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Spermine protects protein kinase C from phospholipid-induced inactivation

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Abstract. Phosphatidylserine (PS), an activator of protein kinase C (PKC) in the assay of protein phosphorylation, inhibited this enzyme in a time-dependent manner following preincubation in the absence of Ca^{2+}. The phospholipid-induced inactivation of kinase activity was dependent on the PS content and on the charge density of liposomes. This inactivation of PKC could be reduced, but not completely eliminated, by addition of Ca^{2+}.
In the present work the effect of a naturally occurring polyamine (spermine) on the PS-induced inactivation of PKC was investigated. The presence of spermine during preincubation without Ca^{2+} was effective in suppressing the PS-induced inactivation of PKC over the period (20 min) required for PS to inhibit the enzyme by 95%.
PKC exists in two membrane-bound states: a reversible one which can be dissociated by Ca^{2+} chelators (membrane-associated form) and an irreversible one which is chelator-stable (membrane-inserted form).
Gel filtration experiments on the PKC-PS complex formed in the presence of Ca^{2+} indicated that less insertion of enzyme into liposomes occurred in the presence of spermine and that the kinase activity of the reversibly membrane-associated PKC was protected from PS inactivation.
Key words. Protein kinase C; polyamines; spermine; protein kinase C inactivation; phospholipid vesicles.

Protein kinase C (PKC) is a phospholipid-dependent group of protein kinases implicated in the regulation of a variety of cellular responses[1]. Interaction of PKC with acidic phospholipids, such as phosphatidylserine (PS), in the presence of Ca^{2+} is believed to be an obligatory step leading to the activation of the kinase. However, the phospholipid-bound enzyme becomes catalytically active only in the presence of diacylglycerol or phorbol esters[1]. In contrast to this positive interaction between acidic phospholipids and PKC, it has been reported that, in the absence of Ca^{2+}, acidic phospholipids can inactivate the kinase activity without influencing the phorbol ester binding activity[2]. The PS-induced inactivation of PKC apparently results from a direct interaction of the phospholipid with the catalytic domain of PKC.
Bazzi and Nelsestuen have shown that PKC can form two membrane-bound states: one is calcium dependent, and membrane binding is reversible upon calcium chelation ('membrane-associated form'); the other is chelator-stable and has the characteristics of an intrinsic membrane protein ('membrane-inserted form')[3]. Previous reports from our laboratory indicated that aliphatic polyamines, such as spermine, interact with acidic phospholipids and greatly decrease the amount of inserted enzyme[4, 5].
The present study was performed to assess the effect of spermine on the PS-induced inactivation of PKC. The results reported here show that spermine, at physiological concentrations, protects the enzyme from inactivation by reducing the insertion of PKC into the hydrophobic core of the membrane.

Materials and methods

Unless indicated, all the chemicals and the reagents were from Sigma Chemical Co. and were of the highest grade of purity. DEAE-cellulose and Sephacryl S-300 were from Whatman and phenyl-Sepharose was from Pharmacia. [^{3}H]phorbol 12,13-dibutyrate (PDBu) (20 Ci/mmol) was from Du Pont-New England Nuclear, [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham Corp. and Soluene-350 was from Packard.
Unilamellar phospholipid vesicles comprising different percentages of phosphatidylserine (PS) and phosphatidylcholine (PC) were prepared by a published method[6] and resuspended in 20 mM Tris-HCl (pH 7.5). Phosphorus was determined by the method of Marinetti[7].
Protein kinase C isolated from Wistar rat brain was routinely partially purified by a method adapted from Huang et al.[8].
This study was conducted on a mixture of the enzyme isoforms since it had previously been demonstrated that PS-induced inactivation is marked in the case of PKC I but rather poor in that of PKC II and III[2].
PKC activity was measured in a reaction mixture (0.25 ml) containing 20 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 60 μM[γ-^{32}P]ATP, 12 μg/ml leupeptin, 0.1 mM EGTA, 160 μg/ml histone III-S, 100 μM phospholipid vesicles, 4 μg/ml diacylglycerol (DG) and 0.05 units of protein kinase C. Incubation was carried out at 30 °C for 5 min. $CaCl_2$ was added to give 0.5 mM free Ca^{2+}. Protamine sulfate kinase activity was measured under the same conditions, but without Ca^{2+},

phospholipid vesicles and DG. 1 unit of kinase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of P_i into protein substrate/min at 30 °C. The phospholipid-induced inactivation assay was performed in two steps: preincubation was carried out at 30 °C for the times indicated in 20 mM Tris-HCl (pH 7.5) containing 12 µg/ml leupeptin, 0.1 mM EGTA, enzyme and phospholipid vesicles at the compositions and concentrations noted in the figure legends; the kinase reaction was then started at 30 °C upon addition of the kinase assay mixture described above. Control experiments were run following the same preincubation step, but in the absence of phospholipids.

The amount of PKC associated with the phospholipid vesicles was estimated by measuring its [^{3}H]PDBu binding activity[9] in a reaction mixture (0.25 ml) containing 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 10 mg/ml bovine gamma-globulins, 12 µg/ml leupeptin, 20 nM [^{3}H]PDBu and PKC-phospholipid complex. The suspension was incubated at 30 °C for 30 min. Samples were then processed as previously described[4].

The formation of PKC-phospholipid complexes was assayed by gel filtration chromatography[10]. In this case, the enzyme (0.25 units) was mixed with 100 µM PS/PC (50:50) vesicles in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin (BSA), 12 µg/ml leupeptin, 0.1 mM EGTA and 0.1 mM free Ca^{2+}. The mixture was incubated at 30 °C for 20 min to allow association, and two aliquots were withdrawn. EGTA was added to one aliquot to a final concentration of 2 mM, to allow dissociation. After an additional incubation of 20 min, the samples were applied on Sephacryl S-300 columns (1.5 × 25 cm) equilibrated and eluted (1 ml fractions) with a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulphonyl fluoride, 0.5 mg/ml BSA, 30 mM β-mercaptoethanol and either 1 mM EGTA (for samples containing EGTA) or 0.1 mM free Ca^{2+} (for samples containing Ca^{2+}). The amount of PKC associated with the phospholipid vesicles was estimated by measuring the [^{3}H]PDBu binding activity of each fraction (0.1 ml) containing PKC-phospholipid complex as described above. The protein kinase activity of each fraction (0.1 ml) was assessed as described above by measuring ^{32}P incorporation into histone IIIS in the presence of Ca^{2+}/PS/DG to determine the activity of the free enzyme.

The concentration of protein was determined as described by Lowry et al.[11] using BSA as a standard.

Results and discussion

PKC is thought to be active when associated with the lipid bilayer[12]. Interaction of PKC with acidic phospholipids, such as PS, was thought to take place only in the presence of Ca^{2+} (cf. ref. 13), but, more recently, it has been reported that PKC can associate with, and subsequently penetrate, the lipid bilayer in the absence of this divalent cation[14]. However, preincubation of PKC in the presence of phospholipid vesicles, before starting the phosphorylation assay by addition of ATP, Ca^{2+}, Mg^{2+}, diacylglycerol and the histone IIIS as substrate, resulted in a time-dependent inactivation of the enzyme. As illustrated in figure 1, nearly 80% of the kinase activity was inactivated after 5 min of preincubation with 100 µM phospholipid vesicles containing PS/PC (50:50). These results are in agreement with those by Huang et al.[2], who found that the catalytic function of the enzyme was irreversibly destroyed by PS.

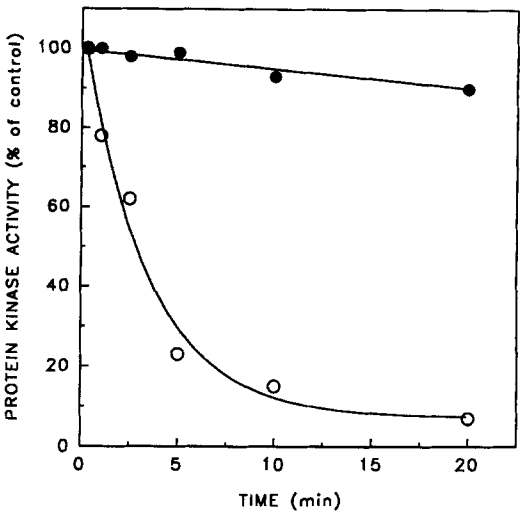

Figure 1. Effect of spermine on phospholipid-induced inactivation of PKC. PKC (0.05 units) was mixed with 100 µM PS/PC (50:50) vesicles in a buffer containing 0.1 mM EGTA and incubated at 30 °C both in the absence (○) and in the presence (●) of 50 µM spermine. At timed intervals, samples were taken for measurements of protein kinase activity with histone IIIS at substrate in the presence of 4 µg/ml DG and 0.5 mM free Ca^{2+}. Incubation of PKC without phospholipid was used as control. Results are means for four separate experiments.

We have previously demonstrated that spermine interferes with the activation of PKC[15,16] by interacting with negatively-charged phospholipids. Accordingly, we set out to establish whether this polyamine might prevent the PS-induced inactivation of the kinase activity. As shown in figure 1, this was indeed the case: spermine protected the enzyme from inactivation over the period (20 min) required for PS to inactivate the enzyme by 95%.

The PS-dependent inactivation of PKC and the protective effect of spermine were also observed when protamine sulfate was the substrate (table). Of the polyamines tested, spermine protected the enzyme completely, spermidine was significantly effective in preventing inactivation, while putrescine had a less potent effect. Addition of $CaCl_2$ or $MgCl_2$ only slightly reduced the effect of PS.

In the experiments reported in figure 2, we investigated the influence of PS concentration and charge density of

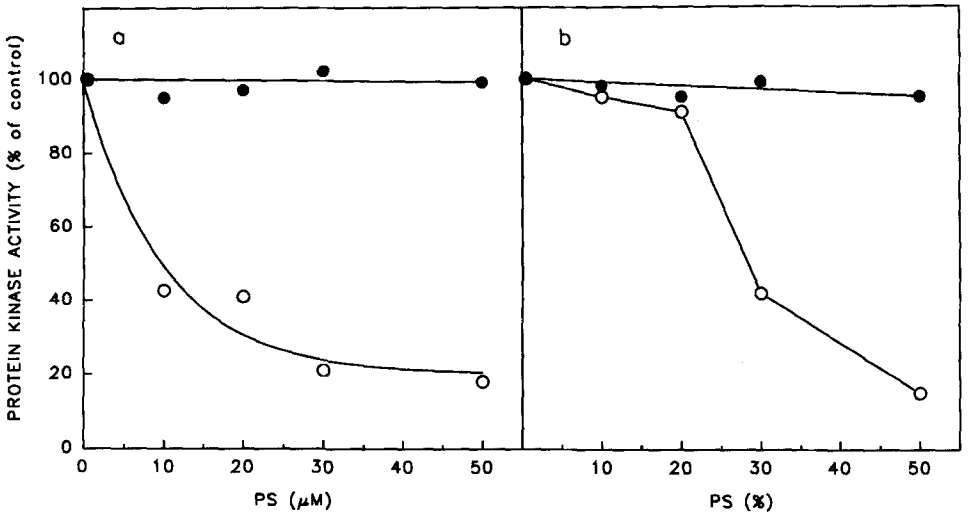

Figure 2. Effect of spermine on inactivation of PKC by phospholipid vesicles as a function of PS concentration (panel *a*) and PS percentage (panel *b*).
Panel *a*. PKC (0.05 units) was mixed with increasing amounts of PS/PC (50:50) vesicles in a buffer containing 0.1 mM EGTA and incubated at 30 °C both in the absence (○) and in the presence (●) of spermine. This polycation was added to give a 1:1 spermine/PS concentration ratio. After 5 min, samples were taken for measurements of protein kinase activity with protamine sulfate as substrate. Incubation of PKC without phospholipid was used as control.
Panel *b*. PKC (0.05 units) was mixed with phospholipid vesicles containing 50 μM PS at the indicated percentage (PC being the other phospholipid present) in the absence (○) or in the presence (●) of 50 μM spermine. The incubation and protein kinase activity assay conditions were as described in panel *a*. Results are means for four separate experiments.

liposomes and the effect of spermine on the PS-induced inactivation of PKC. As shown in panel a, preincubation of PKC with 10 μM PS for 5 min resulted in about 50% inactivation of the kinase. Increasing PS concentration resulted in a higher level of PKC inactivation. The presence of spermine during preincubation was able to prevent inactivation at all PS concentrations tested. The same effect was observed when the liposomes of varying composition were used (panel b). In the present experiments, the enzyme preparation was preincubated for 5 min with phospholipid vesicles containing from 10 to 50% PS, PC being the other phospholipid present. The results show a strong membrane-surface charge effect; vesicles containing 20% PS did not inactivate PKC, whereas vesicles containing 30% PS achieved 60% inactivation (the PS concentration was 50 μM in all cases). Once again, in the presence of spermine, the PS-induced inactivation of kinase activity was prevented.

Table 1. Effect of spermine and metal ions on phospholipid-induced inactivation of PKC

Additions	Protein kinase activity
	%
PKC alone	100
+100 μM PS/PC (50:50) vesicles	20
+25 μM Spermine	97
+50 μM Spermine	98
+25 μM Spermidine	70
+50 μM Spermidine	80
+25 μM Putrescine	20
+50 μM Putrescine	45
+100 μM $CaCl_2$	38
+1 mM $CaCl_2$	48
+2 mM $CaCl_2$	51
+1 mM $MgCl_2$	20
+2 mM $MgCl_2$	52

PKC (0.05 units) was incubated with 100 μM PS/PC (50:50) vesicles and other compounds at 30 °C for 5 min in a buffer containing 0.1 mM EGTA. The protein kinase activity was then assayed with protamine sulfate as substrate. The activity without any addition was taken as 100%. Results are means for three separate experiments.

These results indicate that the inactivation of PKC is caused by high PS content and high membrane charge and suggest that the interaction of spermine with the membrane surface, by decreasing the pool of PS and modulating the surface charge[17], may promote conditions that oppose enzyme inactivation.

It has been reported[3] that PKC can form two different membrane-bound states. The first is calcium-dependent and reversible upon calcium chelation. The second, termed 'irreversible' or 'membrane-inserted', is chelator-stable. Recently, we demonstrated[4,5] that spermine is able to alter the distribution of the two membrane-bound states of the enzyme by reducing the extent of PKC insertion into the bilayer without influencing the total amount of enzyme bound to liposomes. However, the influence of spermine, added during the association of PKC to phospholipid vesicles, on the kinase activity of the two bound forms of the enzyme has not yet been clarified; accordingly, in an attempt to determine whether spermine, by inhibiting the insertion of PKC into liposomes, protects kinase activity, the following experiments were performed.

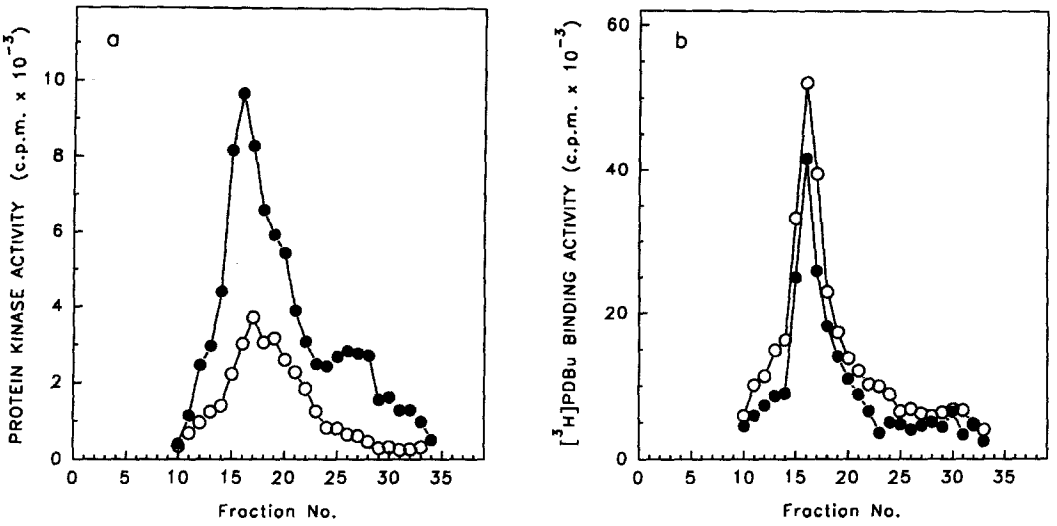

Figure 3. Effect of spermine on PKC-phospholipid complex formed in the presence of Ca^{2+}. PKC (0.25 units) was mixed with 100 μM PS/PC (50:50) vesicles in a buffer containing BSA and 0.1 mM free Ca^{2+} and incubated at 30 °C for 20 min both in the absence (○) or in the presence (●) of 50 μM spermine. Samples were then applied to Sephacryl S-300 columns (1.5 × 25 cm), equilibrated and eluted with a buffer containing BSA and 0.1 mM Ca^{2+}. Fractions of 1 ml were collected for the measurements of protein kinase activity (panel *a*) and [^{3}H]PDBu binding activity (panel *b*) as described in the 'Materials and methods' section. Similar results were obtained in three separate experiments.

Phospholipid vesicles composed of PS/PC (50:50) and a concentration of PS as high as 50 μM were used to generate a high level of the non-dissociable PKC-membrane complex[18]. The complexes formed at 100 μM Ca^{2+} both in the presence and in the absence of spermine were subjected to gel filtration on Sephacryl S-300 columns (fig. 3). As shown, PKC formed complexes with PS regardless of the presence or absence of spermine, as demonstrated by the emergence of both kinase (panel a) and phorbol ester binding (panel b) activities at the exclusion volume of the columns. The addition of spermine during the association process did not affect the total amount of enzyme bound to the vesicles as determined by [^{3}H]PDBu binding activity. However, the PKC-phospholipid complex formed in the absence of spermine exhibited a reduced protein kinase activity; conversely, the inclusion of polyamine in the protein kinase activity assay of the column frac-

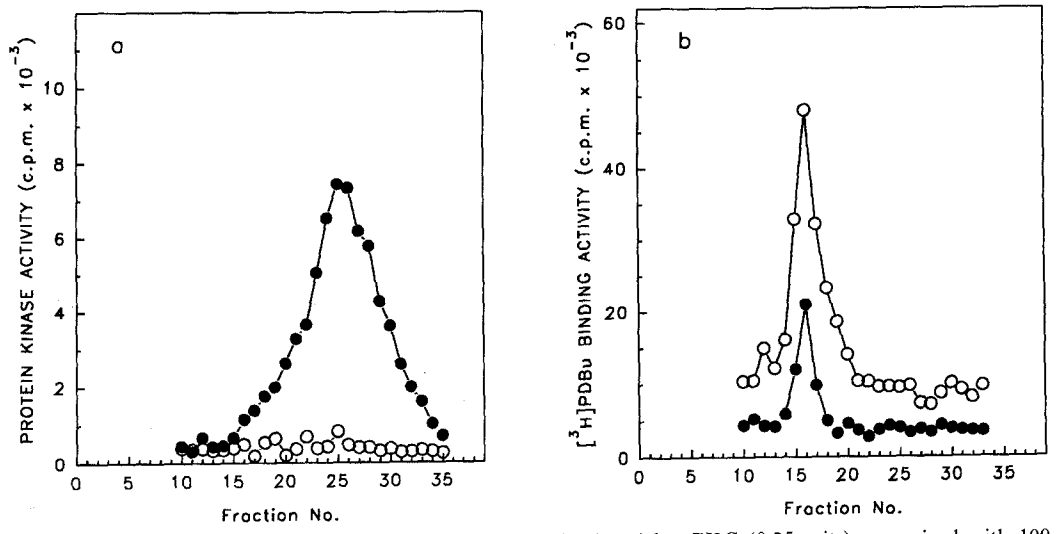

Figure 4. Effect of spermine on the insertion of PKC into phospholipid vesicles. PKC (0.25 units) was mixed with 100 μM PS/PC (50:50) vesicles in a buffer containing BSA and 0.1 mM free Ca^{2+} and incubated at 30 °C for 20 min both in the absence (○) or in the presence (●) of 50 μM spermine to allow association. The extent of enzyme insertion into liposomes was then examined by dissociation with EGTA. Samples were incubated at 30 °C in the presence of 2 mM EGTA for an additional 20 min and then applied to Sephacryl S-300 columns (1.5 × 25 cm), equilibrated and eluted (1 ml fractions) with a buffer containing BSA and 1 mM EGTA. Histone kinase activity (panel *a*) and [^{3}H]PDBu binding activity (panel *b*) of each fraction were assessed as described in the 'Materials and methods' section. Similar results were obtained in three separate experiments.

tions was not able to increase this phosphorylating activity (data not shown). These results confirm that significant inactivation of PKC in the presence of PS took place in the presence of Ca^{2+}, while the addition of spermine during the association process protected the enzyme.

The PKC-phospholipid complexes formed both in the presence and in the absence of spermine were then dissociated by EGTA and subjected to gel filtration (fig. 4). Under the experimental conditions used, in the absence of spermine PKC remained associated with phospholipids after successive additions of EGTA as determined by [^{3}H]PDBu binding activity of the complex eluted at the exclusion volume of the column (panel b). By contrast, an appreciable dissociation of the enzyme from the vesicles was detected when spermine had been added during the association process. In fact, the PKC-phospholipid complex formed in the presence of spermine showed a reduced [^{3}H]PDBu binding activity (about 50%) after addition of EGTA, indicating that PKC insertion into the membranes was inhibited by the presence of spermine. After the 2-h period required for the experiment, the kinase activity of the inserted forms of PKC was completely lost in the absence of Ca^{2+} (panel a). In the sample preincubated in the presence of spermine, a peak of phosphotransferase activity was eluted near the inclusion volume of the column at the position of the free enzyme. The above result confirms that PKC dissociation from liposomes took place under these conditions, and indicates that the kinase activity of reversibly bound enzyme was protected from PS-inactivation.

Early reports from many laboratories, including ours, have shown that the formation of the inserted form of PKC is promoted by high phosphatidylserine content and high membrane charge[5,18]. It is likely that subtle changes in the local concentration of PS may provide a sensitive mechanism for the regulation of the insertion of PKC. The present findings suggest that the formation of membrane-inserted PKC, by bringing PS close to the catalytic domain of the enzyme, facilitates the inactivation of the phosphorylating activity of the enzyme without influencing phorbol ester binding. In these conditions, spermine, by decreasing the pool of PS and shielding the surface charges of the membrane, promotes binding conditions that oppose the penetration of PKC into the lipid bilayer. Thus, the reversible Ca^{2+}-dependent PKC-membrane complex formed in the presence of spermine is protected from inactivation induced by PS.

Assuming that the insertion of PKC into membranes and its inactivation by PS possess physiological relevance, the reported effect of spermine may represent a significant modulatory mechanism in the living cell, where changes in polyamine content and membrane phospholipid microenvironment accompany important variations in major cellular functions.

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