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CALCIUM STIMULATION OF PROTEIN KINASE C IN THE ABSENCE OF ADDED PHOSPHOLIPIDS*

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SUMMARY

The activity of protein kinase C as isolated and described by Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7610-7616, can be markedly stimulated by Ca^{2+} in the presence of 4 mM Mg^{2+}. This Ca^{2+} dependency does not require the presence of phospholipids or exogenous calmodulin. The increase in activity in the presence of Ca^{2+} is blocked by fluphenazine in the presence of 30 mM 2-mercaptoethanol. These results suggest that a calmodulin-like moiety may be a subunit of prokinase C.

INTRODUCTION

Protein kinase C is a proenzyme and acts as a cyclic nucleotide independent protein kinase (1). Protein kinase M can be produced from the proenzyme by limited proteolysis with a Ca^{2+}-dependent protease or trypsin. Recently, it has been observed that the proenzyme is catalytically active in the presence of Ca^{2+} and membrane phospholipids (2). The membrane phospholipids can be replaced with phosphatidylinositol or phosphatidylserine (3). Another group (4,5) has also described a calcium-phospholipid dependent protein kinase in brain and other tissues, but it is not apparent whether the enzyme described by these workers is identical to protein kinase C. Although neither group has reported a role for calmodulin in the calcium activation

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phenomenon, Schatzman et al. (5) report that phenothiazines inhibit the calcium dependent reactions.

Phenothiazines are known to bind to hydrophobic regions of proteins and much interest has evolved from the observation that these compounds interact with calmodulin to powerfully inhibit Ca^{2+}-calmodulin dependent reactions (6).

In this communication we show that protein kinase C isolated from rat brain as described by Inoue et al. (1), is Ca^{2+}-dependent without the addition of a phospholipid fraction under the appropriate incubation conditions. This Ca^{2+} activation is blocked by fluphenazine suggesting that protein kinase C may contain a calmodulin-like region or subunit.

MATERIALS AND METHODS

Experimental Procedure:

Materials: Histone type II was obtained from Sigma. Adenosine 5'-[γ-^{32}P] triphosphate, >2,000 Ci/mmol was purchased from Amersham. Fluphenazine. 2 HCl was obtained from Squibb Inc. and dissolved in water.

Methods: Protein kinase was prepared from rat brain soluble fraction according to the method of Inoue et al. (1). Only the fractions from the isoelectrofocusing step with a pI of 5.6 and a 10 fold activation of kinase activity by trypsin were selected for experiments. Each 3 ml fraction was dialyzed against 500 ml of 50 mM Tris pH 7.5 overnight.

Kinase activity was measured in a reaction mixture of 0.250 ml which contained 1.5 µg of protein kinase 5 µmol of Tris-HCl at pH 7.5, 2-mercaptoethanol (as specified), 10 nmol of [γ-^{32}P] ATP (100-200 dpm/pmol), 100 µg of histone type II as phosphate acceptor, magnesium sulfate (as specified). The reaction was stopped by the addition of 10% trichloracetic acid and the acid-precipitable radioactivity was measured according to the method of De Lange (7).

RESULTS AND DISCUSSIONS

The protein kinase C preparations used in these experiments had a pI of 5.6 and appeared as a single band on 12% polyacrylamide disc gels prepared and run at pH 9.0 under non-denaturing conditions. Before activation the enzyme had a specific activity of 12 nmol/mg/min in the standard kinase assay and was at least 10 fold activated by trypsin. No further attempt was made to assess the absolute purity of these preparations. Figure 1 depicts

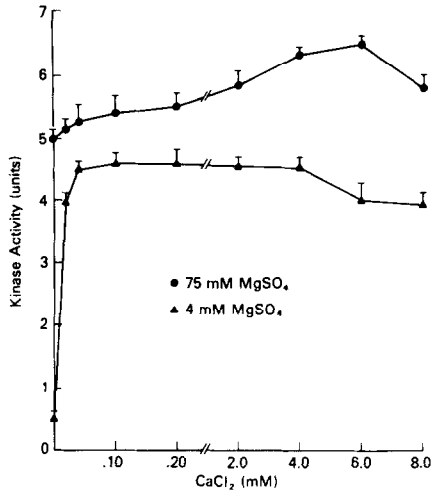

Fig. 1. Prokinase activity as a function of calcium and magnesium in the presence of 30 mM 2-mercaptoethanol. The complete reaction mixture contained all the reactants described in Methods and either 4mM $MgSO_4$ or 75mM $MgSO_4$ (final concentration), 30 mM 2-mercaptoethanol (final concentration), various concentrations of $CaCl_2$ as indicated, and prokinase at 0.4 mg. Kinase activity is expressed as pm/min. Incubation was for 10 min at 30°C.

the activity of this enzyme at two Mg^{2+} concentrations (4 and 75 mM). In the absence of Ca^{2+} the activity of this kinase is increased by about 10 fold by the high Mg^{2+} conditions. In the presence of 75 mM Mg^{2+} additions of increasing amounts of Ca^{2+} show only a slight (25%) stimulation of phosphorylation at relatively high Ca^{2+} concentrations. However, at 4 mM Mg^{2+} there was a dramatic stimulation of kinase activity by as little as 25 μM Ca^{2+}. These results suggest that Ca^{2+} may have an important role in regulating the activity of protein kinase C in the presence of moderate Mg^{2+} concentrations.

The results reported in figure 2 indicate that a relatively high concentration of 2-mercaptoethanol is required to see the full calcium dependency and activation. Of interest is the apparent inhibition of activity of the higher Ca^{2+} concentrations in the presence of 10 mM 2-mercaptoethanol. This would seem to rule out the presence of a contaminating Ca^{2+}-dependent protease as the cause of activation. The phospholipid independent Ca^{2+} sensitivity at 30 mM 2-mercaptoethanol utilizes mixed histone as substrate.

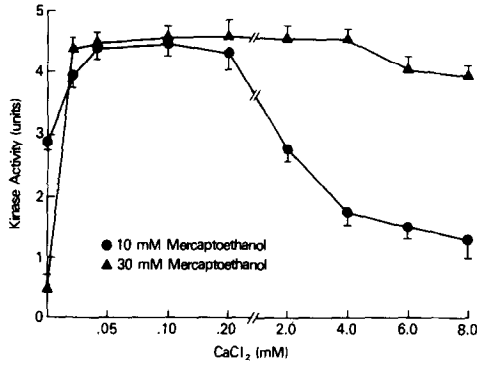

Fig. 2. Prokinase activity as a function of calcium and 2-mercaptoethanol in the presence of 4 mM $MgSO_4$. The complete reaction mixture contained all the reactants described in Methods and either 10 mM 2-mercaptoethanol or 30 mM 2-mercaptoethanol (final concentration), various concentrations of $CaCl_2$ as indicated, 4 mM $MgSO_4$ (final concentration) and prokinase at 0.4 µg. Kinase activity is expressed as pm/min. Incubation was for 10 min at 30°C.

In contrast, the diolein enhanced phospholipid-dependent Ca^{2+} activation first described by Takai et al. (2) utilizes lysine rich histone as substrate. In all reports of phospholipid dependency (2,3,10) the reactions utilized lysine rich histone as substrate and did not contain 2-mercaptoethanol. 2-Mercaptoethanol and phospholipid may facilitate Ca^{2+} activation by opening the catalytic site or the Ca^{2+} binding site. Calmodulin as a hydrophobic subunit of prokinase C may be shielded within a hydrophobic core of the enzyme which is opened to calcium by reduction of disulfide bonds or interaction with phospholipid. Since Ca^{2+} dependent reactions which require calmodulin are strongly inhibited by phenothiazine we tested whether fluphenazine could inhibit the Ca^{2+} activation of prokinase.

The data in Table I show clearly that fluphenazine at 30 nM blocks the Ca^{2+} activation of prokinase C in the presence of 4 mM $MgSO_4$ and 30 mM 2-mercaptoethanol. This effect is specific for blockade of Ca^{2+} activation since 60 nM fluphenazine does not reduce basal kinase activity.

These results show that prokinase C can be activated by physiological levels of calcium in the absence of phospholipid. The non phospholipid-dependent activation is highly dependent on 2-mercaptoethanol when mixed

TABLE I

The Effect of Fluphenazine on C Kinase Activity

Ca^{2+} 40μM	Fluphenazine	Kinase Activity
-	-	5.86
+	-	11.84
-	30 nM	6.80
+	+	6.30
-	60 nM	5.94

Reaction conditions were as described in Methods with the addition of 30 mM 2-mercaptoethanol and 4 mM $MgSO_4$. Incubation was for 5 min at 30°C (±) fluphenazine. Activity represents the mean of duplicates from 2 experiments. Activity is expressed as pm/min.

histone is the substrate. Studies done in this laboratory confirm the work of Kishimoto et al. (3) and show that the level of calcium activation can be enhanced by the addition of phospholipid or a phospholipid/neutral lipid complex utilizing lysine rich histone as substrate. The stimulation by phospholipid occurs in the absence of 2-mercaptoethanol but can be enhanced by the addition of 2-mercaptoethanol (data not shown). It is possible that 2-mercaptoethanol and phospholipid may be serving as modifiers in the exposure of hydrophobic sites required for calcium activation. It is also possible that each modifier is acting at the substrate level rather than by specific interactions with the prokinase.

The results have also shown that fluphenazine, which binds avidly to calmodulin (6), inhibits Ca^{2+} activation of prokinase C in the absence of phospholipid. The work of Schatzman et al. (5) describes fluphenazine inhibition of the phospholipid-dependent Ca^{2+} activation of Ca^{2+}-dependent protein kinase. It is unclear at this time, whether their phospholipid-sensitive Ca^{2+}-protein kinase is prokinase C. The results of Schatzman et al. (5) and Mori et al. (10) indicate that the inhibition of Ca^{2+} activation

by antipsychotics (e.g., trifluoperazine) is through interaction with the phospholipid activator. In contrast, the work described herein shows that the non-phospholipid mediated Ca^{2+} activation of prokinase C can be blocked by the phenothiazine fluphenazine. This suggests that prokinase C may contain calmodulin as a subunit. We are currently investigating this possibility by the use of a fluphenazine-Sepharose affinity column.

Myosin light chain kinase is dependent on Ca^{2+} and calmodulin. Recent studies suggest that the enzyme can also be activated in the absence of Ca^{2+} by limited trypsin proteolysis (11), or sodium dodecyl sulfate (12), phospholipid or arachidonic acid (2). These diverse modes of activation may all affect the enzyme by binding to or exposing a hydrophobic region of the protein.

Prokinase C prepared according to the procedure of Inoue et al. (1) may belong to a class of calcium dependent kinases that vary in sensitivity to proteases, phospholipids, disulfide reagents and substrate specificity. A unifying or primary characteristic to explain these differences would be the presence or absence of calmodulin as a subunit and potential ligand in Ca^{2+} activation.

These results suggest that prokinase C, like phosphorylase kinase (8) and myosin light chain kinase (9) may have a calmodulin regulatory subunit. Such a subunit could mediate the phospholipid Ca^{2+}-activation of the enzyme as well as its inhibition by drugs which bind to lipophilic sites (10).

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