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Phosphorylation of protein kinase C by casein kinase-1

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Because phosphorylation of protein kinase C (PKC) may provide a mechanism for regulation of this enzyme, we have examined the ability of two other kinases to phosphorylate PKC. Our results show that casein kinase 1 (CK-1), but not casein kinase 2 (CK-2), can phosphorylate PKC in the absence of Ca^{2+} and phospholipids. The ^{32}P incorporation into PKC in the presence of Ca^{2+} and phospholipids is also enhanced by CK-1.

Protein kinase C; Casein kinase 1

1. INTRODUCTION

Protein kinase C (PKC) has been shown to undergo Ca^{2+}- and phospholipid-stimulated autophosphorylation in vitro [1–6] and to be phosphorylated in vivo [7]. Accumulating evidence suggests that this phosphorylation may play a role in regulating enzyme activity. Evidence indicates that the autophosphorylation is intramolecular [4,6], that it exhibits a 10-fold lower K_m for ATP than does heterologous phosphorylation [4], that it requires the intact enzyme [5], and that it has a lower K_a for Ca^{2+} in a histone kinase assay [4]. One report suggests that autophosphorylation decreases the K_m for the substrate Histone [5]. Phosphorylation may also alter the membrane binding properties of PKC [4,8,9]. Because trans-phosphorylation could have comparable regulatory effects, we have examined the ability of two other kinases, CK-1 and CK-2, to phosphorylate PKC.

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Abbreviations: PKC, protein kinase C; CK-1, casein kinase 1; CK-2, casein kinase 2

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP, 0.15–1.0 Ci/mmol or 6000 Ci/mmol, was obtained from the Diabetes Research Center Core Laboratory at the University of Virginia or New England Nuclear, respectively. [20-^{3}H]Phorbol 12,13-dibutyrate (10.2 Ci/mmol in ethanol) was also from New England Nuclear. 1,2-*sn*-Dioleoylglycerol was from Sigma. Phospholipids were from Avanti Polar Lipids (Birmingham, AL). Sprague-Dawley rats were from Charles River Breeding Laboratories (Wilmington, MA). Lysine-rich histone was from Sigma (type IIIS). Glycerin, USP, was from J.T. Baker Chemical Co. All other chemicals were reagent grade.

2.2. *Purification and assay of enzymes*

PKC was purified from rat brain and assayed as described previously [11] using lysine-rich histone as substrate. cyclic AMP-independent CK-1 and CK-2 were purified from rat liver as described [12], and kinase activity was assayed at 30°C as in [13] using casein as substrate.

2.3. *Phosphorylation of PKC*

Autophosphorylation of PKC was carried out in a reaction mixture (30 μl) containing 300 μM $CaCl_2$, 100 μM [γ-^{32}P]ATP (3000 cpm/pmol), 5 mM magnesium acetate, 20 mM Tris buffer (pH 7.4 at 30°C), approx. 20 nM PKC, and 25 μg/ml bovine phosphatidylserine, 51 μg/ml egg phosphatidylcholine, and 3 μg/ml dioleoylglycerol (approximate lipid molar ratio of 30:65:5 with a total of 100 μM). Phosphorylation of PKC by

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CK-1 was carried out at 30°C in a reaction mixture (30 μl) containing 10 mM Tris buffer (pH 7.2), 10 mM $MgCl_2$, 100 μM ATP (3000 cpm/pmol), the indicated amounts of PKC and CK-1 with or without 300 μM $CaCl_2$ and lipids as described above. The reaction was stopped by addition of 4× Laemmli sample buffer (6% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 5 min. The resulting mixture was subjected to electrophoresis on 10% polyacrylamide gels in the presence of 0.1% SDS according to the method of Laemmli [14].

For determination of the stoichiometry of phosphorylation, the amount of PKC was quantitated by assay of phorbol 12,13-dibutyrate binding as described previously [15], and the phosphate incorporation was determined by scintillation counting of excised PKC gel bands.

2.4. *Phosphopeptide mapping of PKC*

Radiolabeled PKC was excised from unstained one-dimensional polyacrylamide gels and submitted to oxidation with performic acid. Trypsin digestion was carried out at 30°C with tosylphenylchloromethylketone-trypsin (10 μg/ml added every 8 h) for 24 h. The material was then lyophilized and peptide mapping was carried out on thin-layer cellulose plates with electrophoresis at 1000 V for 30 min at pH 6.5 (pyridine/acetic acid/water, 25:1:225) followed by chromatography in *n*-butyl alcohol/pyridine/acetic acid/water, 75:50:15:60. Phosphopeptides were visualized by autoradiography.

3. RESULTS AND DISCUSSION

To examine the possibility that PKC might be susceptible to regulation by other protein kinases, we first tested the ability of CK-1 and CK-2 to phosphorylate PKC. The casein kinases differ in their structural and kinetic properties [16] but both have rather broad substrate specificities suggesting that they could be involved in the control of a variety of cellular processes [17].

Autophosphorylation of PKC in the absence of Ca^{2+} and phospholipids was only 6% of that in the presence of the cofactors (fig.1 and table 1). Addition of CK-1 caused a 4-fold increase in the ^{32}P incorporation in the absence of Ca^{2+} and phospholipid. The presence of CK-1 and the PKC activators led to a synergistic increase in ^{32}P incorporation into PKC, consistent with the possibility that phosphorylation by CK-1 enhances the autophosphorylating activity of PKC. However, it is also possible that the presence of Ca^{2+} and phospholipids renders PKC a better substrate for CK-1.

The stoichiometry of ^{32}P incorporation in the presence of Ca^{2+} and phospholipids was 2.8 mol/mol PKC. This is in good agreement with the 2–2.5 mol phosphate/mol enzyme reported by Huang et al. [4] and 2.5–3 mol phosphate/mol enzyme reported by Mochly-Rosen and Koshland [5]. Addition of CK-1 with Ca^{2+} and phospholipids resulted in a further 2-fold enhancement of ^{32}P incorporation into PKC.

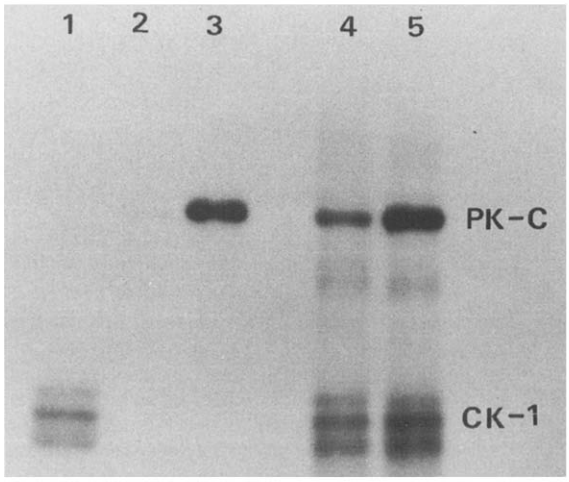

Fig.1. Autophosphorylation and CK-1-mediated phosphorylation of PKC. PKC (50 ng) was incubated with [γ-^{32}P]ATP in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of Ca^{2+} and phospholipids as described in section 2. The incubations were carried out in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of CK-1 (2 U/ml). CK-1 was also incubated with Ca^{2+} and phospholipids in the absence of PKC (lane 1). Phosphorylated enzymes were detected by autoradiography following their electrophoresis on 10% polyacrylamide gels. Positions of migration of PKC and CK-1 are indicated.

Phosphoamino acid analysis of the PKC band excised from one-dimensional gels revealed the presence of phosphoserine and phosphothreonine in preparations that had been autophosphorylated in the presence of Ca^{2+} and phospholipids, consistent with the results of Huang et al. [4]. The

Table 1

Quantitation of PKC phosphorylation with and without CK-1

Enzymes present	% of maximal autophosphorylation[a]	
	− lipids	+ lipids
PKC	6.1 ± 0.4	100
PKC + CK-1	24.3 ± 4.6	204 ± 28.6

[a] Phosphorylation and gel electrophoresis were carried out as in fig.1. The PKC band was excised and radioactivity was determined by scintillation counting. Results represent the means ± SEM of 6 independent experiments

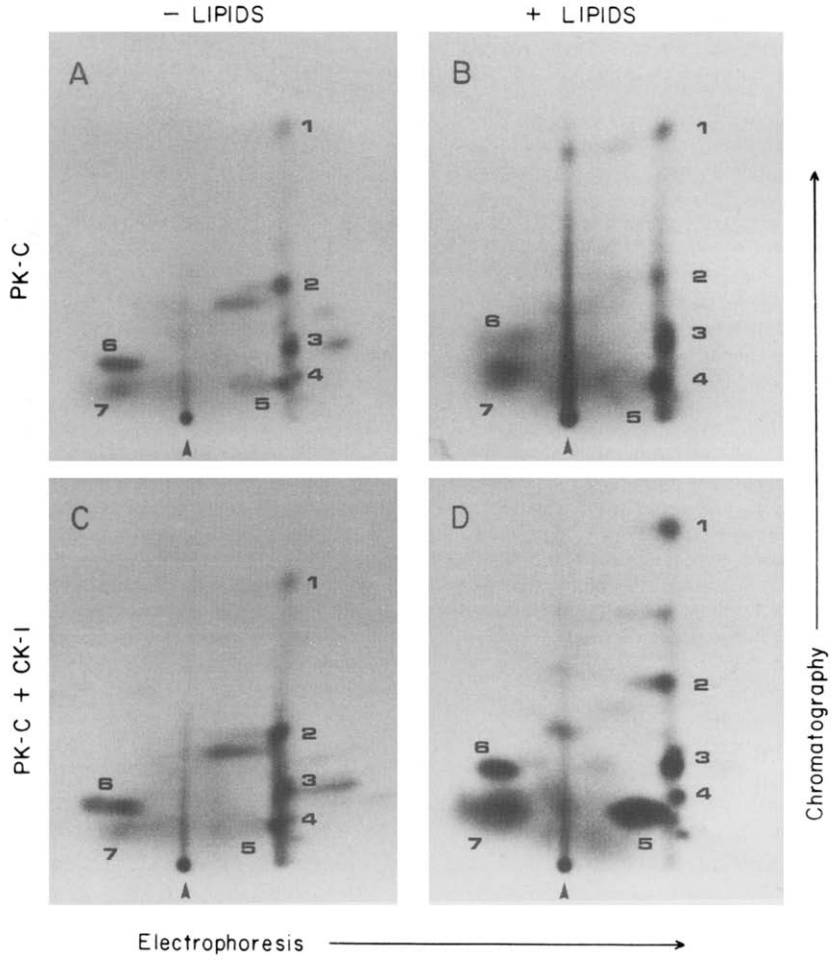

Fig.2. Phosphopeptide mapping of PKC autophosphorylated or phosphorylated by CK-1 in the presence or absence of Ca^{2+} and lipids. PKC was phosphorylated as in fig.1 in the absence (panels A and C) or presence (panels B and D) of Ca^{2+} and lipids and in the absence (panels A and B) or presence (panels C and D) of CK-1. Phosphopeptide fragments were generated and resolved as described in section 2. Autoradiographs shown are from a single experiment representative of four separate phosphopeptide mapping experiments using two different preparations of each enzyme. The arrow in each panel indicates the origin. Although equal radioactivity was used in all conditions, the results cannot be compared quantitatively because different autoradiography exposure times were required to optimize detection of the spots.

CK-1-mediated increase in ^{32}P incorporation in the absence of lipids was predominantly in phosphoserine (data not shown).

To determine whether CK-1 phosphorylated different sites on PKC than were phosphorylated in the autophosphorylation reaction, tryptic peptides derived from the phosphorylated enzyme were analyzed by two-dimensional mapping. Fig.2 shows seven prominent phosphopeptides that were consistently visualized in the autophosphorylated preparations. Although quantitative differences were observed in the peptides phosphorylated by CK-1 vs those affected by autophosphorylation, no new CK-1-phosphorylated peptides were identified. This suggests that trans-phosphorylation may induce the same regulatory changes induced in PKC by autophosphorylation, but without a requirement for diacylglycerol.

The observations made here provide the first evidence for modification of PKC by another kinase, CK-1. Possible specificity of the CK-1-mediated phosphorylation is suggested by

the failure of cAMP-dependent protein kinase [2] and CK-2 to phosphorylate PKC. These results raise the possibility that PKC may be directly regulated by trans-phosphorylation as has been shown for a number of other protein kinases.

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