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PROTEIN KINASE C PHOSPHORYLATES THE SYNTHETIC PEPTIDE ARG-ARG-LYS-ALA-SER-GLY-PRO-PRO-VAL IN THE PRESENCE OF PHOSPHOLIPID PLUS EITHER Ca^{2+} OR A PHORBOL ESTER TUMOR PROMOTER

Catherine A. O'Brian[1], David S. Lawrence[2], E. Thomas Kaiser[2] and I. Bernard Weinstein[1]

[1]Division of Environmental Sciences and Institute of Cancer Research Columbia University, New York, NY 10032

[2]Laboratory of Bioorganic Chemistry and Biochemistry, Rockfeller University, New York, N.Y. 10021

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SUMMARY: The synthetic nonapeptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val is a substrate for in vitro phosphorylation by a partially purified preparation of rat brain protein kinase C, with a Kmapp of about 130μM. The closely related peptide kemptide was a much weaker substrate, bovine serum albumin was not a substrate and the peptide Arg-Arg-Lys-Ala-Ala-Gly-Pro-Pro-Val was a weak inhibitor of the enzyme. Protein kinase C-catalyzed phosphorylation of histone III-S and the nonapeptide are regulated by identical mechanisms since with both substrates the reaction required added phospholipid and either Ca^{2+} (1mM) or TPA (200 nM TPA). Our findings show that polypeptides containing multiple basic residues followed by the sequence Ala-Ser can be substrates for TPA-stimulated phosphorylation by protein kinase C.

The compound TPA[1] and related tumor promoting phorbol esters exert pleiotropic effects on a variety of cells (1). High affinity binding sites for tumor promoting phorbol esters have been observed in the membranes of numerous cell types (2,3,4,5). PKC, a Ca^{2+}- and phospholipid-dependent protein kinase, can be activated by tumor promoting phorbol esters in vitro, and the relative potencies of a series of phorbol esters in tumor promotion tend to correlate with their relative potencies in PKC activation (6). These and other findings suggest that PKC is a major initial cellular binding site and transducer of the pleiotropic effects of phorbol ester tumor promoters (for review see 7). Recent studies indicate that the tumor promoters teleocidin and aplysiatoxin bind to the same cellular receptors as the phorbol esters (8) and also activate PKC (9,10).

[1]Abbreviations: TPA, 12-O-tetradecanoyl phorbol 13-acetate; PKC, protein kinase C; phenylmethylsulfonylfluoride; PI, phosphatidylinositol; PPi, pyrophosphate; BSA, bovine serum albumin; TCA, trichloroacetic acid

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PKC catalyzes the in vitro phosphorylation of a 40KD protein in platelets (11), vinculin (12), ribosomal protein S6 (13), myelin basic protein (14,15), and lysine-rich histone (16); there is indirect evidence that it also phosphorylates the receptors for EGF, insulin and somatomedin (17,18). In this report, the synthetic peptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val, which has the same amino acid sequence as that of the major site of PKC-catalyzed phosphorylation in lysine-rich histone (16), has been examined as a PKC substrate. In addition, the closely related peptide Arg-Arg-Lys-Ala-Ala-Gly-Pro-Pro-Val was assessed as a PKC inhibitor.

MATERIALS AND METHODS

Reagents: [γ^{32}P]ATP was purchased from Amersham. Sigma Chem. Co. supplied the ATP, BSA, Tris-hydrochloride, PMSF, kemptide, DEAE Sephacel, and histone III-S (the lysine-rich histone fraction). AcA-34 Ultrogel was from LKB, and Aquasol was from New England Nuclear. Phosphocellulose paper, grade P81, was from Whatman, and PI was from Avanti Chem. TPA was from LC Services. Biorad protein assay solution was used for all protein determinations. Leupeptin was a gift of the U.S.-Japan Cooperative Cancer Research Program. The synthetic peptides used in this study were synthesized, purified and characterized by previously described procedures (19).

Partial Purification of rat brain PKC: Within thirty seconds after slaughter, eight rat brains were placed in ice-cold Buffer A (20 mM Tris-HCl, 5 mM EGTA, 5mM EDTA,15 mM 2-mercaptoethanol, 0.25 mM PMSF, 10μg/ml leupeptin, at pH 7.5); all subsequent procedures were done at 4°C. The brains were homogenized in 80 ml of Buffer A by seventy-five strokes in a Teflon-glass homogenizer, and the homogenate was centrifuged in an SS34 rotor at 10,000 r.p.m. for twenty minutes. The supernatant fraction (61 ml) was passed through glass wool and loaded onto a DEAE Sephacel column (12 x 2.5 cm) equilibrated in Buffer A. The column was washed with 250 ml of Buffer A and then eluted with a 500 ml linear gradient of 0-0.3M NaCl in Buffer A. Active fractions were pooled, and the resultant solution was made 70% in ammonium sulfate. After stirring the solution for one hour, the solution was centrifuged in an SS34 rotor at 10,000 r.p.m. for one hour. The supernatant fraction was discarded, and the pellet was resuspended with Buffer A to a final volume of seven ml. The resuspended pellet was gel-filtered on an Ultrogel AcA-34 column (66 x 2cm) which was equilibrated and eluted with Buffer A. Active fractions were pooled and concentrated by ultrafiltration using a YM-30 membrane (from Millipore). The enzyme was stored for up to two weeks in Buffer A with 15% glycerol at 4°C. The specific activity of the enzyme was 120 nm/min/mg. Although this preparation is not homogeneous, the marked stimulation obtained with phospholipid and Ca^{2+} (or TPA)(see below) made it suitable for the present studies.

PKC assays: The standard reaction mixture contained in 0.12 ml: 20 mM Tris-HCl, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ or 1 mM EGTA, 40 μg/ml PI (or no phospholipid), 70 μM[γ^{32}P] ATP (250-400 cpm/pmol), 0.67 mg/ml histone III-S or the indicated phosphoacceptor substrate, and 1-4 μg rat brain PKC. Reactions were initiated by the addition of enzyme and were run at pH 7.5 and 30°C. Reactions were terminated by pipetting a 40 μl aliquot onto phosphocellulose paper. The papers were rinsed with three one-liter volumes of water and counted in Aquasol. Where indicated, reactions were terminated by TCA precipitation. This was done by adding 10 ml of ice-cold 20% TCA-1% PP_i to the reaction mixture, filtering the TCA-precipitated material on Millipore HA 0.45 μM filters, rinsing the filters with two ten-ml aliquots of TCA-PP_i solution,

and counting in Aquasol. All assays were done in triplicate and varied by less than 10%.

RESULTS

Regulation of rat brain PKC-catalyzed histone III-S phosphorylation by TPA, phospholipid, and Ca^{2+}. PKC activity was stimulated ten to thirty fold by 1mM Ca^{2+} plus phospholipid and was stimulated to a similar extent when the added Ca^{2+} was replaced with TPA (200 nM) (Table 1). Kinase activity was not greatly stimulated by either TPA or Ca^{2+} in the absence of phospholipid. The activation of PKC by 1 mM Ca^{2+} plus phospholipid was not further enhanced by the addition of TPA and the activation by 200 nM TPA plus phospholipid was not further enhanced by the addition of 1 mM Ca^{2+} (see also refs 9,10).

The velocity of PKC-catalyzed phosphorylation of histone III-S (0.67 mg/ml) was linear over a time course of three to fifteen minutes. We established that the ATP concentration employed in all assays (70 μM ATP) was saturating. The $Km_{app\ histone}$ of PKC was determined from a Lineweaver-Burk plot of five minute reactions run with histone III-S concentrations varying from 3 to 30 μM. The Lineweaver-Burk plots were analyzed by the least square method. The average of two determinations gave $Km_{app\ histone}$ values of 19 ± 5 μM.

PKC-catalyzed phosphorylation of a synthetic nonapeptide. Using our standard PKC assay, we found that the synthetic peptide Arg-Arg-Lys-Ala-Ser-

Table 1

Activation Parameters for PKC-catalyzed Histone III-S-Phosphorylation

Activator Molecules	Chelator	Total picomoles ^{32}P incorporated	Fold Activation
200nM TPA plus 40 ug/ml PI	1mM EGTA	552±51	16
1mM Ca^{2+} plus 40 ug/ml PI	none	474±19	14
200nM TPA	1mM EGTA	95± 7	2.7
40ug/ml PI	1mM EGTA	74±21	2.1
none	1mM EGTA	35±10	1.0
1mM Ca^{2+}	none	29± 1	0.8

Standard reaction mixtures, as described in Materials and Methods, were employed. Reactions were terminated after five minutes. The values represent the total picomoles of ^{32}P retained on phosphocellulose paper minus the background radioactivity obtained from control reaction mixtures, which did not contain PKC. All values represent averages of triplicate assays.

Gly-Pro-Pro-Val (1.4mM) was extensively phosphorylated by PKC in a linear manner for approximately fifteen minutes. Maximal phosphorylation of this peptide required the presence of both Ca^{2+} and phospholipid (Table 2). TPA plus phospholipid stimulated PKC phosphorylation of this peptide as effectively as did Ca^{2+} plus phospholipid (Table 2). The Km_{app} for this peptide, determined from Lineweaver-Burk plots of ten minute reactions run with peptide concentrations that varied from 50 μM to 1.5 mM, was 130 ± 40 μM.

The $Vmax_{app}$ of PKC-catalyzed phosphorylation of this peptide could not be determined, since the absolute concentration of PKC was not known. However, the relative extents of PKC-catalyzed phosphorylation of saturating amounts of histone III-S (32μM) and saturating amounts of peptide (1.4mM) provided a basis for comparing these two substrates. Using a five minute time course we found that the peptide incorporated one-sixth the number of counts incorporated by histone III-S, in a Ca^{2+} -and phospholipid-dependent manner.

PKC phosphorylation of other peptides. BSA was tested as a substrate for PKC at a concentration of 10 μM (approximately 600 μM serine and threonine equivalents), under standard assay conditions in the presence of Ca^{2+} and

Table 2

Activation Parameters for PKC-catalyzed Phosphorylation of Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val

Activator Molecules	Chelator	Total Picomole ^{32}P Incorporated	Fold Activation
Experiment A			
1mM Ca^{2+} plus 40ug/ml PI	none	76±6	19
40ug/ml PI	1mM EGTA	20±3	5.0
none	1mM EGTA	4±0.7	1.0
1mM Ca^{2+}	none	1.8±0.7	0.5
Experiment B			
200nM TPA plus 40ug/ml PI	1mM EGTA	94±2	13
1mM Ca^{2+} plus 40ug/ml PI	none	81±8	12
200nM TPA	1mM EGTA	17±1.0	2.4
none	1mM EGTA	7±1.2	1.0

Standard reaction mixtures, as described in Materials and Methods, were employed; the concentration of the peptide was 0.45mM. Reactions were terminated after ten minutes . The values represent the total picomoles of ^{32}P retained on the phosphocellulose paper minus the background radioactivity obtained from control reaction mixtures, which did not contain protein kinase C. All numbers represent averages of triplicate assays.

phospholipid. No phosphorylation was detected, whether the samples were collected on phosphocellulose paper or by TCA precipitation. Kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly, is a synthetic substrate for the cAMP-dependent protein kinase (20). It has been reported to be practically ineffective as a substrate of bovine heart PKC (14). We found that our preparation of rat brain PKC could phosphorylate kemptide in a Ca^{2+} -and phospholipid-dependent manner, but with a Km_{app} > 500 μM. The low apparent affinity precluded a precise determination. At high substrate concentrations, histone III-S (32μM) and kemptide (1.1mM) were phosphorylated to about the same extent in ten minute standard assays (data not shown here).

Inhibition of PKC by Arg-Arg-Lys-Ala-Ala-Gly-Pro-Pro-Val. It was of interest to determine whether a peptide identical to the active nonapeptide, but lacking a serine residue, would be an inhibitor of PKC. Therefore, the peptide Arg-Arg-Lys-Ala-Ala-Gly-Pro-Pro-Val was synthesized and tested. This peptide was found to be only a very weak inhibitor of PKC since even at 3.5 mM it inhibited PKC by less than 30% in assays containing 6, 12, 24, or 32μM histone III-S (data not shown here).

DISCUSSION

This is the first report to examine synthetic polypeptides which are active substrates of protein kinase C. In this study, the synthetic nonapeptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val, whose primary structure is identical to the sequence in lysine-rich histone that is the major site of PKC-catalyzed phosphorylation (16), was found to be a substrate for PKC, with a Km_{app} of about 130 μM. Kemptide, a closely related peptide, had an extremely weak apparent affinity for PKC, but was an effective substrate at high concentrations. BSA, which contains over fifty serine and threonine residues but no sequences related to that of the nonapeptide substrate, showed no activity as a PKC substrate. The active nonapeptide was approximately forty-fold poorer than histone III-S as a PKC substrate; this may be because the higher molecular weight protein contains additional phosphoacceptor (16) and/or binding sites. Nevertheless, our results indicate that the amino acid sequence immediately surrounding a

PKC-catalyzed phosphorylation site on a target protein is an important determinant of substrate specificity. This is supported by the fact that kemptide was phosphorylated by PKC, since kemptide and the nonapeptide are structurally quite similar. Both peptides contain multiple basic residues followed directly by the sequence Ala-Ser. Multiple basic residues are known to be important determinants in cAMP-dependent protein kinase substrates (20) and our results indicate that they may also be important determinants in PKC substrates. Thus, cAMP-dependent protein kinase and PKC can catalyze the phosphorylation of closely related sequences. Further studies are required to determine whether these two enzymes have significant overlapping specificities for naturally occuring protein substrates. We found that the peptide Arg-Arg-Lys-Ala-Ala-Gly-Pro-Pro-Val, in which the serine residue of the nonapeptide substrate is replaced by alanine, is only a very weak inhibitor of PKC. Similarly, although Leu-Arg-Arg-Ala-Ser-Leu-Gly is a good substrate for the cAMP-dependent protein kinase, the peptide Leu-Arg-Arg-Ala-Ala-Leu-Gly is only a poor inhibitor of this enzyme (21).

The PKC-catalyzed phosphorylations of histone III-S and Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val are regulated by apparently identical mechanisms. Both of these activities are stimulated by Ca^{2+} plus phospholipid, but not by either Ca^{2+} or phospholipid alone. In addition, PKC phosphorylation of both histone III-S and the synthetic peptide were activated by TPA in the presence of phospholipid, but not in the absence of phospholipid. With both substrates, maximal enhancement of PKC activity by TPA was obtained in the absence of added Ca^{2+}, and occured even in the presence of 1 mM EGTA. Our laboratory has previously reported that a preparation of PKC from bovine brain is also stimulated maximally by the tumor promoters TPA, teleocidin, and aplysiatoxin in the absence of added Ca^{2+} (9,10). The present results suggest that synthetic peptide substrates of PKC provide important tools for further probing the mechanism of action of tumor promoters at the enzymatic level, and also for providing clues to cellular target proteins that might be involved in tumor promotion. In addition, the finding that Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val is

a good substrate of PKC allows for the first time the examination of the mechanism of PKC activity with a defined phosphoacceptor substrate containing one phosphoacceptor site. Protein substrates of PKC such as histone H 1 (16) contain multiple phosphoacceptor sites, precluding straightforward kinetic analysis.

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