Affinity Chromatography of Protein Kinase C-Phorbol Ester Receptor on Polyacrylamide-immobilized Phosphatidylserine*

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An affinity column, prepared by immobilizing phosphatidylserine and cholesterol in polyacrylamide, was utilized in the purification of protein kinase C. Protein kinase activity and phorbol ester binding were monitored by assaying Ca** plus phosphatidylserine-dependent phosphorylation of histone H1 and [**H]phorbol dibutyrate binding, respectively. Both activities were present in a cytosolic extract of rabbit renal cortex, eluted together from a DEAE-cellulose column, bound to the affinity column in the presence of Ca**, and eluted symmetrically upon application of EGTA. Recovery from the affinity column was high (30-50%) and resulted in as much as a 6000-7700-fold purification, depending on the region of the DEAE-cellulose peak that was applied. Following affinity column purification, protein kinase and phorbol ester binding activity eluted symmetrically upon gel filtration, with a molecular weight of approximately 89 kDa. A protein of the same size was present in silver-stained gels following sodium dodecyl sulfate-polyacrylamide gel electrophoresis of affinity column purified samples from the DEAE-cellulose peak. From 2-4 other, smaller proteins were also present, their number and relative amounts depending on the region of the DEAE-cellulose peak used. These data indicate that Ca**-dependent binding to a polyacrylamide-immobilized phospholipid provides a useful technique for purification of protein kinase C as well as other, unidentified proteins exhibiting a Ca** plus phospholipid-dependent interaction.

A protein kinase activated by a combination of phosphatidylserine and Ca** with sensitivity to Ca** regulated by diacylglycerol (1-7) has now been demonstrated in many tissues (1-3). Since both diacylglycerol generation and Ca** mobilization occur in response to many hormones and transmitters (8, 9), this protein kinase, termed protein kinase C, appears likely to play a fundamental role in mediating the actions of these agents. In addition, the apparent identity of protein kinase C-phorbol ester receptor from rabbit renal cortex. Ca**-dependent binding of proteins to the dispersed gel is highly specific, permitting rapid, high recovery of the highly purified protein kinase-phorbol ester receptor.

** Experimental Procedures

Materials—Phosphatidylserine was obtained from Avanti and Supelco. Acrylamide and BIS† were from Bio-Rad. Phenylmethylsulfonyl fluoride was from Calbiochem. Histones H1, H2A, H2B, H3, and H4 were purchased from Sigma. γ-[33P]ATP and [**H]phorbol dibutyrate were from New England Nuclear. Fatty acid free bovine serum albumin was from Miles Laboratories.

Methods—Renal cortices dissected from kidneys of New Zealand White rabbits were minced and homogenized with a glass homogenizer with a motor-driven Teflon pestle in 9 volumes of 0.25 M sucrose, 20 mM Tris, pH 7.5, 5 mM dithiothreitol, 2 mM EGTA, 2 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, then further homogenized with three bursts of 15 s each on a Polytron homogenizer at setting 8. The homogenate was centrifuged for 30 min at 35,000 × g and the supernatant was used as starting extract.

DEAE-cellulose Chromatography—Cyclic AMP was added to the cytosolic extract to a final concentration of 1 μM and applied to a column (1.5 × 25 cm) of DEAE-cellulose equilibrated with homogenizing buffer minus sucrose but with 1 mM cyclic AMP. After application of sample and further washing with column buffer, a 0-0.4 M gradient of KCl was applied in a total of 500 ml and 5.0-ml fractions were collected.

Preparation of Affinity Column—Phosphatidylserine (5 mg) and cholesterol (25 mg) dissolved in chloroform were combined in a glass scintillation vial and evaporated under nitrogen. Ethanol (0.2 ml) was added and the vial was capped, placed in boiling water, and swirled until the lipids were dispersed. The vial was quickly removed, 5 ml of a solution of 15% acrylamide, 5% BIS was added and vigorously mixed, followed immediately by addition of 50 μl of 140 mg/ml of ammonium persulfate, 2.5 μl of TEMED, further mixing, and an additional 50 μl of ammonium persulfate. The mixture was transferred to a test tube (13 × 100 mm), covered with parafilm and aluminum foil, and allowed to fully polymerize usually overnight at room temperature.

Gel-containing tubes were broken and the rigid white gel was rinsed with water, minced with a razor blade, and homogenized in 20-30 ml of H2O with three passes of a loose-fitting (0.25-mm clearance) Dounce homogenizer. The homogenized gel was allowed to settle for 5 min, the supernatant was decanted, and this procedure was repeated at least twice after resuspension in water. Settled gel particles were suspended in a column buffer containing 5 mM MES, pH 6.5, 5 mM dithiothreitol, 200 mM KCl, 1 mM CaCl2, 0.1 mM phenylmethylsulfonyl fluoride, packed in a siliconized glass column (1.6 × 50 cm or 1.0 × 40 cm), and equilibrated with 5-10 column volumes of buffer. Flow rates of 30-50 ml/cm/h were used with minimal back pressure and only moderate compaction of the relatively rigid gel particles.

Affinity Chromatography—Leupeptin was added to a final concentration of 0.1 mg/ml. The gel was equilibrated with 50 mM Tris, pH 7.5, 5 mM dithiothreitol, 5 mM CaCl2, and 0.1 mM leupeptin, and 42-phorbol 12-myristate 13-acetate was added to a final concentration of 1 μM. After application of the cytosolic extract to the affinity column, the column was washed with 50 column volumes of the same buffer with 1 mM cyclic AMP. Subsequently, the column was washed with 50 column volumes of buffer minus cyclic AMP, 1 mM leupeptin, and 42-phorbol 12-myristate 13-acetate. Finally, the column was washed with 50 column volumes of buffer minus cyclic AMP, 1 mM leupeptin, and 42-phorbol 12-myristate 13-acetate. The eluted fractions were monitored for protein kinase activity and the purity was monitored by SDS-PAGE.

1. The abbreviations used are: BIS, N,N'-methylenebisacrylamide; PDB, phorbol dibutyrate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MBS, 2-(N-morpholinio)ethanesulfonic acid; TEMED, N,N',N'-tetramethylethylenediamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid.
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Protein Kinase Assay—Protein kinase activity was assayed in a reaction mixture containing 25 mM Tris, pH 7.5, 200 µg/ml of histone H1, 5 mM MgAc, 20 µM ATP, 1–2 x 10^8 cpm [γ-32P]ATP, 400 µM EDTA (cytosol and DEAE-cellulose fractions) or 400 µM EGTA (affinity column fractions), ± 50 µg/ml of phosphatidylserine, 0.5 µg/ml of diolene, and 0.5 mM free Ca^2+ in a total volume of 100 µl. Phosphatidylserine and diolene were combined, evaporated under nitrogen, and dispersed in water or 10 mM Tris, pH 7.5, by sonication for 2–5 min at 60 watts with a Heat System-Ultrasonic sonicator.

Protein determinations on extracts, column fractions, and purified protein kinase were done by a dye-binding assay (17) using 0.01% Serva Blue G in 1.6 M phosphoric acid, 0.5 M methanol with bovine serum albumin as standard.

RESULTS AND DISCUSSION

The protein kinase C activity present in the cytosol of the rabbit renal cortex eluted from DEAE-cellulose at approxi-

tration of 100 µM to a portion (7–14 ml) of individual or combined fractions of the DEAE-cellulose column eluant containing the Ca^2+-dependent protein kinase C and phorbol ester binding activities. This mixture was pumped at 15 ml/h into a closed 0.5 ml mixing chamber, into which a solution containing 10 mM MES, pH 6.5, 14 mM CaCl_2, 5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 300 mM KCl was also pumped at 15 ml/h. The resulting mixture was pumped at 30 ml/h directly onto the affinity column and, after the total sample was applied, was followed by 15 ml of column buffer, which was then followed by 45 ml of the same buffer but with CaCl_2 reduced to 0.1 mM. At this point, eluting buffer, identical to the column buffer but with 2 mM EGTA in place of CaCl_2, was applied and additional 3.0 ml fractions were collected. Plastic tubes were used to collect fractions. Fractions were assayed immediately after collection and bovine serum albumin was subsequently added, to a final concentration of 1 mg/ml, to a portion of the fraction to stabilize protein kinase activity.

Gel Filtration—An aliquot of affinity column purified protein kinase C (100 µl) or a mixture of molecular weight markers (20 µl) was applied to a Spherogel-TSK 3000 SW column (7.5 x 300 mm) connected to a Pharmacia FPLC system. Fractions of 0.22 ml were collected and assayed. Molecular weight markers were measured by agarose column.

SDS-PAGE—Samples for SDS-PAGE were prepared by adding 1 volume of 50% trichloroacetic acid to 9 volumes of affinity column eluate, letting stand 30 min on ice, then centrifuging 30 min at 35,000 × g. The acid supernatant was carefully aspirated, 100 µl of 0.025 M Tris, pH 6.7, 2% SDS, 10% glycerol, 40 mM dithiothreitol, and 0.002% bromophenol blue was added, and the samples were heated for 2 min at 100 °C. Samples were electrophoresed on a 10% polyacrylamide gel essentially as described by Laemmli (15) and were stained using a Bio-Rad silver stain kit.

Phorbol Ester Binding Assay—Phorbol ester binding was assayed by a modification of a fiber glass filtration method (16). The reaction mixture contained 25 mM Tris, pH 7.5, 10 mM MgAc, 1.4 mM CaCl_2, 0.4 mM EDTA and EGTA (DEAE column eluate), or 0.4 mM EGTA (affinity column eluate), 0–50 mM KCl, 5 mg/ml of bovine serum albumin, 100 µg/ml of phosphatidylserine (Avanti), 20 nM [3H]PDB ± 3 µM 45-phorbol 12β-myristate 13α-acetate in a total volume of 200 or 400 µl in glass tubes (12 x 75 mm). After adding 40 µl of sample, the tubes were incubated 2 h or overnight on ice. Bound [3H]PDB was separated from free [3H]PDB by adding 1 ml of 20 mM Tris, pH 7.5, 10 mM MgAc, 1 mM CaCl_2 and filtering the mixture through 2.4-cm Whatman GF/C glass filters by suction. The tubes and filters were washed five times with 1 ml of filtering solution. The filters were counted in 10 ml of Ready Solv MP (Beckman). As with protein kinase activity, some dilution of extract and of column fractions was necessary to achieve proportionality. Specific binding was calculated as total binding minus nonspecific binding observed in the presence of 3 µM 45-phorbol 12β-myristate 13α-acetate, usually less than 2% of total counts.

Protein determinations on extracts, column fractions, and purified enzyme were done by a dye-binding assay (17) using 0.01% Serva Blue G in 1.6 M phosphoric acid, 0.5 M methanol with bovine serum albumin as standard.

RESULTS AND DISCUSSION

The protein kinase C activity present in the cytosol of the rabbit renal cortex eluted from DEAE-cellulose at approxi-

mately 0.1 M KCl (Fig. 1), similar in position to the activities present in the brain (3) and heart (14). Phorbol ester binding activity coeluted with protein kinase C activity. Upon application of a portion of the combined fractions of protein kinase C-phorbol ester binding activity to the affinity column, negligible amounts of both activities passed through the column, while virtually all of the readily detectable protein passed directly through (Fig. 2). Upon eluting with EGTA, protein kinase C and [3H]PDB binding coeluted in a single, sharp peak. Addition of fatty acid free bovine serum albumin (1 mg/ml final concentration) to plastic collection tubes before or immediately after collection was necessary to effectively stabilize an otherwise highly unstable preparation. The enzyme could not be frozen, but remained fully active and responsive for weeks if kept on ice.

The freshly isolated enzyme was almost totally dependent upon Ca^2+ and could be activated 5–10-fold by diolene in the presence of phosphatidylserine and low Ca^2+.

^C. R. Filburn and T. Uchida, manuscript in preparation.
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**Table 1**

**Purification of protein kinase C-phorbol ester receptor**

| Protein kinase activity | Total activity (nmol/min) | Specific activity (nmol/min/mg) | Purification (−fold) | Phorbol ester binding (pmol/mg) | Specific activity (pmol/mg) | Purification (−fold) |
|-------------------------|---------------------------|---------------------------------|----------------------|-------------------------------|---------------------------|----------------------|
| Crude extract           | 946                       | 0.79                            | 1                    | 583                           | 0.49                      | 1                    |
| DEAE-cellulose          | 677 (72)*                 |                                 |                      | 574 (98)*                     | 2.21                      | 4.5                  |
| Early fraction          | 23.8*                     | 5.41                            | 6.8                  | 17.3*                         | 2.35                      | 4.8                  |
| Late fraction           | 33.2*                     | 4.52                            | 5.7                  |                               |                           |                      |
| Affinity column         | Early fraction            | 6.8 (28)*                       | 468                  | 592                           | 335                       | 684                  |
| Late fraction           | 9.7 (29)*                 | 4619                            | 5846                 | 8.9 (51)*                     | 3764                      | 7682                 |

*Numbers in parentheses indicate per cent recovery of activity applied to the column.

The early and late fractions used were located two fractions before and after, respectively, the peak fraction of protein kinase-phorbol ester binding activities eluting from a DEAE-cellulose column similar to that shown in Fig. 1.

A 4.5-ml aliquot of the early fraction and a 7.5-ml aliquot of the late fraction was applied to an affinity column (1.0 × 4.0 cm) and eluted as described under "Experimental Procedures."

**Fig. 3.** SDS-PAGE and gel filtration of protein kinase C purified by affinity chromatography. A, silver stain patterns after SDS-PAGE of preparations derived from an early fraction (lane A, 2 fractions preceding the peak) and a late fraction (lane B) of the DEAE-cellulose peak of activity (Fig. 1). Molecular weight markers were phosphorylase b (93 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa). B, gel filtration of affinity column eluate containing protein kinase C activity. Molecular weight markers were glutamate dehydrogenase (290 kDa), lactate dehydrogenase (140 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome c (12.4 kDa).
cortical tissue have been applied to an affinity column (1.6 × 5.0 cm), with only a minimal amount passing through the column. Recovery was usually reduced (10–25%), probably due in part to a more prolonged exposure of the kinase to a Ca\(^{2+}\)-dependent protease present in the kidney and other tissues (18, 19). This protease is physically similar to protein kinase C, coelutes with the kinase from DEAE-cellulose (18, 20), and is very active against it (20). The lipid-gel matrix described here has, in fact, been used successfully in purification of milligram quantities of protein kinase C from kilogram amounts of bovine brain (20), and is very active against it (20).

It should be appreciated that the total binding capacity of the final gel matrix, which may contain as much as 1.2 μmol of phosphatidylserine/ml of gel, is likely to vary with the surface area of the particles used. Any increase in surface area by reduction in particle size will result in a decrease in maximal flow rate through packed particles. Particles prepared as described here have proven rigid enough to permit a high flow rate, but small enough to provide adequate interaction of the kinase with and binding to the trapped cholesterol-phosphatidylserine micelles. In addition, affinity columns prepared as described here have performed very reproducibly and have been regenerated by simple buffer washes and reused as many as 20 times with no loss in binding efficiency and only moderate reduction in enzyme recovery. Further investigation of various parameters pertaining to preparation and use of lipid-gel matrix may reveal conditions that further enhance its effectiveness. Simply increasing the ratio of cholesterol to phosphatidylserine beyond that described here appears to increase recovery.

In summary, the purification procedure described here provides a simple, rapid means of obtaining a highly purified preparation of protein kinase C-phorbol ester receptor. In addition, it illustrates the usefulness of a technique of embedding a lipid in polyacrylamide for preparation of an affinity gel. It is possible that this method may be extended to preparation of affinity gels with other lipids exhibiting ligand-dependent interactions with soluble cellular components. The demonstration that proteins other than protein kinase C bind in a Ca\(^{2+}\)-dependent manner to such a phosphatidylserine affinity gel (Fig. 3A) raises interesting questions as to the function of these and possibly other proteins and to the role of Ca\(^{2+}\) in these protein-phospholipid interactions. It is possible that some of these proteins are protease-derived fragments of protein kinase C, a question that is currently under investigation.

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