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Ca^{2+}-INDEPENDENT STIMULATION OF CYCLIC GMP-DEPENDENT PROTEIN KINASE BY CALMODULIN

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SUMMARY

Calmodulin purified from bovine brain markedly stimulated cyclic GMP-dependent protein kinase from pig lung in the presence of cyclic GMP. This stimulation by calmodulin did not require Ca^{2+} and was dose-dependent up to optimal amounts, but the extent of stimulation decreased at concentrations over the optimal condition. The concentrations of cyclic GMP and cyclic AMP producing half-maximal stimulation were 4.5×10^{-8} M and 5.0×10^{-6} M respectively, under optimal conditions. Calmodulin increased maximum velocity without altering the Km for ATP. These effects of calmodulin on cyclic GMP-dependent protein kinase were similar to those of the stimulatory modulator described by Kuo and Kuo (J. Biol. Chem. 251, 4283-4286, 1976). Ouf findings indicate that calmodulin regulates enzyme activity both Ca^{2+}-dependently and independently.

INTRODUCTION

Cyclic GMP-dependent protein kinase is considered to mediate the biological effects of cyclic GMP. Kuo et al.(1-3) and Mackenzie and Donnelly (4) reported that this enzyme required a modulator for the stimulatory activity of cyclic GMP. On the other hand, it has also been reported that cyclic GMP-dependent protein kinase is significantly stimulated by cyclic GMP in the absence of a stimulatory modulator (5-10). The enzyme we used herein, was dependent on the stimulatory modulator, as described by Kuo et al.(1-3), and was found to be not only enhanced by the modulator but also by calmodulin in the presence of a low concentration of cyclic GMP, in Ca^{2+}-independent fashion.

Calmodulin, an ubiquitous, heat stable, small (Mw=16,500) acidic and Ca^{2+}-binding protein, plays an important role in Ca^{2+}-regulation of several

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enzymatic systems and cellular reactions including Ca^{2+}-dependent phosphodiesterase (11, 12), brain adenylate cyclase (13, 14), myosin light chain kinase (15-18), erythrocyte (Ca^{2+}-Mg^{2+})ATPase (19, 20), NAD kinase (21), skeletal muscle phosphorylase kinase (22), tryptophan 5-monooxygenase (23), platelet phospholipase A_2 (24), Ca^{2+} transport in the sarcoplasmic reticulum (25), into phosphorylation of membrane protein (26, 27) and the disassembly of microtubules (28). In those systems, the role of calmoudlin was considered to be that of functional Ca^{2+}-receptor. In the present paper, we report that calmodulin stimulates cyclic GMP-dependent protein kinase and that this stimulation was Ca^{2+}-independent. Thus, calmodulin participates not only in the regulation of cyclic nucleotide related enzymes (or other enzymes) in a Ca^{2+}-dependent fashion, but also in the regulation of cyclic GMP-dependent protein kinase, independent of Ca^{2+}.

MATERIALS AND METHODS

Protein preparations : Cyclic GMP-dependent protein kinase was purified from pig lung by the method of Kuo et al.(3). Calmodulin was purified from bovine brain by the method of Wang and Desai (29). Stimulatory modulator of cyclic GMP-dependent protein kinase was purified from bovine brain by the method of Shoji et al.(30). Calf thymus histone (Type II) and bovine serum albumin were purchased from Sigma.

Assay of cyclic GMP-dependent protein kinase : The standard assay system was as follows. The reaction mixture contained, in a final volume of 0.3 ml, potassium phosphate buffer pH 7.0, 15 μmol; theophylline, 0.75 μmol; histone mixture, 60 μg; magnesium acetate, 3 μmol; [γ-^{32}P]ATP, 1.5 μmol containing about 1 x 10^6 cpm; EGTA, 0.6 μmol or $CaCl_2$, 30 nmol; various concentrations of cyclic GMP or cyclic AMP, various amounts of calmodulin or stimulatory modulator and appropriate amounts of protein kinase, as indicated. The incubation was carried out at 30°C for 10 min. The reaction was terminated by the addition of 1 ml of ice cold 20% trichloroacetic acid following addition of 500 μg of bovine serum albumin as a carrier protein. The sample was centrifuged at 3,000 rpm for 5 min, the pellet resuspended in ice cold 10% trichloroacetic acid solution and the centrifugation-resuspension cycle was repeated three times. The final pellet was dissolved in 1 ml of 0.5 N NaOH and the radioactivity was measured by liquid scintillation counter.

RESULTS AND DISCUSSION

The protein kinase used herein was little stimulated by the addition of 1 μM cyclic GMP alone. As shown in Fig. 1, in the presence of 1 μM cyclic GMP, the enzyme was stimulated in a dose-dependent manner up to 86 μg/ml of

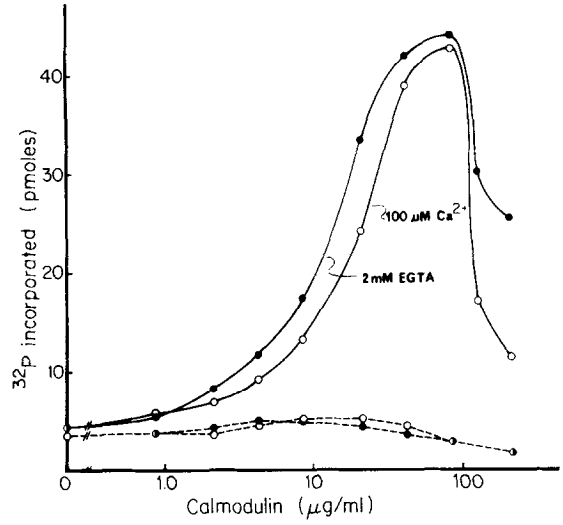

Fig. 1 Effect of calmodulin on cyclic GMP-dependent protein kinase

The assay conditions are as described under "MATERIALS AND METHODS". The enzyme (2.6 μg/ml) was incubated at 30°C for 10 min in the presence of 0.1 mM $CaCl_2$ (○) or 2 mM EGTA (●), and in the presence (—) or absence (---) of 1 μM cyclic GMP with various amounts of calmodulin.

calmodulin, and above this dose, the extent of stimulation decreased. In the absence of cyclic GMP, calmodulin inhibited the basal activity at concentrations above 43 μg/ml. The enzyme activity was dependent on cyclic GMP and calmodulin and independent of Ca^{2+} (Fig. 1). Moreover, Ca^{2+} was to some extent inhibitory as the activity in the presence of EGTA was somewhat higher than in the presence of Ca^{2+}. In the presence of calmodulin (86 μg/ml) producing maximum stimulation, this enzyme was stimulated specifically by a low concentration of cyclic GMP. The concentration of cyclic GMP required to produce half-maximal stimulation was 4.5×10^{-8} M, whereas, that of cyclic AMP was 5×10^{-6} M (Fig. 2A). Similarly, in the presence of 150 μg/ml of stimulatory modulator, (this amount was found to produce maximum stimulation, data not shown), the concentrations of cyclic GMP and cyclic AMP producing half-maximal stimulation were 2.5×10^{-8} M and 4×10^{-6} M, respectively (Fig. 2B). Maximum stimulation of the kinase by cyclic AMP in the presence of calmodulin and stimulatory modulator was about 90% of that seen with cyclic GMP. Kinetic analysis of the enzyme for ATP was then carried out in the presence or absence of calmodulin

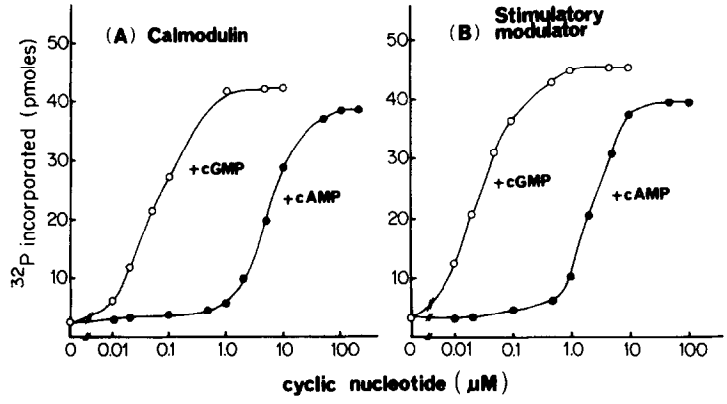

Fig. 2 Effect of cyclic nucleotides in the presence of calmodulin or stimulatory modulator on cyclic GMP-dependent protein kinase

The assay conditions were as described under "MATERIALS AND METHODS". The enzyme (2.6 μg/ml) was incubated at 30°C for 10 min in the presence of 2 mM EGTA, and in the presence of various concentrations of cyclic GMP (○) or cyclic AMP (●). 86 μg/ml of calmodulin (A) or 150 μg/ml of stimulatory modulator (B), which produced maximum stimulation, was used.

or stimulatory modulator (Fig. 3A, B). This figure shows that calmodulin and the stimulatory modulator enhanced the enzyme activity by increasing the maximum velocity without altering the apparent Km for ATP. The value of Km for ATP was 1.1×10^{-5} M. Since calmodulin and the stimulatory modulator

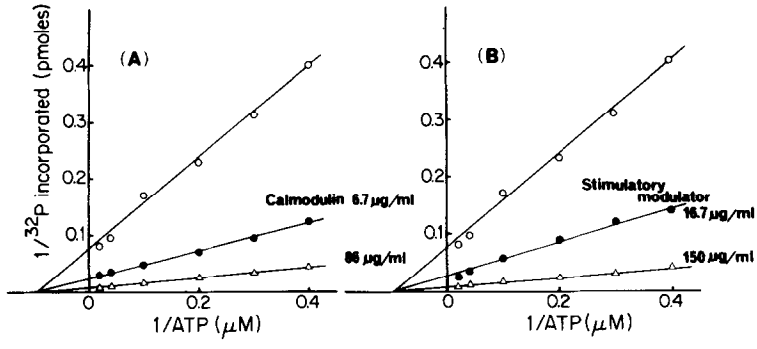

Fig. 3 Double reciprocal plots of phosphorylation of histone catalyzed by cyclic GMP-dependent protein kinase in the presence or absence of calmodulin (A) or stimulatory modulator (B)

The enzyme (2.6 μg/ml) was incubated at 30°C for 10 min in the presence of 2 mM EGTA and 1 μM cyclic GMP. The assay conditions were as described under "MATERIALS AND METHODS" except for the concentration of ATP.
(A) —○— None, —●— 6.7 μg/ml calmodulin, —△— 86 μg/ml calmodulin
(B) —○— None, —●— 16.7 μg/ml stimulatory modulator,
—△— 150 μg/ml stimulatory modulator

TABLE 1

Effect of simultaneous addition of calmodulin and stimulatory modulator on the cyclic GMP-dependent protein kinase activity

	PROTEIN KINASE ACTIVITY (pmoles)		
	no addition	+calmodulin 13 μg/ml	+calmodulin 86 μg/ml
no addition	4.3	26.0	43.0
+stimulatory modulator 30 μg/ml	21.0	34.0	40.0
+stimulatory modulator 150 μg/ml	45.0	44.5	36.0
+stimulatory modulator 300 μg/ml	40.5	38.0	27.5

The enzyme (2.6 μg/ml) was incubated at 30°C for 10 min in the presence of 2 mM EGTA and 1 μM cyclic GMP. The assay conditions are described under "MATERIALS AND METHODS"

showed similar effects on cyclic GMP-dependent protein kinase, experiments were carried out with both activators. As shown in Table 1, calmodulin produced an additive effect when the amount of stimulatory modulator in incubation mixture was less than 150 μg/ml. In these cases maximum activity was not increased by the addition of calmodulin and calmodulin was inhibitory when the amount of the stimulatory modulator was given than 150 μg/ml. These data suggest that calmodulin and stimulatory modulator probably act at the same site on cyclic GMP-dependent protein kinase. Calmodulin and stimulatory modulator have common characteristics, such as, isoelectric point (pH 4.0) and heat stability. Nevertheless, these two proteins are different because purified stimulatory modulator did not stimulate Ca^{2+}-dependent phosphodiesterase and molecular weight of 34,000 (30) was larger. Calmodulin has a molecular weight of 16,500 and stimulates Ca^{2+}-dependent phosphodiesterase. Figs. 2, 3 and Table 1 indicate that the mechanism of action of calmodulin and stimulatory modulator on cyclic GMP-dependent protein kinase are similar. It is likely that acidic properties of both proteins are necessary for their stimulation of the kinase. Recently, we found that calmodulin as well as the modulator

from lobster tail muscle (31) inhibited the cyclic AMP-dependent protein kinase (manuscript in preparation). Cyclic AMP and cyclic GMP-dependent protein kinases are considered to mediate biological effects of these cyclic nucleotides. In addition, calmodulin regulates adenylate cyclase (13, 14) and phosphodiesterase (11, 12), compounds that control the intercellular concentration of cyclic nucleotides. These findings indicate that calmodulin plays an important role in the biological effects of cyclic nucleotides and in Ca^{2+}-regulation of different enzyme systems and cellular reactions (11-28). Nevertheless, calmodulin may not always act as a Ca^{2+}-receptor, our data clearly show that this Ca^{2+}-binding protein is closely linked with cyclic nucleotides in both a Ca^{2+}-dependent and -independent fashion.

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