide phosphodiesterase was assayed by the procedure of Butcher and Sutherland [13]. One unit of enzyme activity is equivalent to the amount of the enzyme which, when maximally activated by the protein activator, hydrolyzes 1 μmol of cAMP per minute at 30°C. Activity of the protein activator was assayed for its ability to stimulate the partially purified cyclic nucleotide phosphodiesterase. One unit of the activator activity is defined as the amount required to activate 0.013 unit of the enzyme to 50% of its maximal activation [8]. Low Ca^{2+} reagents and low Ca^{2+} protein samples were prepared according to previously described procedures using chelax 100 columns [8].

3. Results and discussion

Fig. 1 shows that in solutions containing 10 μM Ca^{2+}, a low concentration of trypsin has little effect on the activity of the protein activator of cyclic nucleotide phosphodiesterase. In contrast, in the presence of 0.1 mM EGTA to chelate the Ca^{2+} the protein activator is rapidly inactivated by trypsin. The protection of the protein activator against tryptic inactivation by Ca^{2+} appears specific. The reaction media contained 1 mM Mg^{2+} yet provided no apparent protection in the absence of free Ca^{2+}.

The low level of Ca^{2+} used in the experiment is not expected to affect the activity of trypsin directly. Although trypsin has been shown to bind Ca^{2+}, there is no direct connection between the trypsin activity and Ca^{2+} binding [14]. Thus the result in fig. 1 suggests

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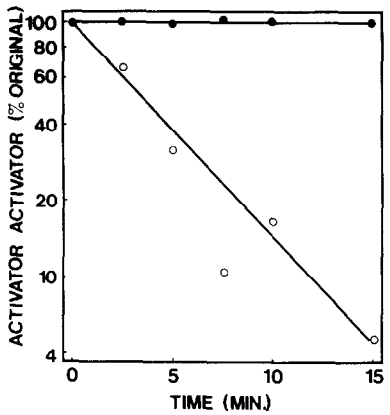

Fig.1. Effect of Ca^{2+} and EGTA on the tryptic inactivation of the protein activator. The protein activator (100 μg/ml) in a pH 7.5 buffer containing 20 mM Tris and 1 mM Mg^{2+} was incubated at 30°C with 1 μg/ml trypsin in the presence of either 10 μM Ca^{2+} (•) or 0.1 mM EGTA (○). At various intervals, aliquots were removed and diluted 10-fold in a buffered solution containing 5 μg/ml egg white trypsin inhibitor and the activity of the protein activator was measured.

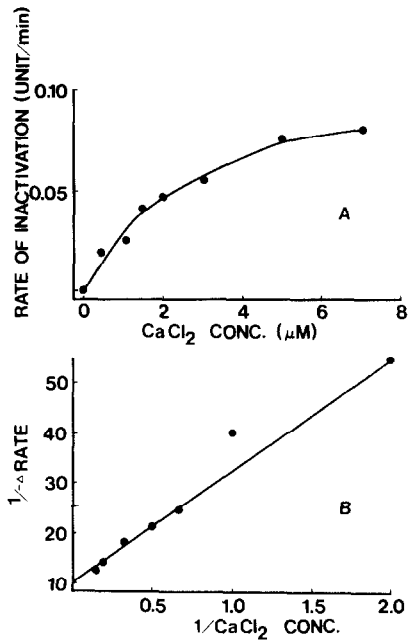

Fig. 2. Effect of Ca^{2+} concentration on tryptic inactivation of the protein activator: Chelax 100 resin purified reagents were used in these experiments. Trypsin treatment was carried out under conditions similar to those described in legend of fig. 1.

that the observed Ca^{2+} effect is due to the binding of Ca^{2+} to the protein activator. Equilibrium binding studies [6–8] have revealed the existence of high affinity Ca^{2+} binding sites on the protein activator.

The initial rate of tryptic inactivation of the protein activator in the presence of 0.1 mM EGTA follows first order kinetics (fig. 1). Using reagents largely freed of contaminating Ca^{2+}, the tryptic inactivations of the protein activator at several concentrations of Ca^{2+} have also been found to follow first order kinetics. Fig. 2A shows that the plot of the decrease in rate of inactivation of the protein activator against Ca^{2+} concentration resembles a ligand saturation curve. Double reciprocal plot of the data gives a straight line (fig. 2B). The dissociation constant calculated from this line is 2.6 μM. Recently, we have shown that the protein activator mediated Ca^{2+} activation of cyclic nucleotide phosphodiesterase has a *K*a value of 2.3 μM for Ca^{2+}. Also, equilibrium binding studies have revealed a high affinity Ca^{2+} binding site in the protein activator with a dissociation constant in the range of 3–4 μM (6–8). The agreement between the dissociation constant determined from fig. 2B and those obtained previously further substantiates the notion that the binding of Ca^{2+} to the protein activator results in an increased resistance of the protein activator to tryptic inactivation.

A number of enzymes retain their activities after limited proteolysis. Thus, the protection of protein activator against tryptic inactivation could be due to the stabilization by Ca^{2+} of an active tryptic derivative of the protein activator. To test this possibility, trypsin treated activator samples have been analyzed by polyacrylamide gel electrophoresis. Fig. 3 shows that the gel patterns for the samples in 10 μM Ca^{2+} before and after incubation with trypsin are almost identical. In contrast, the patterns for the trypsin treated sample in 0.1 mM EGTA shows no protein material at the position corresponding to that of the intact protein activator. The result indicates, therefore that the observed effect of Ca^{2+} on the activity of the protein activator results from its protection of the protein against tryptic hydrolysis.

Protection of a protein by its ligand against proteolysis could be due either to a change in protein conformation or to the blocking of sensitive peptide bonds

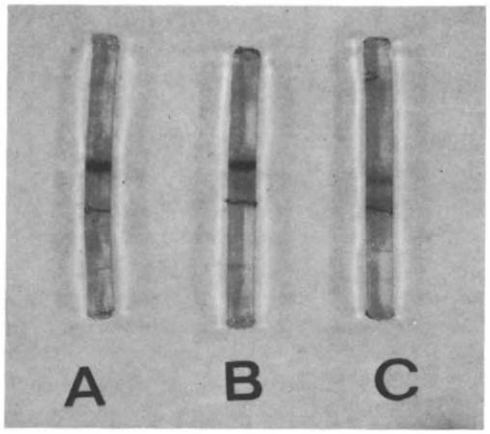

Fig. 3. Disc-gel electrophoretic analysis of trypsin-treated protein activator: Electrophoresis was carried out on 15% polyacrylamide gel at pH 8.3 with a protein activator control sample (A) and samples treated with trypsin for 20 min in the presence of either 10 μM Ca^{2+} (B) or 0.1 mM EGTA (C). Other conditions of trypsin-treatment are similar to those described in legend of fig. 1.

by the ligand. If the mechanism of the protection is the direct blocking of susceptible bonds, inactivation of the protein by a second proteolytic enzyme having a different specificity would probably not be affected by the ligand. Fig. 4 shows that the protein activator

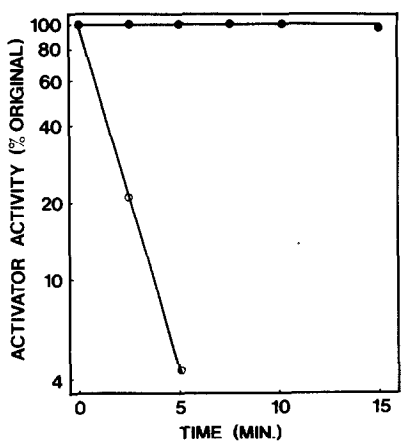

Fig. 4. Effect of Ca^{2+} and EGTA in the chymotryptic inactivation of the protein activator. The experimental conditions were same as those described in the legend of fig. 1 except chymotrypsin and the lima bean protease inhibitor were used in place of trypsin and trypsin inhibitor respectively.

of cyclic nucleotide phosphodiesterase in 0.1 mM EGTA is rapidly inactivated by chymotrypsin. Again, 10 μM of free Ca^{2+} completely prevents this inactivation. The results, therefore suggest that Ca^{2+} binding induces a conformational change in the protein activator. This suggestion is supported by preliminary physical studies. Wolfe and Brostrom [7] stated that they had unpublished results showing a change in circular dichroic spectrum of the protein activator upon Ca^{2+} binding. In our laboratory, preliminary results have indicated that the fluorescence emission of the protein activator is enhanced upon Ca^{2+} binding [12]. Presumably, this change in proteins conformation enables the protein activator to associate with the phosphodiesterase and to bring about the enzyme activation.

Several Ca^{2+} binding proteins have been identified and purified in recent years. Structural studies have led to the suggestion that many of these proteins are homologous [12, 15–17]. Although only a few of the Ca^{2+} binding proteins have identified functional roles, many of them have been shown to undergo conformational changes upon binding of Ca^{2+} [18–20]. Possibly a general action pattern of Ca^{2+} binding proteins involves Ca^{2+}- induced conformational changes.

Acknowledgement

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