N-α-Tosyl-L-lysine chloromethyl ketone and *N*-α-tosyl-L-phenylalanine chloromethyl ketone inhibit protein kinase C

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TLCK (*N*-α-tosyl-L-lysine chloromethyl ketone) inhibits protein kinase C whether or not the enzyme is under the regulation of Ca^{2+} and phospholipid. TLCK ($IC_{50}=1$ mM) is a much more potent inhibitor of protein kinase C than TPCK (*N*-α-tosyl-L-phenylalanine chloromethyl ketone) ($IC_{50}=8$ mM), suggesting that the lysyl moiety of TLCK may be specifically recognized by the active site of protein kinase C. These results extend the evidence that the active site of protein kinase C recognizes basic amino acids, and suggest that the active sites of protein kinase C and the cAMP-dependent protein kinase, which is also inhibited by TLCK and TPCK, are structurally related.

Protein kinase C *Tumor promotion* *Protease inhibitor*

1. INTRODUCTION

Protein kinase C is a Ca^{2+}- and phospholipid-dependent protein kinase which catalyzes the phosphorylation of proteins on serine and threonine residues [1]. Recent studies have suggested a role for this enzyme in the mechanism of action of several growth factors and hormones [2], since certain growth factors and hormones cause the formation of diacylglycerides in responsive cells by stimulating phosphatidylinositol metabolism, and PKC is activated by diacylglycerides [3]. PKC is also activated by tumor promoters, including several phorbol esters [4] as well as the indole alkaloid teleocidin and the polyacetate aplysiatoxin [5]. In addition, the enzyme co-purifies to homogeneity with the high affinity phorbol ester receptor [6]. Thus it appears that this enzyme plays a critical role in tumor promotion.

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Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-α-tosyl-L-phenylalanine chloromethyl ketone; DMSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

[γ-^{32}P]ATP was obtained from Amersham. Histone III-S, phosphatidylserine, TLCK, TPCK, Tris-HCl, PMSF, soybean trypsin inhibitor (type 1-S), antipain, and DEAE-Sephacel were from Sigma. Phosphocellulose paper grade P81 was from Whatman, and leupeptin was a gift of the US-Japan Cooperative Cancer Research Program. Bowman-Birk inhibitor was a gift from Dr Jonathan Yavelow.

PKC was partially purified from rat brain according to a published procedure [7]. The enzyme was assayed by measuring the phosphotransferase reaction between [γ-^{32}P]ATP and histone III-S (lysine rich fraction) in the presence of Ca^{2+} and phosphatidylserine, as described [7]. Reaction mixtures contained either TLCK or TPCK at the designated concentrations. Both TLCK and TPCK were preincubated with PKC for 10 min at 30°C

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prior to their addition to the reaction mixture. All reactions were initiated by the addition of enzyme and incubated at pH 7.5 and 30°C for 10 min. Reactions were terminated on phosphocellulose paper as described [7]. All assays were done in triplicate, and the data are expressed as mean values, with the variations of replicates indicated.

3. RESULTS

Fig.1 indicates the effects of increasing concentrations of TLCK and TPCK on PKC activity in a standard assay done in the presence of Ca^{2+} and phosphatidylserine. TLCK was considerably more potent than TPCK in the inhibition of PKC; their IC_{50} values were approx. 1 and 8 mM, respectively (fig.1). Greater than 90% inhibition was obtained at higher concentrations with each compound. Time course studies employing 5 mM TLCK and 10 mM TPCK indicated that maximal inhibition with each of these compounds was achieved within 10 min at 30°C after the addition of the compound (not shown). Therefore, in all of the studies described here, the enzyme was preincubated with the inhibitor for 10 min at 30°C prior to the addition of the other components of the reaction mixture.

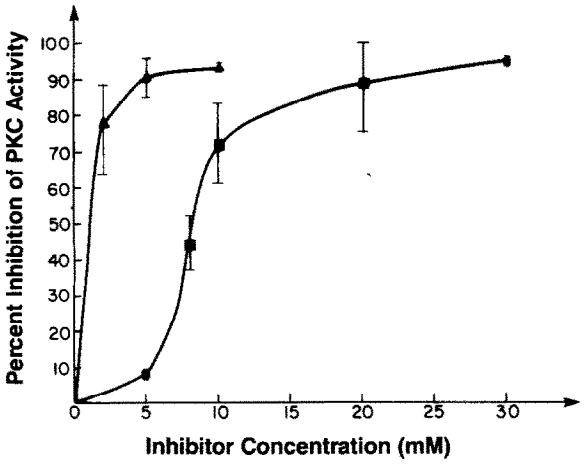

Fig.1. Effects of TLCK and TPCK on PKC activity using histone III-S as the substrate. Rat brain PKC was assayed as described in section 2. An aqueous solution of TLCK was preincubated with PKC for 10 min prior to the phosphotransferase reaction. TPCK was dissolved in DMSO, and was also preincubated for 10 min with PKC. The final concentration of DMSO in the TPCK reaction mixture was 8%. Control studies indicated that this concentration of DMSO did not affect PKC activity. Each data point represents triplicate determinations. Duplicate experiments gave similar results. % inhibition represents [1 − (Ca^{2+}- and phospholipid-dependent histone phosphorylation observed in the presence of inhibitor divided by Ca^{2+}- and phospholipid-dependent histone phosphorylation observed in the absence of inhibitor)] × 100. (▲) Inhibition by TLCK; (■) inhibition by TPCK.

To obtain further information concerning the mechanism of the inhibition of PKC by TLCK, we tested the effects of TLCK on PKC activity using protamine sulfate rather than histone III-S as the phosphoacceptor substrate. The phosphorylation of protamine sulfate which is catalyzed by PKC occurs in a Ca^{2+}- and phospholipid-independent manner [1]. Fig.2 indicates that TLCK is an effective inhibitor of PKC-mediated phosphorylation of protamine sulfate in reactions carried out in the absence of added Ca^{2+} or phospholipid, with an IC_{50} value of about 0.25 mM. These results suggest that TLCK interacts directly with the active site of PKC rather than with the phospholipid cofactor or a regulatory domain on the enzyme.

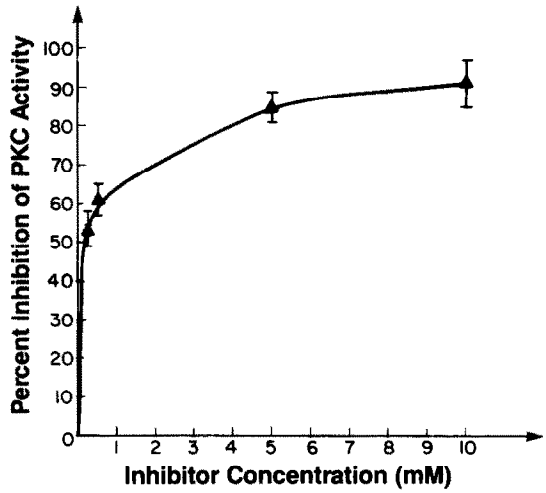

Fig.2. Effects of TLCK on PKC activity using protamine sulfate as the substrate. Rat brain PKC was assayed as described in section 2 except protamine sulfate (0.67 mg/ml) was added rather than histone III-S. % inhibition represents [1 − (protamine sulfate phosphorylation observed in the presence of TLCK divided by protamine sulfate phosphorylation observed in the absence of TLCK)] × 100.

Several studies indicate that the tumor promoting effects of TPA during two stage carcinogenesis on mouse skin can be inhibited by certain protease inhibitors, including TLCK and TPCK [8]. Therefore, we tested some of these compounds, including the Bowman-Birk inhibitor [9] for inhibition of PKC activity in the presence of Ca^{2+} and phospholipid, using histone III-S as the substrate. Leupeptin (4.2 mM), antipain (5 mM), the Bowman-Birk inhibitor (0.2 mM), soybean trypsin inhibitor (0.2 mM) and PMSF (1 mM) failed to produce detectable inhibition of PKC under the assay conditions described in section 2, in which millimolar concentrations of TLCK were inhibitory, providing evidence that the in vitro inhibition of PKC by TLCK and TPCK is not mediated by contaminating proteases.

4. DISCUSSION

Previous studies from this laboratory using synthetic oligopeptides as substrates for PKC suggest that the active site of PKC recognizes basic amino acids that flank the amino terminal side of the phosphoaccecptor residue in substrate proteins [7]. The structures of known PKC protein substrates are consistent with this hypothesis. HMG 17 protein is phosphorylated by PKC at Ser 24 in the sequence Gln-Arg-Arg-Ser 24-Ala-Arg-Leu-Ser 28-Ala-Lys [10], the EGF receptor is phosphorylated by PKC at Thr 654 of the sequence Val-Arg-Lys-Arg-Thr 654-Leu-Arg-Arg [11], and histone H1 is phosphorylated by PKC at Ser 38 in the sequence Arg-Arg-Lys-Ala-Ser 38-Gly-Pro-Pro-Val [12]. The data presented here indicate that TLCK is a more potent PKC inhibitor than TPCK, suggesting that the basic moiety of TLCK may be specifically recognized by the active site of the kinase. We have previously reported that the cAMP-dependent protein kinase and PKC can catalyze the phosphorylation of closely related sequences [7]. The results presented here concerning the inhibition of PKC by TLCK and TPCK extend the evidence that the phosphoacceptor substrate binding sites of the cAMP-dependent protein kinase and PKC are structurally related, since the catalytic subunit of the cAMP-dependent protein kinase is also inhibited by TLCK and TPCK [13]. Our results with TLCK and TPCK suggest that a potent PKC inhibitor can be developed by exploiting active site groups of PKC which recognize phosphoacceptor substrates. Such inhibitors could be extremely valuable for further elucidating the mechanism of action of PKC and the role of this enzyme in hormone action, growth control and tumor promotion.

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