Protein kinase C negatively modulated by phorbol ester

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Pretreatment of protein kinase C with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and phospholipid resulted in complete inhibition of ATP/phosphotransferase activity, irreversibly. The inactivation by TPA required the phospholipid, and TPA alone did not cause inactivation. Ca^{2+} and diacylglycerol mimicked TPA. This action of TPA was not general for all protein kinases as it did not accelerate the inactivation of the catalytic subunit of cAMP-dependent protein kinase by phospholipid. The addition of MgATP to the reaction mixture completely protected protein kinase C from being inactivated by TPA, in the presence of phospholipid. The nucleotide-binding site of the enzyme was probably influenced by the binding of TPA and phospholipid.

Protein kinase C *Phorbol ester* *Enzyme inhibitor* *Negative modulation* *Nucleotide-binding site*

1. INTRODUCTION

Protein kinase C, per se, is usually inactive and is activated in a reversible manner by the simultaneous presence of Ca^{2+} and membrane phospholipids [1]. Among various phospholipids, phosphatidylserine (PS) was the most active and other phospholipids such as phosphatidylinositol, phosphatidylethanolamine and phosphatidic acid were also active. When a small amount of unsaturated diacylglycerol or tumor promoter TPA was added to the reaction mixture, the response of the enzyme to both Ca^{2+} and PS shifted markedly and full enzymatic activity was obtained at much lower concentrations of both this cation and PS [2,3]. These agents modified the protein kinase C activity, but Ca^{2+} diacylglycerol or TPA alone showed little or no effect on enzyme activity. On the other hand, PS was indispensable for activation of the enzyme.

In contrast to this positive interrelation between phospholipid and protein kinase C, it has been reported that phospholipid acts as an inhibitory modulator of other enzymes such as hexokinase, glutamate dehydrogenase and cAMP-dependent and cGMP-dependent protein kinases [4–6]. However, the mechanism underlying the actions of this inactivation has not been clearly identified. We found that the catalytic activity of protein kinase C was also inactivated by acidic phospholipids such as PS and that TPA specifically enhanced this inactivation. These phenomena also suggest a specific interrelation among protein kinase C, PS and TPA. The enzyme cofactors probably inhibit protein kinase C by influencing the nucleotide-binding site of the enzyme.

2. MATERIALS AND METHODS

2.1. *Materials*

Histone III-S and histone H2B were purchased from Sigma. PS (pig liver) was obtained from Serdary Research Laboratories. Chloroform was removed from this phospholipid by a stream of nitrogen, and the phospholipid was sonicated in water for 1 min to produce a suspension of 1 mg/ml. [γ-^{32}P]ATP was obtained from Amersham, England. The catalytic subunit of cAMP-dependent protein kinase II was prepared from bovine heart muscle, by the method of Beavo et al.

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[7]. Protein kinase C was purified from the soluble fraction of 6 fresh rabbit brains according to Inagaki et al. [8].

2.2. *Assay of enzyme activity*

cAMP-dependent protein kinase activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 25 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 2 mM EGTA, 10 μM [γ-^{32}P]ATP (3×10^5 cpm), 100 μg histone H2B and varying amounts of enzyme. Protein kinase C activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 25 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$ or 2 mM EGTA, 10 μg PS, 10 μM [γ-^{32}P]ATP (3×10^5 cpm), 100 μg histone type III-S and varying amounts of enzyme. Assays were performed for 1, 3 and 5 min at 25°C and in all cases demonstrated a linear incorporation of [γ-^{32}P]phosphate into the substrate during the 5 min assay. The reaction was terminated by adding 1 ml ice-cold 20% trichloroacetic acid following addition of 200 μg bovine serum albumin as a carrier protein. The sample was centrifuged at 3000 rpm for 15 min, the pellet resuspended in ice-cold 10% trichloroacetic acid solutions, and the centrifugation-resuspension cycle repeated 3 times. The final pellet was dissolved in 1 ml of 1 N NaOH, and radioactivity was measured by a liquid scintillation counter.

2.3. *Reaction of PS and/or TPA with catalytic subunit of cAMP-dependent protein kinase and protein kinase C*

The catalytic subunit of cAMP-dependent protein kinase (15 μg/ml) was incubated with various concentrations of PS and/or TPA at 37°C in 50 mM potassium phosphate (pH 7.0) and 10^{-8} M Ca^{2+} containing 1.0 mg/ml BSA or 2.0 mg/ml ovalbumin. Protein kinase C (50 μg/ml) was incubated with various concentrations of PS and/or TPA at 37°C in 50 mM potassium phosphate (pH 7.0), 50 mM 2-mercaptoethanol and 10^{-8} M Ca^{2+} containing 1.0 mg/ml BSA or 2.0 mg/ml ovalbumin. Aliquots of the reaction mixture were assayed as described above, throughout the course of the reaction. TPA, which was dissolved in dimethyl sulfoxide (DMSO), was directly mixed with phospholipid suspended in the buffer before being added to the reaction mixture. The final concentration of DMSO in the reaction mixture was 0.01%.

3. RESULTS AND DISCUSSION

Incubation of protein kinase C with PS at 37°C resulted in a progressive decrease in activity to a level that depended on the concentration of added lipid; the results are shown in fig.1A. Phosphatidylinositol, phosphatidic acid and cardiolipin were also active, but phosphatidylcholine and sphingomyelin were ineffective in similar in

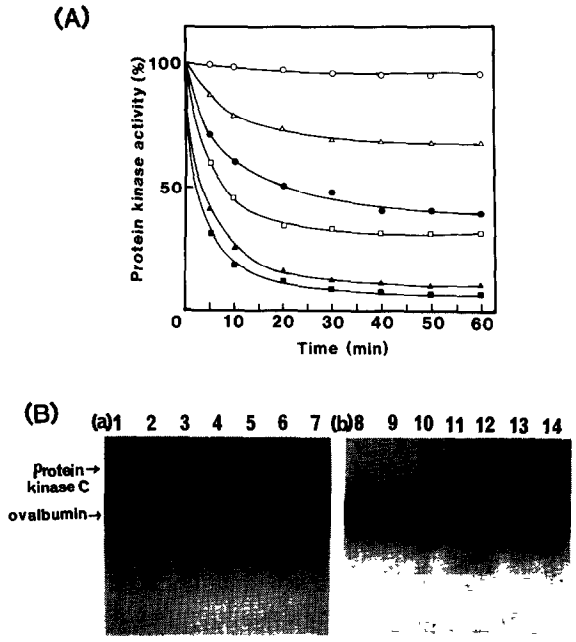

Fig.1. Inhibition of protein kinase C by PS. (A) Protein kinase C was incubated with PS as described in section 2 in the presence (●, ▲, ■) or absence (○, △, □) of 0.1 μg/ml TPA. PS at: (○,●) 10, (△, ▲) 50, (□, ■) 90 μg/ml. (B) SDS-polyacrylamide slab gel electrophoretic profile. SDS-polyacrylamide gel electrophoresis was carried out in 10% gels as described by Laemmli [15]. The gel was stained with Coomassie blue. Protein kinase C was incubated under the conditions described in section 2 in the absence (a) or presence (b) of 50 μg PS and 0.1 μg/ml TPA. Lanes 1–7, in the absence of PS and TPA; lanes 8–14, in the presence of PS and TPA. Periods of incubation were 0, 10, 20, 30, 40, 50 and 60 min. Protein kinase activity was assayed as described in section 2.2. Incubation of protein kinase C with PS and TPA led to inactivation of the enzyme activity.

vitro systems (not shown). Less or no inhibition was seen at 4 and 10°C. Reversal of this inhibition did not occur even when the sample had been diluted 10-fold or more or when the sample had been treated with 1% Triton X-100 added to the assay medium. This temperature-dependent and irreversible inactivation by phospholipid is not specific to protein kinase C. Gorgani and Wilson [4] reported that hexokinase was inhibited by phospholipid and we demonstrate here that cAMP-dependent protein kinase was also inhibited by phospholipid in a similar manner (fig.2). When TPA was directly added to the reaction mixture, the phospholipid-induced inactivation of protein kinase C was greatly enhanced (fig.1A). PS was indispensable, and TPA alone was unable to inactivate the enzyme. Ca^{2+} or diacylglycerol mimicked TPA (not shown). In similar in vitro systems, cAMP-dependent protein kinase was not affected by TPA (fig.2). As shown in fig.1B, the M_r of protein kinase C (84000) is unchanged, even after inactivation by TPA. These results suggest that this inactivation is not caused by proteolysis of protein kinase C molecules. Kinetic analysis indicated that TPA greatly increased the apparent affinity of the enzyme for PS, and thus enhanced the inactivation of the enzyme. Namely, in the presence of PS alone, relatively higher concentrations of PS were needed. The concentration needed for half-maximum inactivation was 60 μg/ml. If, however, there was a small amount of TPA, this value was

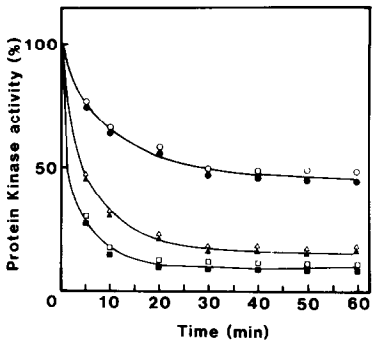

Fig.2. Inhibition of the catalytic subunit of cAMP-dependent protein kinase by PS. The catalytic subunit of cAMP-dependent protein kinase was incubated with PS as described in section 2 in the presence (●, ▲, ■) or absence (○, △, □) of 0.1 μg/ml TPA. PS at: (○,●) 10, (△, ▲) 50, (□, ■) 90 μg/ml.

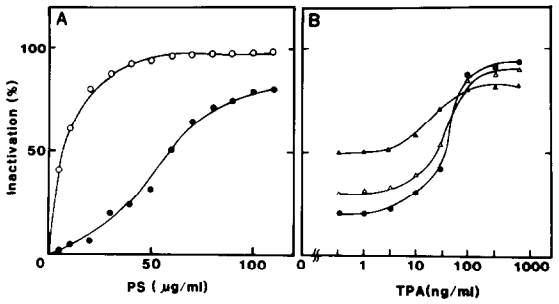

Fig.3. Inactivation of protein kinase C by TPA and PS at various concentrations of PS and TPA. (A) Protein kinase C was incubated with various concentrations of PS in the presence (○) or absence (●) of 0.1 μg/ml TPA as described in section 2. (B) Protein kinase C was incubated with various concentrations of TPA in the presence of 30 μg/ml PS, as described in section 2 in the presence of Ca^{2+} at: (●) 10^{-8}, (△) 10^{-7}, (▲) 10^{-6} M. Values given are percentages of activity lost after 40 min incubation under the various conditions indicated.

6 μg/ml (fig.3A). TPA produced a dose-dependent inactivation of protein kinase C in the presence of PS at concentrations of 10 ng/ml and above (fig.3B).

It has been reported that TPA greatly enhances protein kinase C activity with concomitant decreases in PS and Ca^{2+} concentrations required for enzyme activation [3]. Thus, our results suggest that the negative effects of TPA on protein kinase C activity may be mediated through mechanisms similar to those related to the activation processes of the enzyme molecule.

The inactivation reaction was carried out in the presence or absence of nucleotides, protein substrates, metal ion and the lipid interacting agents, trifluoperazine and chlorpromazine. Table 1 shows the extent of the protecting effect of these reagents. Trifluoperazine and chlorpromazine were reported to interact with phospholipid and thereby block the enzyme activating process [10,11]. These agents protected the enzyme from inactivation over the period (40 min) required for TPA and PS to inhibit the enzyme by 85%. Such evidence supports the proposal that enzyme cofactors inactivate protein kinase C through mechanisms similar to those involved in the reversible activation of the enzyme activity.

The free nucleotide ATP at 0.3 mM proved to be ineffective in preventing inactivation. In the

Table 1

Effect of various compounds on inactivation of protein kinase C by PS and TPA

Additions to reaction mixture	Percent residual activity
None	13
Trifluoperazine (0.3 mM)	82
Chlorpromazine (0.3 mM)	64
H-7 (0.3 mM)	79
GTP (0.3 mM)	13
ATP (0.3 mM)	13
ADP (0.3 mM)	13
$MgCl_2$ (1 mM)	21
$MgCl_2$ (3 mM)	38
$MgCl_2$ (1 mM) + H-7 (0.3 mM)	83
$MgCl_2$ (1 mM) + GTP (0.3 mM)	21
$MgCl_2$ (1 mM) + ATP (0.3 mM)	100
$MgCl_2$ (1 mM) + ADP (0.3 mM)	27
$MgCl_2$ (1 mM) + AMP (0.3 mM)	23
$MgCl_2$ (1 mM) + AMP-PNP (0.3 mM)	66
Histone III-S (0.4 mg/ml)	30
Smooth muscle myosin light chain (0.4 mg/ml)	13

Incubations were carried out as described in section 2 using 30 μg/ml PS and 0.1 μg/ml TPA. Aliquots were removed after 40 min incubation (under various conditions indicated) and assayed for ATP phosphotransferase activity

presence of 1 mM $MgCl_2$ and 0.3 mM nucleotide, ATP completely protected the enzyme from inactivation. Other nucleotides could not substitute for ATP; thus ADP (0.3 mM), AMP (0.3 mM) and GTP (0.3 mM) were minimally effective in preventing inactivation, whereas AMP-PNP (0.3 mM) was significantly effective. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7), a competitive inhibitor of protein kinase C with respect to MgATP [12,13], also prevented the inactivation of the enzyme. Mg^{2+} itself had a less potent effect on this inactivation. Protein substrates such as myosin light chain and histone III-S were examined for their possible protection. Myosin light chain which has a large net negative charge, was ineffective in preventing this inactivation, while histone III-S partially protected against the TPA and PS-induced enzyme inactivation. This effect is probably due to the large net positive charge of histone III-S near neutral pH.

It has been reported that for binding of ^{3}H-labelled phorbol ester to protein kinase C there is an absolute requirement for Ca^{2+} and phospholipid, and that this quaternary complex is enzymatically fully active for protein phosphorylation [14]. We suggest that this enzymatically active form of protein kinase C may be very unstable and that an adequate concentration of MgATP would act as a stabilizing factor of the active protein kinase C.

In summary, our data indicate that the mode of interaction of the enzyme cofactors with protein kinase C observed in negative modulation was similar to that in the case of enzyme activation; (i) in the presence of TPA, the PS-induced inactivation of protein kinase C was greatly enhanced; (ii) PS was indispensable and TPA alone was unable to inactivate the enzyme; (iii) Ca^{2+} or diacylglycerol mimicked TPA; (iv) PS, phosphatidylinositol, phosphatidic acid and cardiolipin but not phosphatidylcholine and sphingomyelin were active; (v) trifluoperazine and chlorpromazine, which interact with phospholipid and block the enzyme activating process, also prevented this inactivation. It is interesting to note the ATP analogues which are capable of affording protection from inactivation. In the series ATP, AMP-PNP, ADP and AMP, a significant protection was observed only with the two nucleotides containing the γ-P (ATP and AMP-PNP). In the absence of the γ-P (ADP), the protection was considerably lower, and the absence of β-P as well (AMP) resulted in an analogue that hardly afforded protection from inactivation.

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