

A protein kinase C inhibitory activity is present in rat brain homogenate

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The partial purification and characterization of (a) factor(s) from rat brain which inhibit(s) the activity of calcium and phospholipid-dependent protein kinase from the same tissue is described. This factor, present in $100\,000 \times g$ rat brain homogenate supernatant, is inactivated upon treatment by trypsin and pepsin and is therefore assumed to be a protein. It was partially purified by ion-exchange chromatography on DEAE-cellulose, ammonium sulfate precipitation and gel filtration. This inhibitor is not stable to heating at 70°C for 10 min, however partial renaturation of the inhibitory activity can be observed after incubation of the denatured inhibitor for 24 h at 4°C . It is precipitable by 10% trichloroacetic acid and by 2 M ammonium sulfate. It exhibits a Stokes radius of 20 Å by gel exclusion chromatography, corresponding to a molecular mass of 20 kDa assuming a globular shape. Kinetic analysis of the inhibition of calcium-phospholipid-dependent histone kinase activity indicates that the inhibitor is competitive with respect to the protein substrate. No change was observed in the kinetic values of the kinase for ATP, Ca^{2+} and phospholipids.

Calcium Phospholipid Kinase Kinase inhibitor Phosphoinositide

1. INTRODUCTION

A calcium-dependent protein kinase activity which does not require the presence of calmodulin but requires phospholipids as an activator has been described [1] and purified from rat brain [2,3] or bovine heart [4]. This new Ca^{2+} -dependent protein kinase has been found in a wide variety of tissues and phyla. Many protein substrates have been described but thus far there is no evidence that these phosphorylations are involved in cell regulation. Some oncogene products were recently reported [5,6] to act as phosphatidyl inositol kinases. The breakdown of phosphatidyl inositol diphosphate, on the receptor activated pathway, and the resulting activation of protein kinase C by diacyl glycerol might induce cellular responses

such as cell proliferation. These findings brought more interest to the study of regulation of this kinase. Here we report the presence of protein kinase C inhibitory activity in rat brain and briefly describe some of its properties.

2. MATERIALS AND METHODS

2.1. Materials

Histones (III-S), diolein and phospholipids were from Sigma. Protease inhibitors – leupeptin, pepstatin, aprotinin – and Hepes buffer were from Boehringer Mannheim. Heavy metal free DTT was obtained from Calbiochem. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 10 mCi/ml and 3000 Ci/mmol was purchased from Amersham. Rialuma scintillant was from Kontron Analytique.

Ultrogel AcA 34 was obtained from the Industrie Biologique Française (IBF), diethylaminoethylcellulose and P-81 phosphocellulose paper from Whatman. Protein determination reagents were from Biorad Laboratories. Trypsin was ob-

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tained from Mann Research Laboratories and pepsin from Worthington.

2.2. Methods

Protein concentrations were determined by the Coomassie blue technique [7] using bovine serum albumin and/or immunoglobulins as standards. SDS (0.1%)-polyacrylamide (10% or 5–20% gradient) gel electrophoreses were performed essentially as in [8]. Gels were stained using Coomassie blue or the silver stain method [9].

Assays for the Ca^{2+} -phospholipid-dependent protein kinase were performed essentially as in [3]. The standard reaction mixture contained 20 mM Hepes–NaOH buffer (pH 7.5), 10 mM MgCl_2 , 0.2 mM Ca^{2+} , 60 $\mu\text{g/ml}$ phosphatidylserine, 6 $\mu\text{g/ml}$ diolein, 5 mM DTT, 1 mg/ml lysine-rich histones, 100 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (spec. act. 70–150 cpm/pmol). Reactions were initiated by the addition of ATP and were carried out for up to 15 min at 20°C. Reactions were terminated by pipetting 40 μl of the reaction mixture onto Whatman P-81 phosphocellulose papers (1.5 \times 1.5 cm). Papers were then washed and processed as in [10] and the radioactivity measured in 5 ml of Rialuma scintillant.

Assay for the Ca^{2+} -phospholipid-dependent protein kinase inhibitor was an adaptation of the assay for the kinase described above. Reaction mixtures contained all the components as listed, but the amount of protein kinase was fixed and varying amounts of inhibitor were included.

2.3. Enzymatic treatments of the inhibitor

Inhibitor adjusted to a concentration giving about 50% inhibition of the kinase C activity in the standard assay conditions was incubated in the presence of 0.2 mg/ml trypsin, pepsin, phospholipase or DNase for various lengths of time. Inhibitor was then added to the phosphorylation reaction mixtures. Incorporation of ^{32}P into histones III-S was measured over a short time in order to avoid any effect of the proteolytic enzymes on the kinase itself. Soybean trypsin inhibitor or changes of pH were also used in order to inhibit the proteolytic activity of trypsin and pepsin respectively, at the end of incubation. The activity of phospholipases was tested by their ability to hydrolyze phosphatidylserine and abolish the

activation of protein kinase C by this phospholipid.

3. RESULTS

3.1. Partial purification of the protein kinase C inhibitor

For each preparation, 4 rats (male, Wistar) were killed by decapitation. Brains were removed and washed in ice-cold buffer A (20 mM Hepes–NaOH buffer, pH 7.5, containing 0.3 M sucrose, 2 mM EDTA, 10 mM EGTA, 2 mM DTT, 2 mM PMSF, 10 $\mu\text{g/ml}$ pepstatin, 10 $\mu\text{g/ml}$ soybean trypsin inhibitor, 10 $\mu\text{g/ml}$ leupeptin and 25 $\mu\text{g/ml}$ aprotinin). Most of the membranes and blood vessels were removed and brains homogenized in 8–10 vols of buffer A using a glass teflon pestle homogenizer. The homogenate was centrifuged at $100000 \times g$ (35000 rpm, Kontron 50.38 rotor) for 1 h. The pellets were discarded and the supernatant adjusted to ionic strength 1.0 mmhos or lower.

3.2. DEAE-cellulose chromatography

The $100000 \times g$ supernatant was applied to a DEAE-cellulose column (2.5 \times 2.5 cm, flow rate 30 ml/h) equilibrated with buffer B (20 mM Hepes–NaOH buffer, pH 7.5, containing 2 mM DTT, 2 mM EDTA and 2 mM EGTA). The column was washed with 10 vols of buffer B. The proteins retained on the column were eluted with a linear gradient of NaCl (0–0.3 M) in 2 \times 250 ml buffer B and 5 ml fractions were collected. As shown in fig.1, the Ca^{2+} -phospholipid-dependent protein kinase activity was resolved from its inhibitor(s). Two peaks of inhibitory activity could be observed. The first peak was eluted at 0.18 M NaCl and the second peak at about 0.28 M NaCl. Therefore, it seems that the inhibitory activity is heterogeneous due to either the presence of more than one molecular species or heterogeneity (e.g., due to aggregation) of one inhibitor.

Calmodulin as well as protein S-100 have been recently shown to inhibit protein kinase C activity [11]. Given the ionic strength required for the elution of these calcioproteins, the second peak of inhibitory activity observed on fig.1 can be due to the presence of these proteins. The first and main peak of inhibitory activity was eluted at lower ionic strength and the presence of calcioproteins such as

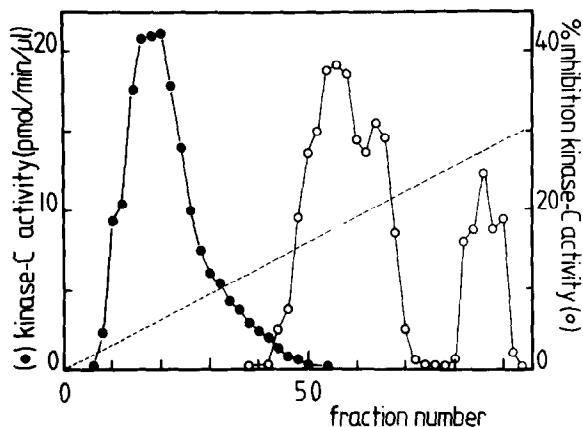


Fig. 1. DEAE cellulose chromatography of kinase C and its inhibitor(s) from rat brain homogenate $100\,000 \times g$ supernatant. A linear gradient $0 \rightarrow 300$ mM NaCl (---) was used. An aliquot of each fraction was assayed for kinase C activity (●—●) and for kinase C inhibitory activity (○—○).

calmodulin or protein S-100 is not likely. Furthermore, no inhibition of protein kinase C activity by 10^{-5} M calmodulin was observed using histones III-S as substrate. The fractions containing the inhibitory activity (48–68) were pooled, made 0.2 mg/ml soybean trypsin inhibitor and then adjusted to 70% saturation in ammonium sulfate. Proteins were allowed to precipitate for 10 min at 4°C and then centrifuged at $15\,000 \times g$ for 15 min. Pellets were dissolved in a minimum volume of 20 mM Hepes–NaOH buffer (pH 7.5) containing 0.5 mM EGTA, 0.5 mM EDTA and 2 mM DTT (buffer C).

3.3. Gel exclusion chromatography

Concentrated solutions of inhibitor (vol. < 2 ml) were then applied to either an Ultrogel AcA 34 or a Sephacryl S-200 filtration column (2.5×200 cm) equilibrated with buffer C. Proteins were eluted at 10 ml/h with the same buffer. Fractions were tested for the presence of inhibitor. Fig. 2 presents the elution profile of kinase C inhibitory activity from the Sephacryl S-200 column. The same overall pattern was obtained using Ultrogel AcA 34 instead of Sephacryl S-200. Two peaks of activity were eluted just after the void volume of the column corresponding to high molecular mass species (300 kDa) and one peak was eluted just before the total volume corresponding to a Stokes

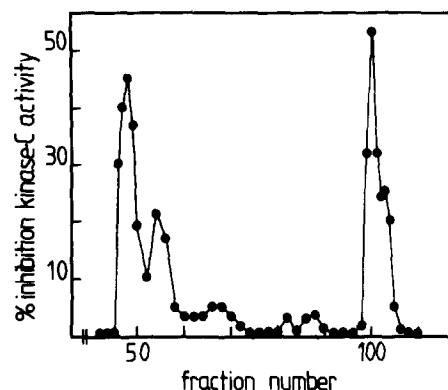


Fig. 2. Sephacryl S-200 chromatography of the kinase-C inhibitor. Inhibitory activity (●—●) was assayed as described in section 2.

radius of about 20 Å. Traces of activity were also found along the whole column pointing to the possibility of association between the inhibitor on the one hand and various proteins on the other hand. Further studies are underway to determine whether the same protein is responsible for all inhibitions observed. The ratio of low and high molecular mass forms of inhibitory activity was constant and did not depend on the conditions used in the previous steps (e.g., presence or absence of protease inhibitors), therefore the low molecular form is not likely to be a degradation product of the high molecular form(s). The following results were obtained using the inhibitor which exhibits a low molecular mass (~ 20 kDa assuming a globular shape).

Since almost no protein was detectable after the gel filtration step by either protein determination or by SDS-gel electrophoresis it was not possible to make an estimation of the purification factor for the inhibitor. Since almost no protein kinase C activity was detected in the crude extract, it can be assumed that the concentration of inhibitor present in these extracts is close to the concentration of kinase C. Furthermore, the high purification factors observed in first purification steps can be affected by removal of inhibitor and did not correlate directly with the purification of the enzyme.

3.4. Biochemical nature of the inhibitor

The inhibitor was not affected significantly upon incubation for as long as 24 h in the presence of phospholipases C and A or DNase. The study of

the behaviour of the inhibitor in the presence of proteolytic enzymes was made difficult by the fact that the very same proteolytic enzymes are able to activate the kinase C by its conversion into kinase M [1]. Therefore, it was necessary to use proteolytic enzymes for which inhibitors or specific conditions can be used to abolish their activity. Fig.3 presents the effect of trypsin on the inhibitor activity. After 24 h incubation the inhibitor was destroyed by trypsin. The same type of result was obtained with pepsin. Controls were made with kinase C inhibitor incubated under the same conditions without proteolytic enzymes. In the absence of pepsin, a 30% decrease of inhibitor activity was observed due to the low pH conditions used in these experiments. However, in the presence of pepsin the extent of denaturation (78%) was larger and 48% of the denaturation observed can be ascribed to proteolytic degradation.

Therefore, it can be assumed that the inhibitor or at least the part required for its activity, is a polypeptide. Preliminary kinetic analysis of the ef-

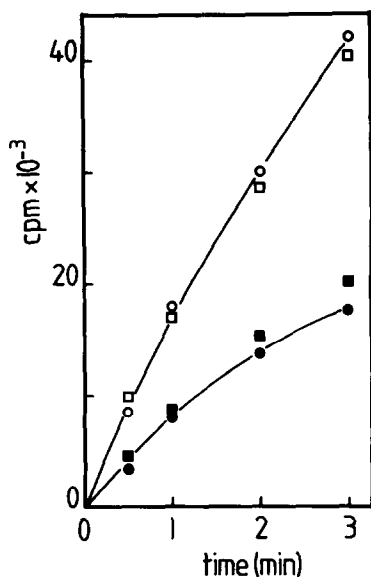


Fig.3. Effect of inhibitor on the rate of phosphorylation of histones III-S by kinase C, and effect of previous incubation of the inhibitor in the presence of trypsin. The extent of phosphorylation was examined in the presence (●—●) or absence (○—○) of inhibitor and in the presence of inhibitor incubated with trypsin in the absence (□—□) or presence (■—■) of soybean trypsin inhibitor.

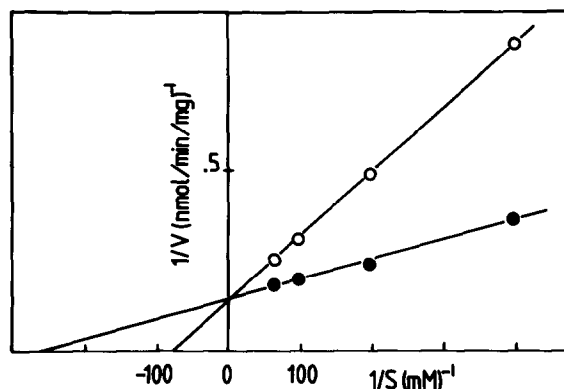


Fig.4. Double reciprocal plot of the initial velocity of the phosphorylation of histones III-S by kinase C vs histones concentration in the presence (○—○) or absence (●—●) of inhibitor.

fect of the inhibitor on the catalysis of histones III-5 phosphorylation by kinase C indicated competition with respect to the protein substrate and histones (fig.4) and no competition with respect to ATP and to activators, phosphatidyl serine, diolein or calcium (not shown). The hypothesis of inhibition by direct interaction between an acidic inhibitor and basic histones can be dismissed for the following reasons: from the results of DEAE experiments reported above, it can be assumed that the inhibitor is less acidic than calmodulin or protein S-100. Moreover, the concentration of inhibitor required for 50% inhibition (see above) is very low compared with substrate concentration, therefore the competitive inhibition due to interaction between the inhibitor and basic histones is not likely to occur.

From these experiments it can be assumed that an inhibitor of protein kinase C is present in rat brain homogenate supernatants. This protein is distinct from the calproteins already described as possible regulators of kinase C activity [11]. Further experiments are underway to purify this inhibitor to homogeneity and to define more precisely its properties.

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