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Modulation of cofactor requirement for the activation of protein kinase C by heparin

Possible effect at the regulatory domain

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Heparin was found to stimulate the phosphorylation of histone H1 but not protamine sulfate catalyzed by Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C or PKC). The effect of heparin on histone H1 phosphorylation appeared to be due to an increase in phosphatidylserine affinity for PKC activation in the presence of heparin. This effect of heparin was abolished when trypsinized, cofactor-independent, PKC was employed to phosphorylate histone H1. These studies suggest that heparin acts at the regulatory domain of PKC, and emphasize the importance of the negative charge in influencing the accessibility of the substrate to PKC action.

Protein kinase C; Heparin; Phosphorylation; Regulatory domain

1. INTRODUCTION

Protein kinase C (PKC) requires Ca^{2+}, phospholipids, and diacylglycerol for activation and translocation from cell cytosol to membranes. The three most commonly studied isozymes of PKC (types I, II and III) are closely related in structure and properties. They contain regulatory and catalytic domains at the amino terminal half and at the carboxyl terminal half, respectively [1,2]. The former domain has been shown to contain a conserved region with features of a pseudosubstrate which has been proposed to maintain the enzyme in the inactive form [3]. It has been suggested that the binding of the allosteric cofactors to the regulatory domain of the enzyme relieves the inhibition exerted at its catalytic domain [1,2]. Furthermore, it appears that, in the membrane environment, polybasic structures may contribute to an attenuation of PKC activity [3], whereas the presence of negatively charged environment on the surface of the membrane may markedly influence the accessibility of the substrate to PKC action [13]. Thus, the nature of the substrate also plays an important role in the cofactor interaction with the regulatory domain. For example, it has been shown that phosphorylation of histone H1 catalyzed by PKC is significantly enhanced by phospholipids and diolein, whereas that of protamine sulfate or the synthetic peptide for PKC is uninfluenced by these allosteric cofactors [1,2,4,5]. Analogous to this is the observation that partial proteolytic degradation of PKC results in the release of a fragment of the enzyme that retains its catalytic activity but lacks the requirement for allosteric cofactors [1,2].

In the present communication we provide new evidence to suggest that charge properties at the regulatory domain of PKC play a major role in relieving the enzyme inhibition. For this, we utilized heparin which is a well known negatively charged molecule of considerable biological significance [6]. Heparin is a potent inhibitor of certain messenger-independent protein kinase reactions [7,8]; however, we demonstrate here that PKC catalyzed phosphorylation of histone H1 but not of protamine sulfate is markedly enhanced in the presence of heparin. This effect of heparin appears to be mediated via its interaction with phosphatidylserine binding to PKC and/or histone H1 substrate.

2. MATERIALS AND METHODS

2.1. *Animals*

Male Sprague-Dawley rats weighing 295–325 g, purchased from Harlan Sprague-Dawley Co., Indianapolis, IN, were used as a source of ventral prostate as described previously [9].

2.2. *Chemicals*

Lysine-rich histone H1 was obtained from Worthington Biochemical Corp., Freehold, NJ. Diolein, phosphatidylserine, pro-

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tamine sulfate, and heparin were purchased from Sigma Chemical Co., St. Louis, MO. Phenylmethylsulfonyl fluoride and leupeptin were supplied by US Biochemical, Cleveland, OH.

2.3. *Purification of PKC*

The procedure employed for partial purification of rat ventral prostate PKC isozymes was as described previously [10,11]. Two main isoforms of PKC (type II or β, and type III or α) were identified in the prostatic cytosol [11]. Cofactor-independent form of the PKC was prepared by treatment of the purified enzyme (35 μg/ml) with trypsin (1 μg/ml for 3 min at 30°C) as described previously [12]. Trypsinization was terminated by the addition of soybean trypsin inhibitor (25 μg/ml).

2.4. *Determination of PKC activity*

PKC activity assays were carried out at 30°C over a time course of 0, 3, and 6 min in a reaction medium consisting of 10 mM magnesium acetate, 1.2 mM $CaCl_2$, 1 mM EGTA, 50 μg/ml phosphatidylserine, 10 μg/ml diolein, 0.04% NP-40, 0.1 mM [γ-^{32}P]ATP (spec. act. of 100–500 dpm/pmol of ATP), 20 mM Tris-HCl, pH 7.5, and 1 mg/ml histone H1 or protamine sulfate as substrate, in a final volume of 0.15 ml. The reaction was initiated by the addition of the enzyme and terminated at the time periods indicated. PKC activity was expressed as nmol ^{32}P incorporated in the substrate/h/mg of enzyme protein in the presence of Ca^{2+}/phospholipids minus that in the absence of Ca^{2+}/phospholipids [11]. The experimental data presented are representative of type II isozyme of PKC; however, type III isozyme also gave similar results.

3. RESULTS AND DISCUSSION

It is known that maximal histone H1 phosphorylation catalyzed by PKC requires the presence of Ca^{2+}/phospholipids, whereas that of protamine sulfate can occur in the absence of these cofactors. The regulation of substrate phosphorylation by anionic lipids (phosphatidylserine and diolein) is believed to relate to their charge properties in providing a favorable environment for interaction of PKC with the substrate [2,4,5]. Heparin, a naturally occurring negatively charged polymer with numerous biological activities [6], is known to influence certain protein kinase reactions such as those catalyzed by messenger-independent casein kinase II (see e.g. [7,8]). We therefore examined the effects of heparin on phosphorylation of histone H1 and protamine sulfate catalyzed by purified PKC from rat ventral prostate cytosol. As shown in Table I, the presence of heparin (at 0.2–5 μg/ml) evoked a marked concentration-dependent stimulation of phosphorylation of histone H1 but not of protamine sulfate. Further, this observation was apparent for both the type II and type III isozymes of PKC (Fig. 1).

Experiments were undertaken to determine if the stimulatory effect of heparin on histone phosphorylation was mediated via its action at the catalytic or at the regulatory domain of PKC. It was observed that heparin stimulation of histone H1 phosphorylation was independent of the ATP concentration, suggesting that the effect of heparin was not due to its interaction with the catalytic domain of PKC. The apparent k_m for ATP

Table I

Effect of heparin on phosphorylation of histone H1 and protamine sulfate catalyzed by PKC

Heparin (μg/ml)	PKC activity (nmol ^{32}P/mg/h)	Percent change
Histone H1		
0.0	4079	0
0.2	4803	+18
0.5	5109	+25
2.0	8173	+100
5.0	9057	+122
Protamine sulfate		
0.0	496	0
5.0	501	+1

The experimental details for measurement of PKC activity towards histone H1 and protamine as substrates were as described in section 2. Concentration of heparin was varied as shown

(10 μM observed by us) remained unchanged in the presence of 5 μg/ml of heparin in the reaction. Likewise, the heparin stimulation of PKC activity occurred only in the presence of Ca^{2+} and heparin did not replace the Ca^{2+} requirement for the enzyme activity (data not shown).

Allosteric cofactors such as diolein and phosphatidylserine produce an activation of PKC (depending on the nature of the substrate) through effects at the Ca^{2+} site (for diolein) or through modification of the regulatory domain via substrate-lipid complex formation for substrate interaction with the catalytic site. We therefore examined the effects of heparin on diolein and phosphatidylserine requirement for PKC activity towards histone H1 as substrate which

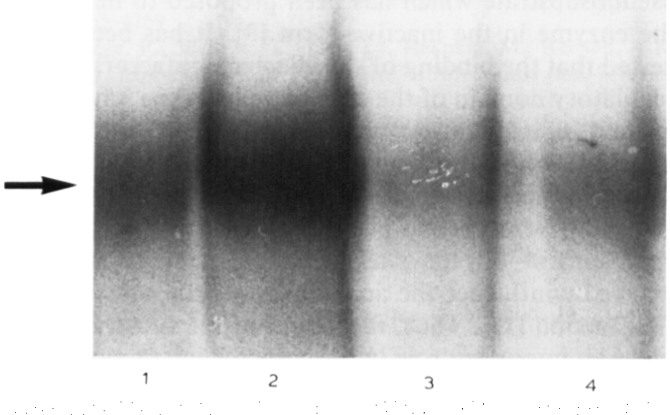

Fig. 1. Effect of heparin on phosphorylation of histone H1 catalyzed by type II and type III isozymes of PKC. Autoradiogram of gel electrophoretically separated histone H1 substrate after phosphorylation catalyzed by PKC isozymes in the absence and presence of heparin (5 μg/ml) is shown. Lanes 1 and 2, PKC type II isozyme; lanes 3 and 4, PKC type III isozyme; lanes 1 and 3, control without heparin; lanes 2 and 4, with heparin. Arrow indicates the position of histone H1.

Table II

Effect of heparin on PKC activity towards histone H1 at varying concentrations of phosphatidylserine (PS)

PS (μg/ml)	PKC activity (nmol ^{32}P/mg/h)		
	Control	Heparin (5 μg/ml)	% Change
0.0	975	1647	+69
10	2691	7455	+177
25	4710	8708	+85
50	5177	9712	+88
100	8120	10054	+24
200	10392	12266	+18

The experimental details for measurement of PKC activity were the same as described in section 2, except that phosphatidylserine (PS) was added at the concentrations shown

Table III

Effect of heparin on the activity of trypsinized PKC towards histone H1 as substrate

Additions	PKC activity (nmol ^{32}P/mg/h)		
	No cofactors	With cofactors	ΔPKC
Intact enzyme			
None	200	3200	3000
Heparin (5 μg/ml)	270	7270	7000
Trypsinized enzyme			
None	3100	3100	0
Heparin (5 μg/ml)	3200	3200	0

PKC activity of the intact and trypsinized enzyme was measured in the presence of the complete assay system as described in section 2, except that phosphatidylserine, diolein, and Ca^{2+} were omitted when assays were carried out in the absence of various cofactors as shown. ΔPKC represents the difference of enzyme activity with or without cofactors added under each experimental condition

requires both of these cofactors for maximal phosphorylation. In results not shown, it was observed that at varied concentrations of diolein, the stimulatory effect of heparin on phosphorylation of histone H1 by PKC was essentially the same at all concentrations of diolein, i.e., it was enhanced by about 2-fold, as also observed in the absence of added diolein. This result suggested that heparin did not substitute for diolein at its site(s) of action. It is generally believed that diolein activates PKC by reducing the requirement for Ca^{2+}; a lack of effect of heparin on diolein requirement may be related to its lack of effect at the Ca^{2+} site.

Since activation of PKC with histone H1 as substrate also requires phosphatidylserine, the effect of heparin was studied at varying concentrations of phosphatidylserine (Table II). Heparin stimulated the PKC activity towards histone H1 in the absence of phosphatidylserine; however, in the presence of heparin, a maximal stimulation of PKC activity was obtained at significantly lower concentrations of phosphatidylserine (10 μg/ml phosphatidylserine plus 5 μg/ml of heparin as compared with that at 100 μg/ml of phosphatidylserine in the absence of heparin). Kinetic analysis showed that heparin produced a 6-fold decrease in the apparent k_m for phosphatidylserine, and as expected, at relatively higher concentrations of phosphatidylserine the effect of heparin was attenuated.

The above-described observations suggested that heparin-induced lowering of the requirement for phosphatidylserine in the reaction may relate to an effect of heparin at the regulatory domain. To test this hypothesis, effect of heparin on trypsinized PKC was studied. Trypsinization of PKC results in its cleavage into the catalytic (~51 kDa) and regulatory fragments; however, the former behaves as a cofactor-independent PKC. It is clear from the data in Table III that heparin stimulation of histone H1 phosphorylation was abolished when trypsin-treated enzyme was used.

It has been suggested that the interaction of a given substrate with various domains of PKC may determine the activity of the enzyme towards the particular substrate [2,5]. With regard to protamine sulfate as substrate, it is known that its phosphorylation catalyzed by PKC is not significantly enhanced in the presence of phosphatidylserine and diolein [2], giving rise to the proposal that protamine sulfate interacts strongly with PKC resulting in its aggregation in a binary mixture. This results in the presentation of the substrate to the proximity of the active sites even without the cofactors. On the other hand, histone H1 phosphorylation by PKC may depend on its association with Ca^{2+}/phosphatidylserine/diolein complex [2]. We propose that heparin action on the regulatory domain of PKC is mediated largely via its lipid-binding domain. This is based on the following observations on heparin action: (1) it lowers the $K_{m\,app}$ for phosphatidylserine activation of PKC; (2) it has no effect on the activity of PKC towards protamine sulfate as substrate; (3) it has no effect on the kinase activity of the cofactor-independent catalytic fragment of PKC. Thus, heparin may alter the interaction of certain substrate/phospholipid complexes and contribute an additional negatively charged surface resulting in greater accessibility of the substrate to the enzyme.

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