Purification of Stable Protein Kinase C from Mouse Brain Cytosol by Specific Ligand Elution Using Fast Protein Liquid Chromatography

Arco Y. Jeng, Nancy A. Sharkey, and Peter M. Blumberg

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ABSTRACT

The Ca2+- and phospholipid-dependent protein kinase (protein kinase C) has been purified to electrophoretic homogeneity from mouse brain cytosol. [20-3H]Phorbol 12,13-dibutyrate binding activity was located quantitatively with the protein kinase activity throughout the purification procedure. The crude extract was first run over a DE52 column. Fractions containing peak activities were then chromatographed using a fast protein liquid chromatography system with a Mono Q column followed by chromatography on the same column run in the presence of 1 mM adenosine triphosphate. The adenosine triphosphate specifically shifted the elution position of the Ca2+- and phospholipid-dependent protein kinase, providing a powerful step in the purification procedure. The remaining minor contaminants were removed by hydrophobic chromatography on a TSK-phenyl-5-PW column. This purification procedure required less than 2 days after the initial large batch DE52 column chromatography. The molecular weight of the purified receptor was estimated to be 81,000 by its mobility on sodium dodecyl sulfate/polyacrylamide gels, in agreement with the published values. Optimal conditions for the storage of the purified receptor were sought. Both protein kinase and phorbol ester binding activities were stable for 2 mo when stored in the presence of 0.01% Triton X-100 at -70°C. Polyclonal antibodies to the purified receptor have been prepared from rabbits. These antibodies recognized the purified receptor in electroblotting as says and were able to immunoprecipitate the purified receptor.

INTRODUCTION

Phorbol ester tumor promoters have been shown to play an important role in skin carcinogenesis (1-3) as well as to affect many other biological responses in in vitro systems (4-6). The high potency of the phorbol esters and their structure-activity relationships for the induction of biological responses (7) strongly suggested that the phorbol esters functioned through action at a specific receptor. Such receptors were demonstrated first in chick embryo fibroblasts (8) and subsequently in a variety of tissue preparations and intact cells (see Ref. 9 for a review) using [3H]PDBu.2 The receptor was found to share many properties in common with a protein kinase C first identified by Nishizuka (10). These properties included tissue distribution, high activities in brain, evolutionary conservation, high Ca2+ sensitivity, phospholipid association, and interaction with the substrate specificity of protein kinase C, its interaction with

MATERIALS AND METHODS

Materials. Preswollen diethylaminoethyl cellulose (DE52) and cellulose phosphate F81 papers were obtained from Whatman (Clifton, NJ). Mono Q columns (HR 5/5 and 10/10), FPLC system, and electrophoresis molecular weight calibration kit were purchased from Pharmacia (Piscataway, NJ). [20-3H]Phorbol 12,13-dibutyrate ([3H]PDBu, 10.8 to 13.4 Ci/mmol) and [γ-32P]ATP were from New England Nuclear (Boston, MA). Bovine γ-globulins and fatty acid-free BSA were obtained from Sigma (St. Louis, MO). Bio-gel TSK-phenyl-5-PW column (75 x 7.5 mm), SDS, protein dye reagent, DEAE-Affigel Blue, and electrophoresis grade acrylamide were purchased from Bio-Rad (Richmond, CA). ATP and Pansorbin were products of Calbiochem (LaJolla, CA).

Buffers Used in the Purification of Protein Kinase C. The buffer solutions used for the purification were: buffer A, 2 mM EGTA, 5 mM EDTA, 0.3 M sucrose, 5 mM DTT, and 60 mM Tris HCl for the preparation of crude extract; buffer B, 0.5 mM EGTA, 0.5 mM EDTA, 5 mM DTT, and 20 mM Tris-HCl for DE52 column chromatography; buffer C, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT, and 20 mM Tris-HCl for Mono Q column fractionation; buffer D, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT, 1.5 M NaCl, and 20 mM Tris-HCl for TSK-phenyl-5-PW column elution; buffer E, 0.125 mM EGTA, 0.125 mM EDTA, 0.25 mM DTT, and 5 mM Tris-HCl also for TSK-phenyl-5-PW column elution. Buffers were adjusted to pH 7.4 at room temperature (for those used in Mono Q and TSK-phenyl-5-PW column chromatography) or 4°C (for DE52 column chromatography).

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centrifuged at 40,000 rpm for 60 min in Beckman 50.2 Ti rotors. The supernatant was pooled and diluted with 4 ml of chilled 5 mM DTT/g of brain. This diluted cytosol was then loaded on the DE52 column in the next step of purification.

DE52 Column Fractionation. DE52 resin (50 g/150 mice) was prepared according to the manufacturer’s instruction and was equilibrated in buffer B overnight. The diluted cytosol was loaded and the column was washed with 7- to 10 bed vol of buffer B. Proteins were then eluted with a linear gradient containing 0 to 300 mM NaCl in buffer B (total gradient volume, 500 ml for cytosol from 150 mice). One hundred fractions were collected and [3H]PDBu binding or protein kinase C activity of the fractions was determined. The fractions containing high protein kinase or [3H]PDBu binding activity were pooled and then concentrated and desalted with Amicon stirred cells (Danvers, MA) using PM30 membranes. The final protein concentration was about 25 mg/ml in buffer B.

Mono Q Column Chromatography. The concentrated DE52 peak material was further fractionated by a Mono Q (HR 10/10) column using a Pharmacia high resolution FPLC system. The Mono Q column was equilibrated with buffer C and about 80 mg of protein was loaded onto the column in each of the several repetitive column runs. The column was washed with 5 bed vol of buffer C and proteins were eluted with a linear gradient containing 0 to 300 mM NaCl in buffer C (total gradient volume, 160 ml). The flow rate was 4 ml/min; 2-ml fractions were collected and the protein kinase activity was assayed. The peak kinase activity was pooled and again concentrated and desalted.

The desalted Mono Q pool was then run over the same Mono Q column under identical conditions except that 1 mM ATP was included in all of the buffers. In order to minimize the interference of ATP in the protein kinase assay, protein samples from each fraction were diluted at least 1000-fold in the final reaction mixture. The peak protein kinase activity was pooled.

TSK-phenyl-5-PW Column Elution. The salt concentration of the protein pool from the previous Mono Q/ATP column run was adjusted to 1.5 M NaCl and then loaded onto a hydrophobic column, TSK-phenyl-5-PW, preequilibrated in buffer D using the Pharmacia FPLC system. The column was washed with 5 vol of buffer D and proteins were eluted with a linear gradient containing 0 to 300 mM NaCl in buffer D (total gradient volume, 160 ml). The flow rate was 4 ml/min; 2-ml fractions were collected and the protein kinase activity was assayed. The peak kinase activity was pooled. Fractions containing high protein kinase activity were pooled.

Preparation of Polyclonal Antibodies against Purified Protein Kinase C. The purified protein (100 μg) was emulsified with an equal volume of complete Freund’s adjuvant and injected i.m. into New Zealand white rabbits (Denver, PA) at 3 to 4 spots. The same procedure was repeated at 7-day intervals except that the incomplete Freund’s adjuvant was used. Rabbits were bled weekly after the third administration of antigen. The titer of antibodies was checked using the Bio-Rad Immun-Blot facility, University of Michigan. The content of cysteic acid was determined using duplicate samples oxidized with performic acid before hydrolysis.

RESULTS

Purification of Protein Kinase C. In previously published protocols, chromatography on DEAE-cellulose has proven to be a useful, high capacity step for the purification of protein kinase C. Accordingly, mouse brain cytosol was loaded onto a DE52 column, the column was washed with buffer B, and proteins were eluted by a 0 to 300 mM NaCl gradient in buffer B. Fig. 1 shows a typical elution profile. The major Ca2+- and phospholipid-dependent protein kinase activity was eluted between 80 and 120 mM NaCl. Only minimal protein kinase activity was detected in the absence of Ca2+ and phosphatidylserine (Fig 1). [3H]PDBu binding activity was found to coelute with the Ca2+- and phospholipid-dependent protein kinase activity, and additional peaks were not seen within the limits of the elution gradient (data not shown). Protein concentration in each fraction was measured (Fig. 1), and fractions containing the peak ratio of the protein kinase activity per mg of protein were pooled.

The pool from the DE52 column was loaded onto a Mono Q column. Fig. 2A shows the elution profile from the Mono Q column using a 0 to 300 mM NaCl gradient in buffer C. Peak protein kinase C activity was eluted consistently at 180 mM NaCl (Fig. 2A). [3H]PDBu binding activity was also found to coelute with protein kinase C activity (results not shown). Since protein kinase C interacts with ATP, we reasoned that inclusion of ATP in the elution buffer might selectively shift the elution position of the kinase, leaving most of the contaminating proteins unaffected. Given the high resolution of the Mono Q column, a relatively small shift in elution position would suffice for a substantial separation. Fractions for the Mono Q column containing high protein kinase activities were therefore pooled, desalted, and rerun over the same Mono Q column in the presence of 1 mM ATP and 3.75 mM magnesium acetate. Under this new condition, the peak protein kinase C activity was shifted to 150 mM NaCl (Fig. 2B). A shift was not observed in the presence of 1 mM GTP or in 1 mM ATP but without
Table I  Amino acid composition of the purified receptor

The amino acid composition shown was the average from two different preparations. The content of cysteic acid was determined using duplicate samples oxidized with performic acid before hydrolysis. Negligible protein kinase activities were detected in the absence of Ca²⁺ and phosphatidylserine.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mouse brain</th>
<th>Pig Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>5.1 ± 0.3</td>
<td>7.8</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>2.0 ± 0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.4 ± 0.1</td>
<td>8.6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10.1 ± 0.3</td>
<td>10.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.1 ± 0.1</td>
<td>6.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.0 ± 0.5</td>
<td>10.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.4 ± 0.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.8 ± 0.1</td>
<td>4.4</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.6 ± 0.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.4 ± 0.3</td>
<td>10.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.9 ± 0.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Proline</td>
<td>6.0 ± 0.2</td>
<td>6.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.5 ± 0.6</td>
<td>5.7</td>
</tr>
<tr>
<td>Serine</td>
<td>7.4 ± 0.8</td>
<td>6.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.2 ± 0.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Valine</td>
<td>6.2 ± 0.2</td>
<td>8.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.7 ± 2.4</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Taken from Ref. 33.

† Mean ± range.

A major loss of the receptor appears to be due to the first Mono Q column run (Table 2). The Mono Q column used in this report was a preparative column. Previous runs with analytical Mono Q columns gave a consistent 50% total recovery.
mined in the presence of 30 pM nonradioactive phorbol 1,2,13-dibutyrate. Binding activity was found to be very stable when stored in 50% glycerol and 0.01% Triton X-100 was routinely used. Storage at —70°C for 4 days in 50% glycerol at —20°C, binding activity was 84 or 100%, respectively, of the original value (Table 3), indicating that the majority of the antibodies was not directed against the site of [3H]PDBu binding or the catalytic site for the protein kinase activity (Fig. 4).

Stability of the Purified Receptor. The partially purified receptor was found to be very stable when stored in 50% glycerol at −20°C. No significant decrease of protein kinase C and [3H]PDBu binding activities was observed for several months (results not shown). In contrast, the purified receptor was not stable. Stored in 50% glycerol at −20°C for 10 days resulted in an 80% loss of the phorbol ester binding activity. Various agents were added to prevent the loss of [3H]PDBu binding activity due to either surface adsorption or possible minor protease contaminants. Table 3 shows that the inclusion of protease inhibitors (leupeptin and soybean trypsin inhibitor) did not stabilize [3H]PDBu binding activity satisfactorily, suggesting that the major loss of binding activity was not due to protease contamination. On the other hand, in the presence of bovine γ-globulins (0.4 mg/ml) or low concentrations of Triton X-100 for 4 days in 50% glycerol at −20°C, binding activity was 84 or 100%, respectively, of the original value (Table 3), indicating that the loss of activity might be due to surface absorption.

Further investigation revealed that a low concentration of Triton X-100 alone was sufficient to stabilize both kinase and binding activities for prolonged periods if samples were first frozen with liquid nitrogen then kept at −70°C. The optimal concentrations of Triton X-100 was between 0.01 and 0.05%, and 0.01% Triton X-100 was routinely used. Storage at −70°C for 1 mo caused no apparent loss of the binding activity while there was only a 28% decrease in the kinase activity (results not shown). The effects of rapid freeze and thaw were also studied. No loss of [3H]PDBu binding activity was observed after two cycles of freeze and thaw during a period of storage for 2.5 mo and about one-half of the kinase activity was still retained after 4 mo (results not shown).

Immunoprecipitation of the Purified Receptor by Polyclonal Antibodies. Polyclonal antibodies were prepared as described in “Materials and Methods.” These antibodies were able to recognize the purified receptor. Fig. 4 shows that the antibody-receptor complex could be precipitated by Pansorbin and resulted in a decrease of the available receptor for [3H]PDBu binding and protein kinase activities remaining in the supernatant. This decrease was dose dependent and the preimmune serum at the highest dose tested did not complex with the receptor (Fig. 4). There was little decrease in either activity when the precipitation by Pansorbin was omitted, indicating that the majority of the antibodies was not directed against the site of [3H]PDBu binding or the catalytic site for the protein kinase activity (Fig. 4).

γ-Immunoglobulins obtained from antiserum recognized purified protein kinase C (Fig. 5, lane d). Among the numerous protein bands shown in the pooled material from DE52 column chromatography (Fig. 5, lane b), these γ-immunoglobulins reacted strongly with a double band of molecular weight of about 80,000 and a band of 60,000 (Fig. 5, lane c). In addition, one...
minor band of molecular weight of about 42,000 and two minor bands of 36,000 were also observed (Fig. 5, lane c).

DISCUSSION

The recent convergence of two originally independent research fields, Ca²⁺-activated phospholipid-dependent protein kinase and phorbol ester tumor promoter receptor, has had profound impact on our understanding of the mechanism of action of the tumor promoting phorbol esters. However, the difficulty in obtaining adequate quantities of purified stable receptor had seriously impeded study of numerous aspects of the biochemistry of the phorbol ester receptor. We have, therefore devoted effort to the methodological development of a suitable purification protocol. The purification scheme reported in this paper yielded up to 170 μg of the purified receptor within 2 days after the initial DE52 column chromatography. In addition, conditions for stabilization of the purified receptor were determined. Under these conditions, protein kinase C and phorbol ester binding activities were not significantly lost in 2 mo.

The advantages of the purification protocol presented in this paper include the purity of the isolated protein and the reproducibility of the methodology. The SDS-polyacrylamide gel electrophoresis indicated electrophoretic homogeneity of the purified receptor and the amino acid compositions obtained from two different preparations gave consistent results. The fact that the purified receptor had a blocked NH₂-terminus further substantiates its purity. Based on the average stoichiometry of binding, 0.45:1, we think it most probable that the purified receptor has been partially inactivated during the course of purification. Although it is not likely, the possibility that more than one protein with blocked NH₂-terminus and with identical mobility in the SDS/polyacrylamide gel can not be excluded. Sequence analysis should provide definitive evidence.

A methodological concept in the development of the purification scheme is the shift of elution position from the same column in the presence of a ligand which interacts specifically with the protein of interest. This approach is a variant of the usual technique of specific substrate elution chromatography (see Ref. 34 for an early review). Le Peuch et al. (22) used a similar approach with Ultragel AcA34 in EGTA in the purification of the protein kinase C. The peak activity from the first column was pooled and rerun on the same column in the presence of phosphatidyserine and Ca²⁺ which shifted the protein kinase C peak to the void volume. However, the protein kinase activity obtained through this protocol was highly unstable. In addition, it was difficult to remove phosphatidyserine for biochemical studies of protein kinase C. In the purification scheme reported here, the binding of ATP to protein kinase C would have been expected both to give an additional negative charge to the complex and also to induce a conformational change in the protein. Since the inclusion of ATP resulted in the elution of protein kinase C at a lower salt concentration, this latter effect appeared to predominate. Our method was carried out under nonequilibrium conditions. ATP was accumulated in the Mono Q column and eluted by salt in the course of the gradient (Fig. 2B). Nevertheless, the shift in the elution position in the presence of ATP was specific, because GTP, which should not interact specifically with protein kinase C, did not cause a shift in the elution profile (data not shown).

A constant ratio of protein kinase activity to phorbol ester binding activity was found at different stages of purification and matched that of the purified receptor. Technically, this result means that either assay can be used to predict activity in the other assay and that the choice of assay is a matter of convenience. In addition, this result and the observation that both activities could be immunoprecipitated to the same extent in the presence of polyclonal antibodies (Fig. 4) agree with the findings of others that both kinase and binding activities reside in the same protein entity. For mouse brain cytosol, this enzyme appears to be of M₆, 81,000, larger than that reported for the enzyme from mouse brain membranes (13) but similar in size to the enzyme from rat brain cytosol. The basis for this difference is not presently known.

Although the polyclonal antibodies we obtained recognized the purified receptor with a good titer (1:1500 dilution) in the electrophoretic assay and immunoprecipitated the purified receptor in a dose-response manner (Fig. 4), they did not react uniquely with the intact receptor with molecular weight of 81,000. In addition to a double band of molecular weight of about 80,000, the purified γ-immunoglobulins from the antisera recognized in the DE52 pool another major band of 60,000 and three other minor bands. Recently, the preparation of polyclonal antibodies to Ca²⁺/phospholipid-dependent protein kinase reported by Girard et al. (35) also showed a dominant recognition of a M₆, 67,000 protein from the brain extract. Whether the M₆, 60,000 protein is a degradation product of the purified receptor remains to be investigated.

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REFERENCES


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