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A 40 kDa inhibitor of protein kinase C purified from bovine brain

Ferdinand Hucho, Helmut Krüger, Iris Pribilla and Ulrich Oberdieck

Institut für Biochemie, Freie Universität Berlin, Thielallee 63, 1000 Berlin 33, Germany

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An inhibitor of protein kinase C has been purified to homogeneity from bovine brain cytosol by a four-step method. It is heat stable, has an apparent molecular mass of 40 kDa and is composed of two polypeptide chains of 19 kDa.

Protein kinase C; Enzyme inhibitor; (Striatum)

1. INTRODUCTION

Protein kinase C (PKC) is one of the protein kinases involved in signal transduction from extracellular space to intracellular targets [1]. Besides Ca^{2+}, diacylglycerol and phosphatidylserine, a variety of natural [2,3] and artificial [4] activators of PKC have been described. Little is known about inhibition of this enzyme: a synthetic inhibitor has been reported (R59002 [5]), and two reports of endogenous brain proteins affecting PKC activity appeared: Schwantke and Le Peuch [6] described a heat-labile inhibitory fraction which was not further purified or characterised. In particular, the presence of protein phosphatases counteracting histone phosphorylation by PKC was not excluded. Protein phosphatases would mimic inhibition of protein phosphorylation. Calmodulin, S-100, and troponin C have also been shown to inhibit protein kinase C to various extents [7].

More recently McDonald and Walsh [8] detected several Ca^{2+}-binding proteins in bovine brain, one of which, a heat-stabile Ca^{2+}-binding protein of 17 kDa inhibited PKC. Here we describe a PKC inhibitor which is similar in some and different in other aspects. In analogy to the inhibitor of the cAMP-dependent protein kinase it may be involved together with protein phosphatases in the regulation of target proteins of PKC.

Correspondence address: F. Hucho, Institut für Biochemie, Freie Universität Berlin, Thielallee 63, 1000 Berlin 33, Germany

2. MATERIALS AND METHODS

2.1. *Partial purification of protein kinase C from bovine corpus striatum*

The procedure is a modification of a published purification of protein kinase C from whole rat brain [9]. Bovine striatum was homogenized in 10 vols of a buffer containing 20 mM triethanolamine (TEA), pH 7.4, 0.3 M sucrose, 10 mM EGTA, 2 mM EDTA, 5 mM dithioerythritol (DTE), and the protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 2 mM). Homogenization was accomplished by 20 strokes (1000 rpm) in a type S teflon homogenizer. The homogenate was centrifuged for 1 h at 100000 × *g*. The supernatant of this centrifugation was loaded on a column filled with the ion-exchange resin Whatman DE 52 equilibrated with 20 mM TEA, 2 mM DTE, 10 mM EGTA and 2 mM EDTA. Subsequently the column was washed with 20 mM TEA, pH 7.4, containing 50 mM mercaptoethanol, 1 mM EGTA and 1 mM EDTA. Elu-

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tion was performed with a 0–150 mM NaCl gradient in washing buffer. All manipulations were performed at 4°C. Enzyme activity was assayed according to Roskowski [10]. Protein was determined as described by Bradford [11].

2.2. *Materials*

Histone (type III S), L-α-phosphatidyl-L-serine and phorbol 12-myristate, 13-acetate were purchased from Sigma, and [γ-^{32}P]ATP from NEN. The FPLC apparatus and the Superose 12 HR 10/30 and Mono Q HR 515 columns were Pharmacia. DEAE-cellulose and P 81 ion-exchange chromatography paper were from Whatman. Concentration of protein fractions was performed by ultrafiltration with an Amicon cell using the PM-10 filter type. All other reagents were of the highest purity commercially available.

2.3. *Protein kinase C inhibitor (PKC-I)*

PKC-I was assayed by adding 10 μg protein of a sample to the PKC assay mixture. Free calcium concentration was adjusted to 10^{-7} M with EDTA/$CaCl_2$ buffers. Inhibitor activity was expressed as percent inhibition of PKC.

3. RESULTS AND DISCUSSION

3.1. *Purification of a protein kinase C inhibitor from bovine striatum*

After separating PKC from the soluble fraction of bovine striatum as described in section 2 the DE 52 ion-exchange column was washed with a solution of 500 mM NaCl in 20 mM TEA buffer, pH 7.4, containing 50 mM mercaptoethanol, 1 mM EDTA and 1 mM EGTA. The pooled protein-containing fractions were heated for 30 min at 100°C (boiling water bath) and subsequently centrifuged for 20 min at 100000 × g. The supernatant was extensively dialysed against 20 mM TEA buffer, pH 7.4, containing 1 mM EDTA and 1 mM EGTA, and concentrated about 5-fold by pressure-filtration (Amicon PM-10 filter). This sample containing PKC-I but no protein phosphatase activity was further purified via FPLC chromatography using a Superose size-exclusion column (fig.1). The inhibitory activity eluted as a single peak corresponding to a molecular mass of about 40 kDa. The inhibitory fractions from 7 Superose runs were pooled, concentrated (Amicon PM 10), and dialysed against 20 mM phosphate buffer, pH 7.5, containing 1 mM EDTA, 1 mM EGTA. The concentrated sample was applied to an FPLC ion-exchange column (Mono Q) and eluted with an NaCl gradient (0–800 mM, two slopes). The inhibitory activity again elutes as a single peak (fig.2).

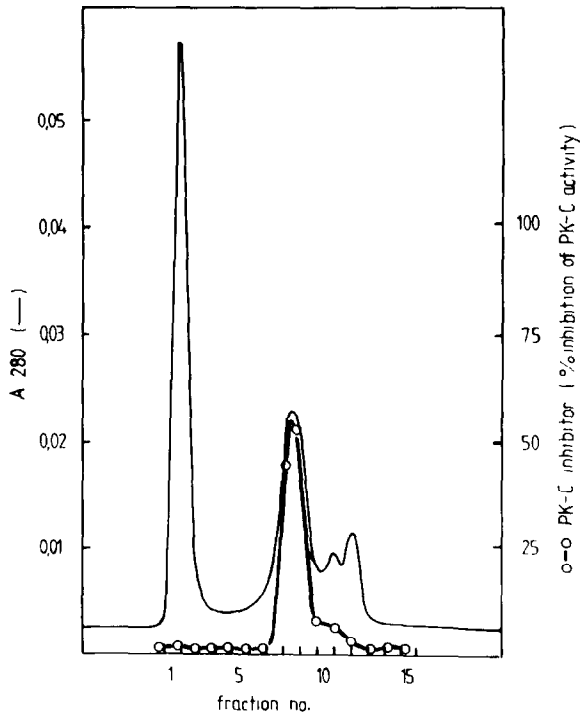

Fig.1. Gel-permeation chromatography: FPLC chromatography on a Superose 12 column. (——) Protein, as determined by light absorption at 280 nm; (o——o) PKC inhibitor activity. Eluant: 20 mM Hepes, pH 7.5, 1 mM EDTA, 150 mM NaCl, degassed and filtered; flow rate 0.8 ml/min.

3.2. *Characterization of PKC-I*

The peak fraction from the Mono Q run contained only one protein of 19 kDa, as judged from SDS-polyacrylamide gel electrophoresis (fig.3). Molecular mass determination with the native protein on a carefully calibrated Superose column (fig.4) gave about twice this molecular mass (40 kDa), indicating that the PKC-I is a dimer of two similar polypeptide chains. Fig.5 shows the inhibition of PKC by increasing concentrations of

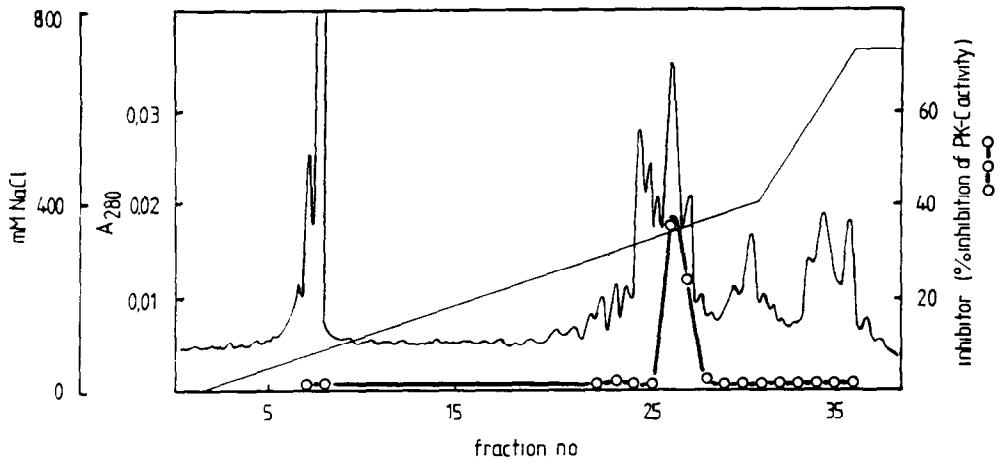

Fig.2. Ion-exchange chromatography: FPLC chromatography on a Mono Q column. NaCl gradient (0.800 mM, two slopes). (——) Protein, (○—○) PKC inhibitor activity.

PKC-I. Strikingly, complete inhibition could not be obtained. Whether or not this hints at different PKC entities in our preparation remains to be determined.

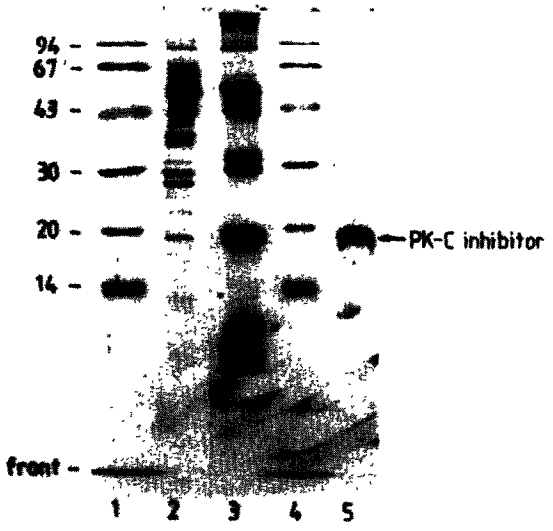

Fig.3. SDS-polyacrylamide gel electrophoresis. Lanes: 1, marker proteins; 2, crude soluble bovine brain fraction; 3, heat-resistant proteins from the high-salt wash of the DEAE column; 4, marker proteins; 5, pure PKC inhibitor after FPLC-Mono Q, migrating with an apparent molecular mass of 19 kDa. 15% polyacrylamide Laemmli gel, Coomassie blue stained.

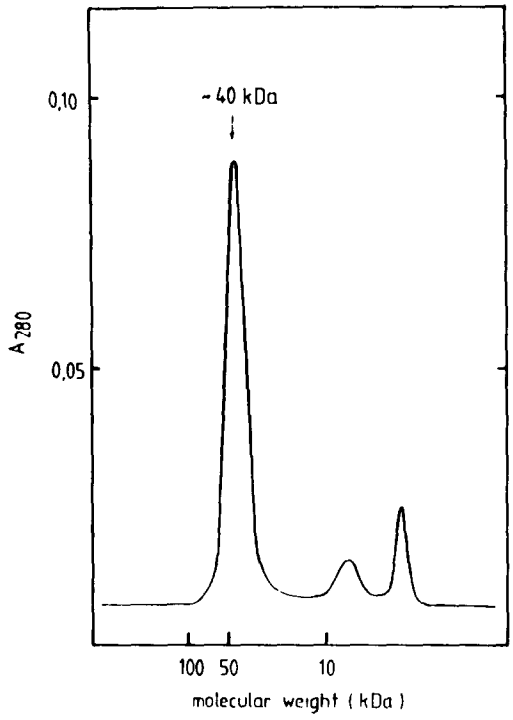

Fig.4. Molecular mass determination of native protein kinase C. FPLC-Superose column calibrated with BSA (68 kDa), ovalbumin (45 kDa), carboanhydrase (30 kDa), chymotrypsinogen (26 kDa), cytochrome *c* dimer (23 kDa), and myoglobin (18 kDa). The native PKC inhibitor elutes with an apparent molecular mass of 40 kDa. The two minor low molecular mass components eluting from this column have no inhibitory activity.

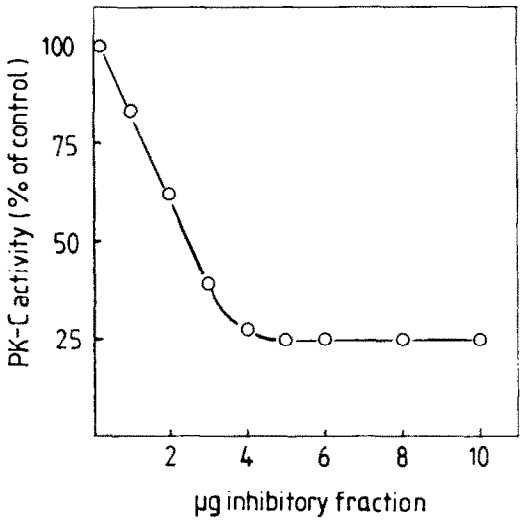

Fig.5. Dependence of inhibition of protein kinase C on PKC-I concentration.

ACKNOWLEDGEMENTS

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