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Immunological Evidence for Two Physiological Forms of Protein Kinase C

JAMES R. WOODGETT* AND TONY HUNTER

Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California 92138-9216

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Our recently described purification scheme for rat brain protein kinase C yields an enzyme consisting of a 78/80-kilodalton (kDa) doublet upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (submitted for publication). Antisera against this preparation were raised in two rabbits. One of the antisera detected only the 80-kDa component by immunoblotting of purified protein kinase C and immunoprecipitated an 80-kDa [^{35}S]methionine-labeled protein from a variety of human, rodent, and bovine cells, which was shown to represent protein kinase C by comparative one-dimensional peptide mapping. In contrast, the second antiserum detected both 78- and 80-kDa enzyme forms by immunoblotting and immunoprecipitated a [^{35}S]methionine-labeled 78/80-kDa doublet from mammalian cells. One-dimensional peptide maps of these 78- and 80-kDa proteins were similar to those derived from the 78- and 80-kDa forms of purified protein kinase C, respectively. The two forms were not related by either partial proteolysis or differential phosphorylation, showing that two distinct forms of this enzyme exist in mammalian cells. Treatment of mouse B82 L cells with 2.5 μg of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml for 18 h resulted in complete loss of immunoprecipitable protein kinase C with a half time of disappearance of 48 min. Since the normal half-life of protein kinase C was >24 h and the biosynthetic rate of the protein was not decreased after 18 h by TPA treatment, TPA induces down-regulation by increasing the degradation rate of the enzyme. Treatment of cells with 50 ng of TPA per ml followed by resolution of the membrane and cytosol in the presence of ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) promoted an apparent translocation of both 78- and 80-kDa proteins from the cytosol to the membrane fraction. A similar translocation was effected by cell lysis in the presence of Ca^{2+}, indicating the subcellular localization of protein kinase C to be sensitive to the presence of both activators and micromolar amounts of Ca^{2+}.

Since its discovery in 1977 by Nishizuka and colleagues (26), two developments have dramatically changed perceptions of the role of the Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C). The first was the realization that the enzyme was activated by diacylglycerol (DAG), which reduces the K_a values for Ca^{2+} and phosphatidylserine (32, 55). Since DAG is one of the transient products of phosphatidylinositol (PI) turnover, protein kinase C was implicated in the signal transduction mechanism of the many agonists of this communication pathway (for reviews, see references 5 and 50). The second was the identification of protein kinase C as the major cellular receptor for tumor-promoting phorbol esters that substitute for DAG and cause protracted activation of the enzyme (8, 37, 49, 51). This revelation suggested a pivotal role for protein kinase C in mediating the pleiotropic effects of tumor promoters on tumorigenesis and growth control (2).

Although considerable progress has been achieved in elucidating the mechanisms of activation of protein kinase C, such studies have relied on enzymological techniques to monitor activity and identify physiological substrates. There has been a dearth of immunological reagents for probing the regulation of the enzyme in vivo, owing, in part, to difficulties in purifying large quantities of the protein kinase and to the extreme lability of purified preparations (see, for example, reference 38). Recently, we developed a 2-day procedure that allows the isolation of milligram quantities of protein kinase C from rat brain (submitted for publication). This preparation was remarkably stable, with up to 50% of activity remaining after 12 months of storage at 0°C. The enzyme exhibited a 78/80-kilodalton (kDa) doublet upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, unlike conventional preparations, which have been reported to comprise a single polypeptide of 77 to 83 kDa (30, 38, 51, 62). Although we presented data suggesting that the 78- and 80-kDa polypeptides were not simply related by proteolysis and, hence, an artifact of the purification procedure (submitted), we did not know whether the two forms existed in vivo. To address this question, we used this preparation to raise polyclonal antisera to protein kinase C. In this paper, we describe the characterization of these antisera and present evidence for two physiological forms of protein kinase C in mammalian cells. We have also used the antisera to probe the regulation of the enzyme by tumor-promoting phorbol esters, Ca^{2+}, and phosphorylation.

MATERIALS AND METHODS

Generation of antibodies to protein kinase C. Protein kinase C was purified to homogeneity from rat brain as described previously (submitted). Two New Zealand White female rabbits were each immunized by intradermal injection of 250 μg of protein kinase C mixed 1:1 (vol/vol) with complete Freund adjuvant. After 4 weeks, the animals were each boosted by intrapopliteal lymph node injection of 50 μg of protein in complete Freund adjuvant. At subsequent 4- to 6-week intervals, the animals were each boosted again with 150 to 250 μg of protein in adjuvant by intradermal and intramuscular injection. Antiserum was collected 1 week after each booster injection. Titers of antibodies in serum were determined by the enzyme-linked immunosorbent assay with 50 ng of protein kinase C per microtiter plate well as antigen, alkaline phosphatase-conjugated goat anti-rabbit

* Corresponding author.

immunoglobulin G (affinity purified; Sigma Chemical Co.) as second antibody, and *p*-nitrophenylphosphate as substrate.

Immunoblotting. Protein samples to be immunoblotted were denatured by being heated at 100°C for 2 min in SDS sample buffer and resolved on 12.5% SDS-polyacrylamide gels (36). After being heated, brain extracts were sonicated for 10 to 20 s to fragment the DNA. Following electrophoretic transfer overnight onto a nitrocellulose membrane in the presence of 0.1% SDS (59), the membrane was incubated for 2 h in 10 mM Tris hydrochloride (pH 7.5)–150 mM NaCl (buffer A) containing 0.02% (wt/vol) NaN_3 and 2% (wt/vol) dehydrated low-fat milk (Carnation Co.) to block nonspecific binding sites. The membrane was briefly rinsed with buffer A and then incubated for 4 h with a 200-fold dilution of antiserum in buffer A containing 0.02% (wt/vol) NaN_3 and 0.25% (wt/vol) gelatin. The nitrocellulose was washed once with buffer A, twice with buffer A containing 0.05% (vol/vol) Nonidet P-40, and once again with buffer A for a total of 40 min.

Bound rabbit immunoglobulin G was detected either by using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (affinity purified; Cooper Biomedical, Inc.) followed by staining with horseradish peroxidase color reagent (Bio-Rad Laboratories) by using goat anti-rabbit immunoglobulin G coated with colloidal gold (Janssen) followed by counterstaining with silver lactate as specified by the manufacturer. Molecular weights were estimated by concurrent electrophoresis and transfer of protein standards that were visualized by staining appropriate strips of membrane with amido black.

Affinity purification of antibodies. Homogeneous protein kinase C (0.5 mg) was coupled to 0.5 g of CNBr-activated Sepharose 4B (Pharmacia, Inc.) by the procedure recommended by the manufacturer. The coupling efficiency was 98%. Crude, heat-treated (56°C for 30 min) antiserum (4 ml) or an equivalent amount of immunoglobulin fraction, prepared by passage of dialyzed antiserum through a 30-ml column of DEAE–Affi-Gel Blue (Bio-Rad), was mixed by rotation with the affinity resin for 1 to 2 h at 4°C in the presence of 4 μg of leupeptin per ml, 4 μg of soybean trypsin inhibitor per ml, and 0.1% (vol/vol) aprotinin per ml. The resin was collected by brief centrifugation and transferred to two 1-ml spin columns. After being washed with 10 ml of 10 mM Tris hydrochloride (pH 7.5)–0.1% (wt/vol) NaN_3–500 mM NaCl, the resin was washed twice with 10 mM Tris hydrochloride containing 0.2% (wt/vol) sodium deoxycholate. Specifically bound antibodies were desorbed with six 0.2-ml washes of 50 mM diethylamine (pH 11.5) containing 0.2% (wt/vol) sodium deoxycholate. Eluates were collected by brief centrifugation and immediately neutralized by the addition of 1/2 vol of 2 M glycine. Combined eluates were dialyzed against 10 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–0.02% (wt/vol) NaN_3–0.1% (vol/vol) 2-mercaptoethanol, concentrated two- to fivefold by reverse dialysis against solid polyethylene glycol 6000, and redialyzed. By this procedure, 85 to 90% of the antibodies to protein kinase C bound to the column and were eluted with a final recovery of 30 to 35% as determined by the enzyme-linked immunosorbent assay.

Cells and labeling. Murine NIH 3T3 and Abelson murine leukemia virus-transformed NIH 3T3 (ANN-1) fibroblasts, B82 L cells (hereafter called B82 cells), and rat F208 fibroblasts were grown in Dulbecco-Vogt modified Eagle medium (DMEM) supplemented with 10% calf serum and plated at a density of 2×10^5 cells per 35-mm dish 24 h prior to labeling. Human A431 epidermal carcinoma cells, Rous sarcoma virus-transformed A431 cells, Snyder-Theilin feline sarcoma virus-transformed A431 cells, rat B15 and B49 neuroblastoma cells, and Madin-Darby bovine kidney cells were grown similarly, except that DMEM was supplemented with 10% fetal calf serum in place of calf serum. Unless otherwise noted, labeling was performed with 150 μCi of [^{35}S]methionine (>1,000 Ci/mmol; Amersham Corp.) per ml for 16 to 18 h in methionine-free DMEM supplemented with 5% DMEM, 5% calf serum, or 5% fetal calf serum as appropriate and 10 mM HEPES-OH (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4) or with 1.5 to 2.5 mCi of [^{32}P]phosphate (ICN Pharmaceuticals, Inc.) per ml for 16 to 20 h in phosphate-free DMEM supplemented with 4% calf serum or 4% fetal calf serum as appropriate and 10 mM HEPES-OH (pH 7.4). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) or 4-β-phorbol in dimethyl sulfoxide was added at a concentration of 50 ng/ml for 5 min at the end of the labeling period unless otherwise specified.

Pulse-labeling of mouse B82 cells was performed by washing 35-mm dishes with methionine-free DMEM followed by incubation in 0.5 ml of this medium. After 15 min, 300 μCi of [^{35}S]methionine was added for a further 15 min. The cells were then washed with chase medium, which consisted of DMEM supplemented with 10% calf serum, 2 mM unlabeled methionine, and 10 mM nonessential amino acids, and incubated in this medium for 0 to 6 h as indicated.

[^{3}H]myristic acid labeling of B82 cells was performed by the method of Buss and Sefton (6) for 16 h in DMEM supplemented with 10% calf serum, 0.2 mCi of 9,10-[^{3}H]myristic acid (12.9 Ci/mmol; New England Nuclear Corp.) per ml, 10 mM nonessential amino acids, and 5 mM sodium pyruvate.

Immunoprecipitation. All procedures were carried out at 4°C unless otherwise noted. Cells were rinsed twice with ice-cold 10 mM Tris hydrochloride (pH 7.4) and lysed in 35-mm dishes each containing 0.4 ml of ice-cold RIPA buffer (54) modified by the addition of 2 mM EDTA, 50 mM NaF, and 100 μM Na_3VO_4. Cells were scraped into 1-ml screw-cap tubes (Eppendorf), 10 μg of bovine pancreatic RNase was added to ^{32}P-labeled lysates, and the extracts were clarified by centrifugation at 25,000 × *g* for 1 h. The supernatant was decanted, and an aliquot (50 to 150 μl), normalized for incorporated radioactivity, was added to tubes containing antiserum and adjusted to a final volume of 0.5 ml with modified RIPA buffer. After 1 h on ice, 10 to 20 μl of Formalin-fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring) prewashed twice in RIPA buffer was added for a further 30 min. Immunocomplexes were collected by centrifugation at 6,000 × *g* for 15 min at 4°C over a 1-ml cushion of modified RIPA buffer containing 10% (wt/vol) sucrose. Pellets were resuspended in 1 ml of modified RIPA buffer and recentrifuged for 5 min at 6,000 × *g*. After three such washes, pellets were rinsed with 0.8 ml of 20 mM HEPES-OH (pH 7.5)–2 mM EDTA–50 mM NaF–100 μM Na_3VO_4, suspended in 30 μl of sample buffer, heated at 100°C for 2 min, and centrifuged in a microfuge for 2 min. Proteins were resolved on 12.5% SDS-polyacrylamide gels (36). When indicated as boiled lysates, cells were lysed at room temperature in 0.1 ml of 0.5% (wt/vol) SDS–20 mM Tris hydrochloride (pH 8.0)–10 mM dithiothreitol–4 μg of leupeptin per ml–4 μg of soybean trypsin inhibitor per ml–0.1% aprotinin, immediately scraped into an Eppendorf tube, and heated for 2 min at 100°C. The samples were then diluted with 0.4 ml of modified RIPA buffer and clarified by centrifugation as described above.

Unless noted otherwise, immunoprecipitations were performed in antibody excess. Dried analytical gels containing

[^{35}S]methionine or [^{3}H]myristate were enhanced by impregnation with diphenyloxazole, and radioactivity was detected by autoradiography with presensitized film. For quantification, gel pieces were excised, hydrolyzed with 70% perchloric acid–30% hydrogen peroxide (2:1) for 24 h at 60°C, and subjected to scintillation counting. After background counts were subtracted, counts were divided by the total amount of radioactivity used in each immunoprecipitation as measured by acid precipitating a volume of lysate on a fiber glass disk followed by counting in scintillant. All quantitations are expressed as a percentage of total cell protein.

Gels containing ^{32}P were analyzed by autoradiography (with intensifying screens when indicated), and bands were quantified by counting Cerenkov radiation.

Cell fractionation. Dishes (35 mm) of growing cells (approximately 70% confluent) labeled for 16 h with [^{35}S]methionine were treated with 50 ng of TPA per ml for various times. After being rinsed twice with ice-cold 10 mM Tris hydrochloride (pH 7.4), the cells were allowed to swell for 5 min on ice in 0.3 ml of 5 mM HEPES-OH (pH 7.5)–0.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]–2 mM $MgCl_2$–5 mM 2-mercaptoethanol–4 μg of leupeptin per ml–4 μg of soybean trypsin inhibitor per ml–0.1% (vol/vol) aprotinin. Lysis of cells in the presence of various Ca^{2+} concentrations was performed with Ca^{2+}-EGTA buffers, which consisted of 20 mM HEPES-OH (pH 7.4), 10 mM EGTA, 10 mM $MgCl_2$, 50 mM NaCl, 0.5 mM dithiothreitol, 1 mM NaN_3, 4 μg of leupeptin per ml, 4 μg of soybean trypsin inhibitor per ml, 0.1% aprotinin, and 0 to 11 mM $CaCl_2$. These buffers were treated with Chelex 10 resin (Bio-Rad) before $CaCl_2$ was added. The free Ca^{2+} concentration was then determined with a Ca^{2+} electrode before and after cell lysis.

Cells were scraped into Eppendorf tubes and sonicated on ice for two 10-s bursts at setting 3 of a Heat Systems sonicator fitted with a microprobe. The cell lysate was centrifuged at 100,000 × *g* for 20 min at 4°C in a Beckman Airfuge, and the supernatant was decanted into 0.3 ml of 2× concentrated modified RIPA buffer (cytosolic fraction). The pellet was gently resuspended in 0.3 ml of modified RIPA buffer, incubated on ice for 20 min, and recentrifuged at 100,000 × *g* for 20 min. The supernatant was transferred into 0.3 ml of modified RIPA buffer and was considered to be the membrane (particulate) fraction.

Assay of protein kinases. Cyclic AMP-dependent protein kinase was purified from rabbit skeletal muscle by the method of Beavo et al. (4). Assays of protein kinase C and cyclic AMP-dependent protein kinase were performed exactly as described previously (21), with lysine-rich histones as substrate (type IIIS; Sigma Chemical Co.).

Treatment with potato acid phosphatase. Purified or immunoprecipitated protein kinase C was incubated at 30°C with 0.25 to 2.0 μg of potato acid phosphatase (P 0157; Sigma) in 10 mM MES-OH (morpholineethanesulfonic acid) (pH 5.5)–1.0 mM $MgCl_2$–50 mM NaCl–4 μg of leupeptin per ml–4 μg of soybean trypsin inhibitor per ml–0.1% aprotinin–0.1 mM dithiothreitol in a total volume of 25 μl for various times. Control incubations were performed in the absence of phosphatase or in the presence of 10 mM potassium phosphate, a potent inhibitor of this enzyme. Reactions were stopped by the addition of hot SDS-sample buffer.

One-dimensional peptide mapping. One-dimensional peptide maps were prepared as described previously (9), with 15% gels and 5 or 25 ng of *S. aureus* V8 protease. Protein was detected by silver staining (65), and radioactivity was detected by autoradiography.

Phosphoamino acid analysis and two-dimensional peptide mapping. Phosphoamino acid determinations were performed as described previously (11, 25). [^{35}S]methionine-labeled proteins were resolved on SDS-polyacrylamide gels, extracted, and subjected to tryptic digestion as described previously (25). Tryptic peptides were separated in two dimensions on 100-μm cellulose thin-layer plates by electrophoresis at pH 4.72 followed by ascending chromatography (20). Before autoradiography, plates were treated with En-^{3}Hance (New England Nuclear).

RESULTS

Characterization of antibodies. The preparations of rat brain protein kinase C used to immunize rabbits consisted of a 78/80-kDa doublet when subjected to SDS-polyacrylamide gel electrophoresis (submitted). These two polypeptides are closely related structurally as determined by partial proteolytic mapping but do not appear to be generated by proteolytic cleavage during isolation (submitted). Antisera collected from two rabbits after the second booster injection exhibited titers in excess of 10^4 by the enzyme-linked immunosorbent assay with purified protein kinase C as antigen. The characterization of one of these antisera, termed antiserum A, will be considered first. Immunoblotting analysis of this antiserum with purified protein kinase C detected predominantly an 80-kDa band which comigrated with the 80-kDa polypeptide from purified protein kinase C (Fig. 1, lane 2).

Following affinity purification on a column of immobilized protein kinase C, antiserum A specifically immunoprecipitated an 80-kDa polypeptide from two [^{35}S]methionine-labeled rat neuroblastoma cell lines (Fig. 2A). The amount of 80-kDa protein immunoprecipitated from rat B15 cells was about five times higher than that from rat B49 cells. This correlates with the number of [^{3}H]phorbol dibutyrate-binding sites on these cells, which is 5- to 10-fold higher in B15 than in B49 cells (A. Horowitz and I. B. Weinstein, personal communication). Preabsorption of antiserum A with 2 μg of purified protein kinase C reduced the subsequent yield of immunoprecipitated 80-kDa protein by 60 to 70% (data not shown). Furthermore, this affinity-purified antiserum inhibited the activity of purified protein kinase C by 40%, whereas the activity of cyclic AMP-dependent protein kinase was unaffected (data not shown).

To confirm that the 80-kDa polypeptide was protein kinase C, immunoprecipitates from [^{35}S]methionine-labeled rat B15 cells were resolved on SDS-polyacrylamide gels and detected by direct autoradiography, and the 80-kDa band was excised and subjected to one-dimensional proteolytic mapping with *S. aureus* V8 protease (Fig. 3B). The pattern of ^{35}S-labeled peptides so generated was compared with similarly treated digests of the 80- and 78-kDa components of purified protein kinase C visualized by silver staining (Fig. 3A). Overall, the peptide map of the immunopurified 80-kDa band was very similar to both of the digests of the 80- and 78-kDa components of purified protein kinase C, although closer homology was exhibited to the 80-kDa species. On the basis of these results, the immunoprecipitated 80-kDa protein was identified as the 80-kDa component of protein kinase C.

Quantitative analysis of the amount of this protein immunoprecipitated from a variety of cell lines of various species with an excess of antiserum A (Table 1) showed cross-reactivity with rat, mouse, bovine, and human protein kinase C. The levels of this protein were low in fibroblast cell

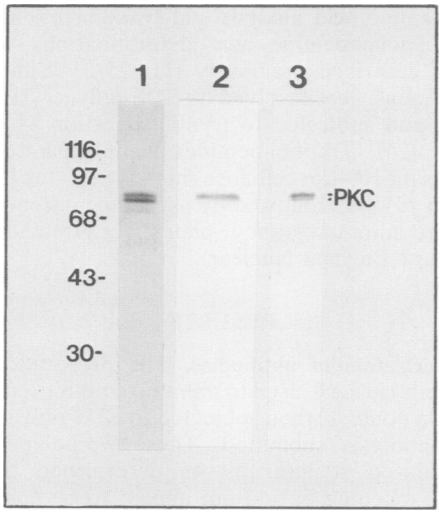

FIG. 1. Immunoblotting of protein kinase C. Rat brain protein kinase C (1 μg) was resolved on a 12.5% polyacrylamide gel and either stained with Coomassie blue (lane 1) or electrophoretically transferred from the gel to nitrocellulose and probed with antiserum A (lane 2) or antiserum B (lane 3) as described in Materials and Methods. Molecular masses were calibrated from protein standards: β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa); PKC indicates protein kinase C.

lines, as expected. Three clonal lines of A431 cells, characterized by differing numbers of epidermal growth factor receptors expressed at the cell surface (G. N. Gill, personal communication), varied 10-fold in their protein kinase C content. The highest levels of protein kinase C were observed in rat B15 neuroblastoma cells and mouse B82 cells, which were therefore used for most of the subsequent studies.

In B15 cells labeled with $^{32}P_i$, antiserum A immunoprecipitated an 80-kDa phosphoprotein (Fig. 4A). Treatment of these cells with 50 ng of TPA per ml resulted in a two- to threefold increase in ^{32}P label associated with this band. Partial acid hydrolysis revealed mostly phosphoserine with a smaller amount of phosphothreonine and no phosphotyrosine, with little change following TPA treatment (Fig. 4B). To determine whether protein kinase C was a substrate for protein tyrosine kinases in vivo, A431 cell lines transformed with either Rous sarcoma virus or Snyder-Theilen feline sarcoma virus were labeled with $^{32}P_i$, treated for 10 min with 50 ng of TPA per ml, and then lysed. After normalization for incorporated radioactivity, the lysates were immunoprecipitated with antiserum A. These viruses encode protein-tyrosine kinases and cause elevated phosphotyrosine levels in their host cells (for a review, see reference 24). Again, an 80-kDa phosphoprotein was specifically immunoprecipitated, although the protein from the two virally transformed cell lines contained two- to threefold more phosphate label than did that from the control A431 cells (data not shown). Both cell lines had similar levels of protein kinase C as determined by immunoprecipitation of cells labeled in parallel with [^{35}S]methionine (not shown). However, phosphoamino acid analyses indicated that the increase in incorporation was confined to phosphoserine and phosphothreonine, implying that protein kinase C is not a direct substrate of these protein-tyrosine kinases (data not shown).

Recently, several proteins, including three protein kinases, were demonstrated to contain a myristate group at their N termini (1, 6, 7, 41). In the case of pp60$^{v\text{-}src}$, the presence of this fatty acid is required for membrane attachment and transforming function (29). Although protein kinase C associates tightly with membranes (see below) and has a blocked N terminus (L. E. Gentry, personal communication), we were unable to immunoprecipitate an 80-kDa protein from [^{3}H]myristate-labeled B82 cells with antiserum A, indicating that this protein kinase is not quantitatively modified by myristylation (data not shown).

Immunoprecipitation of two forms of protein kinase C. Like antiserum A, antiserum B from a second rabbit immunoblotted the 80-kDa species of purified protein kinase C; however, it also recognized the 78-kDa component present in the purified preparations of protein kinase C just as efficiently (Fig. 1, lane 3). Moreover, upon affinity purification, antiserum B specifically immunoprecipitated a 78/80-kDa doublet from [^{35}S]methionine-labeled mouse B82 cells and rat B15 cells (Fig. 2B). One-dimensional peptide mapping of individually excised 78- and 80-kDa from immunoprecipitates of mouse B82 cells revealed the 80-kDa protein to have an identical cleavage pattern to that of the 80-kDa protein recognized by antiserum A in rat B15 cells (Fig. 3C). Although the map of the 78-kDa component exhibited a similar pattern to that of the 80-kDa protein, the few differences were reminiscent of those between the

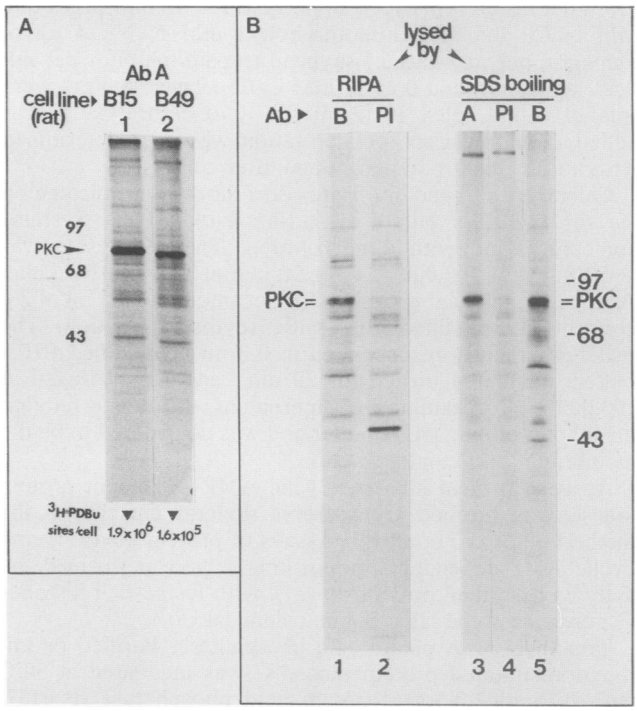

FIG. 2. Immunoprecipitation from [^{35}S]methionine-labeled cell lines. Cells were labeled for 18 h with [^{35}S]methionine and immunoprecipitated with antiserum A (panel A, lanes 1 and 2; panel B, lane 3), antiserum B (panel B, lanes 1 and 5) or preimmune serum (panel B, lanes 2 and 4), as described in Materials and Methods. (A) Immunoprecipitates from two rat neuron-like cell lines differing significantly in numbers of [^{3}H]phorbol dibutyrate-binding sites per cell (given below each lane): lane 1, B15 cells; lane 2, B49 cells. (B) Immunoprecipitates from mouse B82 cells. Lanes 1 and 2 contain immunoprecipitates from cells lysed with RIPA buffer, while lanes 3 to 5 contain immunoprecipitates from cells lysed with SDS and subsequently boiled. Molecular masses were calculated as for Fig. 1. Exposure times for the enhanced gels with presensitized Kodak XAR 5 film were 30 h for panel A and 18 h for panel B.

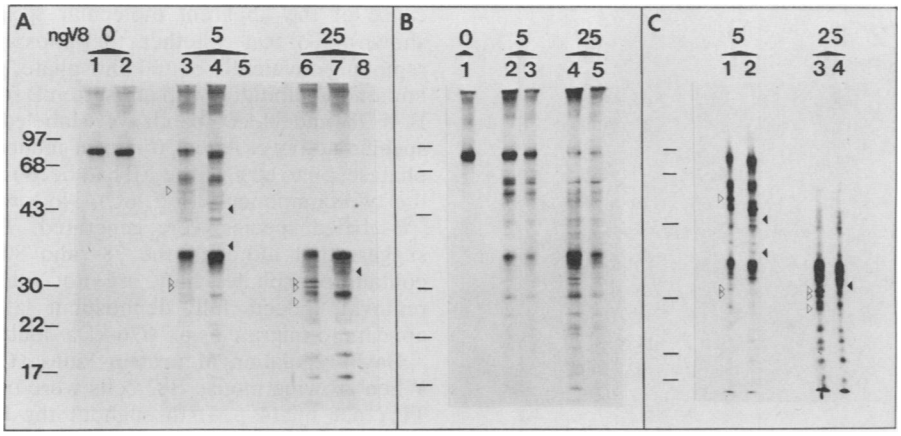

FIG. 3. One-dimensional peptide maps of purified and immunoprecipitated protein kinase C. (A) The 80-kDa (lanes 1, 3, and 6) and 78-kDa (lanes 2, 4, and 7) components of purified protein kinase C were excised from a 12.5% gel and reelectrophoresed on a second 15% gel in the absence (lanes 1 and 2) or presence of 5 ng (lanes 3 and 4) or 25 ng (lanes 6 and 7) of *S. aureus* V8 protease. Protease alone was added to lanes 5 and 8 (5 and 25 ng, respectively). Peptides were visualized by silver staining by the method of Wray et al. (65). (B) The 80-kDa [^{35}S]methionine-labeled band immunoprecipitated with antiserum A from rat neuron-like B15 cells as in Fig. 2 was excised and reelectrophoresed as described for panel A in the absence (lane 1) or presence of 5 ng (lanes 2 and 3) or 25 ng (lanes 4 and 5) of *S. aureus* V8 protease. Lanes 2 and 4 differ from lanes 3 and 5, respectively, in representing immunoprecipitations with two separate isolates of antiserum A. (C) The 80-kDa (lanes 1 and 3) and 78-kDa (lanes 2 and 4) [^{35}S]methionine-labeled bands immunoprecipitated by antiserum B from mouse B82 cells as in Fig. 2 were separately excised and reelectrophoresed as in panel A in the presence of 5 ng (lanes 1 and 2) or 25 ng (lanes 3 and 4) of *S. aureus* V8 protease. [^{35}S]methionine-labeled peptides were detected by autoradiography with enhancement. Exposure times were 5 days for panel B and 8 days for panel C. Molecular masses (indicated by lines in panels B and C) were calibrated from protein standards as in Fig. 1, except that soybean trypsin inhibitor (22 kDa) and myoglobin (17 kDa) were included. In panels A and C, peptide differences between the 80- and 78-kDa proteins that appear to be conserved between the purified and immunoprecipitated 80-kDa bands are marked with open arrowheads, while those for the 78-kDa bands are marked with closed arrowheads.

peptide maps of the 78- and 80-kDa components of purified protein kinase C (Fig. 3A). Hence, the cultured cells contained 80- and 78-kDa proteins very similar to those of the purified enzyme, providing evidence for the existence of multiple forms of protein kinase C in vivo.

Two-dimensional tryptic peptide maps of the immunoprecipitated 78- and 80-kDa proteins confirmed the close relationship between the polypeptides, since only two differences were seen in 28 [^{35}S]methionine-labeled peptides (Fig. 5, marked with arrowheads). Two-dimensional peptide mapping of ^{125}I-labeled 78- and 80-kDa polypeptides of the purified enzyme had previously shown these two components to be highly related but also revealed that each had some unique peptides (submitted). The similarity of the two forms suggested that the 78-kDa species might be generated from the 80-kDa protein by proteolytic cleavage during isolation. However, immunoprecipitation was carried out below 4°C in the presence of protease inhibitors. Furthermore, lysis in SDS followed immediately by boiling at 100°C did not affect the ratio of the 78-kDa band to the 80-kDa band (Fig. 2B) (see Discussion). Unlike antiserum A, antiserum B failed to inhibit protein kinase activity, suggesting that the two antisera were directed against different epitopes (data not shown). Immunoprecipitation of ^{32}P-labeled cells with antiserum B generated a doublet of 80 and 78 kDa (Fig. 6C).

TABLE 1. Quantification of the 80-kDa protein in various cell lines[a]

Cell line	Species	Amt of 80-kDa protein (% of total cell protein)
Whole brain	Rat	0.04–0.08
B15 neuroblastoma	Rat	0.015
B49 neuroblastoma	Rat	0.003
F208 fibroblast	Rat	0.0015
B82 cell	Mouse	0.03
NIH 3T3 fibroblast	Mouse	0.002
ANN-1 Ab-MuLV-transformed NIH 3T3 fibroblast[b]	Mouse	0.002
MDBK kidney epithelial cell	Bovine	0.001
A431 epidermoid carcinoma	Human	0.0013
A431 clone 29_R	Human	0.003
A431 clone 18_0	Human	0.0004

[a] Cells were labeled for 18 h with [^{35}S]methionine, immunoprecipitated with antiserum A, and resolved on 12.5% SDS-polyacrylamide gels, and the amount of [^{35}S]methionine associated with the 80-kDa protein kinase C band was quantified as described in Materials and Methods. The amount of protein present in rat brain was estimated from the yield of purified protein (submitted).

[b] Ab-MuLV, Abelson murine leukemia virus.

Affinity-purified antiserum A from later bleeds immunoprecipitated a 78/80-kDa doublet from [^{35}S]methionine-labeled cells rather than the 80-kDa protein alone (for example, see Fig. 7). This could be explained either by the delayed generation of antibodies to the 78-kDa protein in this rabbit or by the selective purification of antibodies to determinants shared by both forms of the enzyme. With regard to the latter possibility, since the protein kinase C affinity columns were eluted under denaturing conditions and used repeatedly, it is probable that antibodies recognizing the denatured protein were preferentially selected, consistent with the ability of these affinity-purified sera to immunoprecipitate protein kinase C from SDS-boiled lysates (Fig. 2B).

Are the two forms of protein kinase C related by phosphorylation? Many proteins, when modified by phosphorylation, exhibit retardation upon SDS-polyacrylamide gel electrophoresis (see, for example, reference 43). We previously

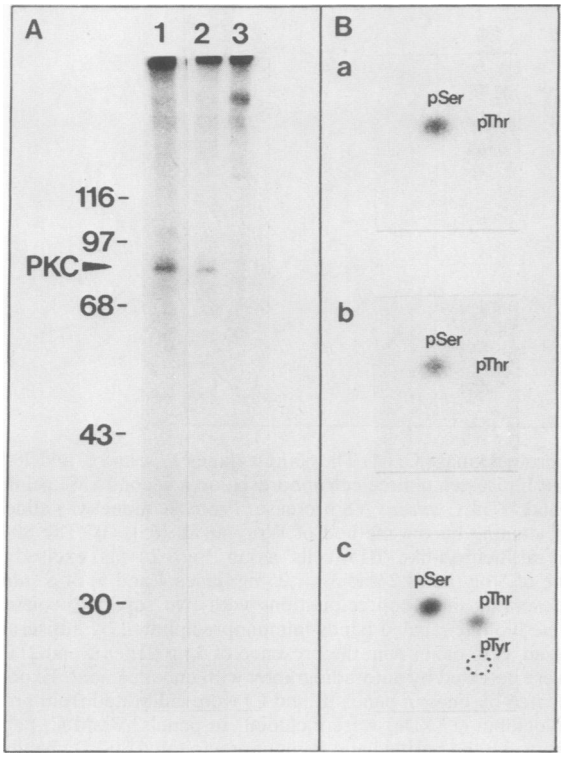

FIG. 4. Immunoprecipitation of protein kinase C from ^{32}P-labeled cell lines. (A) Rat B15 cells were labeled with $^{32}P_i$ for 18 h and immunoprecipitated with antiserum A (lanes 1 and 2) or preimmune serum (lane 3). Cells were treated with 50 ng of TPA per ml (lanes 1 and 3) or 50 ng of 4-β-phorbol per ml (lane 2) for 5 min prior to lysis. All lanes were run on the same gel. (B) Phosphoamino acid analysis of 80-kDa phosphoprotein from panel A: (a) lane 1; (b) lane 2; (c) purified protein kinase C (80-kDa polypeptide) autophosphorylated in vitro. Phosphoserine and phosphothreonine are denoted by pSer and pThr, respectively: in panel B, section c, the position of the phosphotyrosine (pTyr) standard is indicated by a broken circle. Exposure times (with intensifying screens) were 20 h for panel A; 4 days (120 cpm) for panel B, section a; 4 days (100 cpm) for panel B, section b; and 20 h (1,000 cpm) for panel B, section c.

demonstrated that both 78- and 80-kDa bands of purified protein kinase C contain phosphate as isolated (submitted). It was therefore possible that the 80-kDa species represented a phosphorylated form of the 78-kDa protein, particularly since all immunoprecipitations were conducted in the presence of phosphate buffer containing NaF and Na_3VO_4, all of which are potent phosphatase inhibitors. To investigate whether the 78- and 80-kDa polypeptides of protein kinase C were related by phosphorylation, [^{35}S]methionine-labeled B82 cells were lysed with RIPA buffer containing 10 mM Tris hydrochloride (pH 7.4) in place of phosphate in the presence and absence of NaF and Na_3VO_4 (Fig. 6A). As expected, in the presence of the phosphatase inhibitors, a 78/80-kDa doublet was observed. However, in their absence, a 76/78/80-kDa triplet was apparent. Incubation of protein kinase C, prepared by immunoprecipitation in the presence of phosphatase inhibitors, with potato acid phosphatase generated several faster-migrating bands. After a 15-min treatment, two predominant polypeptides of 74 and 76 kDa were apparent (Fig. 6B). Inclusion of 10 mM potassium phosphate, to inhibit the phosphatase, prevented formation of the faster-migrating bands, ruling out proteolysis as the cause of the apparent molecular weight shifts (data not shown). To test whether the phosphatase was actually removing covalently bound phosphate, protein kinase C was immunoprecipitated with antiserum B from ^{32}P-labeled cells. Both 78- and 80-kDa bands were labeled with ^{32}P to a similar specific activity. After 30 min of incubation with the phosphatase, 50 to 60% of the ^{32}P had been removed from each of the two components (Fig. 6C). No lower-molecular-weight ^{32}P-labeled species were generated. Together, these data suggest that although the 78- and 80-kDa proteins both contain phosphate, they are not interconvertible by its removal. Indeed, fully dephosphorylated protein kinase C appears to migrate as a 74/76-kDa doublet (Fig. 6B, lane 4).

Down-regulation of protein kinase C by phorbol esters. When growing mouse B82 cells were treated with 2.5 μg of TPA per ml (4 μM) throughout the labeling period with [^{35}S]methionine (18 h), a complete loss of immunoprecipitable 78- and 80-kDa proteins was noted (Fig. 7A). A similar result was obtained with A431 cells (clone 29R). Treatment of B82 cells labeled overnight with [^{35}S]methionine with 2.5 μg of TPA per ml for various times demonstrated half-maximal disappearance of the 80-kDa protein after 48 min (Fig. 7B). In contrast, pulse-chase experiments established the half-life of protein kinase C in untreated cells to be >24 h (Fig. 7C). Until this point, our experiments have measured the net effect of TPA on synthesis and turnover of protein kinase C. To determine whether TPA influenced the synthetic rate of protein kinase C, B82 cells were incubated for 18 h in the presence or absence of 2.5 μg of TPA per ml and then pulse-labeled with [^{35}S]methionine for 15 min and chased with 2 mM unlabeled methionine for 10 min (Fig. 7D). Rather than causing a decrease in the synthetic rate of the 78/80-kDa doublet, TPA appeared, if anything, to stimulate synthesis. Polypeptides of lower molecular weight were observed in immunoprecipitates specifically from cells treated with TPA. These bands possibly represent degradation products of protein kinase C and, if so, reflect enhanced proteolysis of the enzyme in TPA-treated cells.

Translocation of protein kinase C in response to TPA and Ca^{2+}. [^{35}S]methionine-labeled B82 cells were lysed in hypotonic buffer containing 0.5 mM EGTA and fractionated by centrifugation into cytosol and particulate (membranous) fractions. Under these conditions, 90 to 95% of immunoprecipitable protein kinase C (78- and 80-kDa bands) was present in the cytosol fraction (Fig. 8A, lanes 3, 4, 7, and 8). Hence, in resting cells, little, if any, protein kinase C is stably associated with membranes, nuclei, or other organelles. Immunofluorescence experiments with these cells showed diffuse cytoplasmic fluorescence with no nuclear staining (data not shown). Treatment of the cells with 50 ng of TPA per ml induced a rapid disappearance of the protein from the cytosol fraction and proportionately increased the fraction partitioning with the particulate fraction (Fig. 8A, lanes 1, 2, 5, and 6). Half-maximal translocation occurred 5 min after TPA treatment and was complete after 20 min (Fig. 8B). Subsequently, the amount of precipitable particulate-associated enzyme decreased without a corresponding increase in the soluble fraction, indicating net loss of protein (Fig. 8B). Immunofluorescence of TPA-treated cells again revealed no nuclear staining, although the cytosolic fluorescence appeared less diffuse than in control cells (not shown).

Since protein kinase C requires EGTA for efficient extraction from brain tissue (30), the effect of Ca^{2+} on membrane binding of this enzyme was investigated. Lysis of cells in the presence of different concentrations of Ca^{2+} in the absence of TPA showed that Ca^{2+} alone induced translocation of

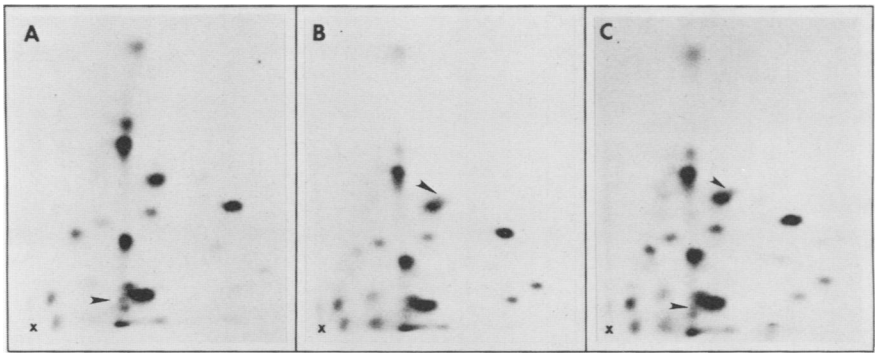

FIG. 5. Two-dimensional tryptic maps of immunoprecipitated protein kinase C. The 78- and 80-kDa polypeptides of protein kinase C were immunoprecipitated with antiserum B from mouse B82 cells labeled for 18 h with [^{35}S]methionine, separately extracted, digested with trypsin, and subjected to two-dimensional peptide mapping as described in Materials and Methods. Crosses mark the point of sample application. Electrophoresis at pH 4.7 was performed in the horizontal direction, with the anode on the left. (A) 80-kDa band; (B) 78-kDa band; (C) mixture of digests of A and B. Differences between the 80- and 78-kDa polypeptides are indicated by arrowheads. Radioactivity loaded onto thin-layer plates and exposure times (−70°C with enhancement and sensitized film) were 2,600 cpm and 9 days for panel A; 3,000 cpm and 9 days for panel B; and 1,500 cpm of each, and 9 days for panel C.

both forms of protein kinase C to the particulate fraction, half-maximal redistribution occurring at 4 μM Ca^{2+} (Fig. 8C).

DISCUSSION

Our isolation procedure for protein kinase C from rat brain yields a protein consisting of a 78/80-kDa doublet upon denaturing gel electrophoresis (submitted). The two bands are closely related structurally and functionally, but the 78-kDa polypeptide does not seem to be derived from the 80-kDa protein by proteolysis (submitted). Because we could not judge the physiological relevance of the two forms, we used this preparation to raise antisera against protein kinase C to see whether similar species in other cells could be recognized. The two antisera we generated differed in

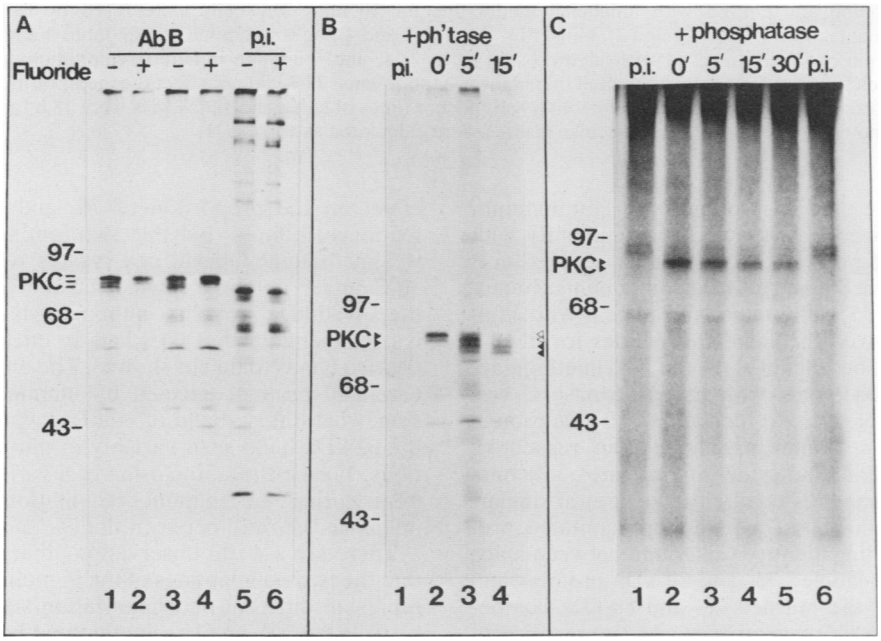

FIG. 6. Dephosphorylation of immunoprecipitated protein kinase C. (A) [^{35}S]methionine-labeled mouse B82 cells lysed in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of the phosphatase inhibitors NaF and Na_3VO_4 were immunoprecipitated with two isolates of antiserum B (lanes 1 to 4) or preimmune serum (lanes 5 and 6). (B) [^{35}S]methionine-labeled B82 cells were lysed with RIPA buffer containing 50 mM NaF and 100 μM Na_3VO_4 and immunoprecipitated with preimmune serum (lane 1) or antiserum B (lanes 2 to 4). The immunoprecipitates were washed twice with 10 mM MES-OH (pH 5.5) and incubated at 30°C in the presence of 0.5 μg of potato acid phosphatase for 0 min (lane 2), 5 min (lane 3), or 15 min (lanes 1 and 4) as described in Materials and Methods. (C) $^{32}P_i$-labeled B82 cells were treated for 5 min with 50 ng of TPA per ml, lysed by being boiled in SDS, and immunoprecipitated with antiserum B (lanes 2 to 5) or preimmune serum (lanes 1 and 6). The immunoprecipitates were washed with MES buffer as for panel B and then treated with 0.5 μg of potato acid phosphatase in the presence of 10 μg of bovine pancreatic RNase per ml (to degrade any contaminating ^{32}P-labeled RNA) as described in Materials and Methods for 0 min (lanes 1, 2, and 6), 5 min (lane 3), 15 min (lane 4), or 30 min (lane 5). All samples were analyzed on 12.5% gels. Exposure times for enhanced gels were 36 h for panel A, 30 h for panel B, and 5 days (without intensifying screen and with Kodak XS film in place of XAR) for panel C.

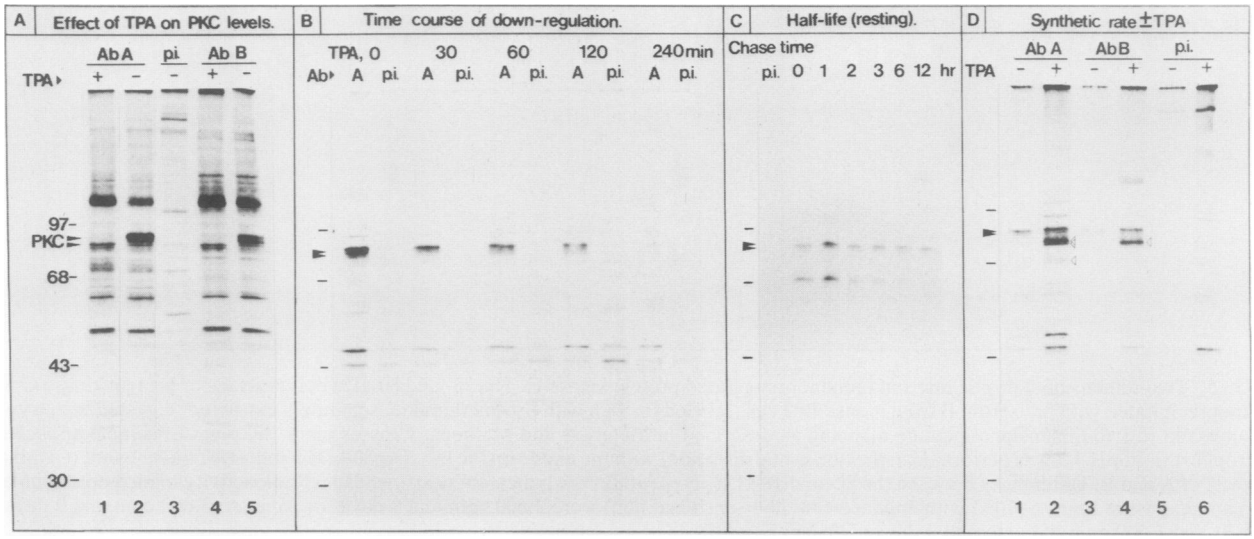

FIG. 7. (A) Effect of TPA treatment on levels of immunoprecipitable protein kinase C. Mouse B82 cells were concurrently labeled with [^{35}S]methionine and treated for 18 h with 2.5 μg of TPA per ml (lanes 1 and 4) or 2.5 μg of 4-β-phorbol per ml (lanes 2, 3, and 5) and immunoprecipitated with antiserum A (lanes 1 and 2), antiserum B (lanes 4 and 5), or preimmune serum (lane 3). All samples were run on the same gel. (B) Time course of disappearance of protein kinase C from mouse B82 cells. Mouse B82 cells were labeled for a total of 20 h with [^{35}S]methionine. Toward the end of the labeling period, 2.5 μg of TPA per ml was added to the medium for the times indicated. Following lysis in SDS and subsequent boiling, the cell supernatants were immunoprecipitated with antiserum A (A) or preimmune serum (p.i.). (C) Half-life of protein kinase C in untreated cells. Mouse B82 cells were pulse-labeled for 15 min with [^{35}S]methionine and then chased with excess unlabeled methionine as described in Materials and Methods. At the times indicated, the cells were lysed with RIPA buffer and immunoprecipitated with antiserum A or preimmune serum (labeled p.i.). Immunoprecipitates were resolved on a 12.5% SDS-polyacrylamide gel, and proteins were detected by autoradiography. (D) Effect of TPA on the biosynthetic rate of protein kinase C. Mouse B82 cells were incubated in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 2.5 μg of TPA per ml for 18 h. The cells were then labeled for 15 min with [^{35}S]methionine and chased with unlabeled methionine for 10 min. The cells were lysed by being boiled in SDS and immunoprecipitated with antiserum A (lanes 1 and 2), antiserum B (lanes 3 and 4), or nonimmune serum (lanes 5 and 6). To aid visualization of the putative degradation products (marked with arrowheads) lanes 2, 4, and 6 contain immunoprecipitations of twice the amount of radioactivity used for lanes 1, 3, and 5. It should be noted that in these experiments, TPA did not affect the incorporation of labeled methionine into cell protein as measured by acid-precipitable radioactivity. Exposure times of enhanced 12.5% gels were 18 h for panel A, 30 h for panel B, 5 days for panel C, and 6 days for panel D. Molecular masses were calculated as for Fig. 1.

their specificity for the purified protein kinase by immunoblotting analysis. Antiserum A reacted predominantly with the 80-kDa band of the protein kinase, whereas antiserum B immunoblotted both the 80- and 78-kDa components. Immunoprecipitation from [^{35}S]methionine-labeled cells with affinity-purified serum showed the same specificities for the 80- and 78-kDa bands. The identity of the [^{35}S]methionine-labeled 80- and 78-kDa bands with protein kinase C was demonstrated by four criteria: (i) the yield of 80-kDa protein from two neuronlike cell lines paralleled their respective numbers of [^{3}H]phorbol dibutyrate-binding sites; (ii) one-dimensional peptide maps revealed close structural similarity between the 78- and 80-kDa immunoprecipitated proteins; (iii) a corresponding identity was found between maps of the [^{35}S]methionine-labeled 78- and 80-kDa proteins and silver-stained maps of the purified 78- and 80-kDa components of protein kinase C, respectively; and (iv) preabsorption of the antisera with purified protein kinase C reduced subsequent precipitation of [^{35}S]methionine-labeled 78- and 80-kDa proteins (data not shown).

This raises the critical question of whether these two forms are products of distinct mRNAs or are derived from a single primary translation product. If the latter were the case, there are at least two possible explanations for the existence of the 78/80-kDa doublet: partial proteolysis and differential phosphorylation. However, conditions of immunoprecipitation were designed to inhibit proteolysis, and boiling of lysates immediately following extraction had little effect on the proportion of 78- and 80-kDa proteins immunoprecipitated by the two antisera. In addition, if [^{35}S]methionine-labeled cell lysates were briefly heated at 30°C under conditions which should promote proteolysis, the 78/80-kDa doublet, although less intense than from lysates maintained at 0°C, had an unchanged ratio between the two bands (data not shown). The only new protein kinase C-related protein detected by immunoprecipitation was a somewhat diffuse band of 60 kDa, which may be similar to the 62-kDa band seen variably in purified enzyme preparations. Furthermore, inclusion of a variety of protease inhibitors during the immunoprecipitation procedure did not influence the ratio between the 78- and 80-kDa proteins.

There are several observations that refute the possibility that the two polypeptides of the immunoprecipitated enzyme represent different phosphorylation states of protein kinase C. (i) If the ^{32}P- and [^{35}S]methionine-labeled immunoprecipitates are compared, the specific activity of the in vivo ^{32}P-labeled 78- and 80-kDa bands appears to be similar, indicating similar amounts of phosphate are associated with each protein. Furthermore, TPA treatment of cells, while increasing the amount of phosphate associated with protein kinase C, did not alter the relative specific activity of the two bands (not shown). (ii) Treatment of in vivo ^{32}P-labeled protein kinase C with phosphatase did not affect the ratio of the two bands but did cause their dephosphorylation. (iii) Antiserum A recognized both the phosphorylated and dephosphorylated forms of the 80-kDa polypeptide in lysates

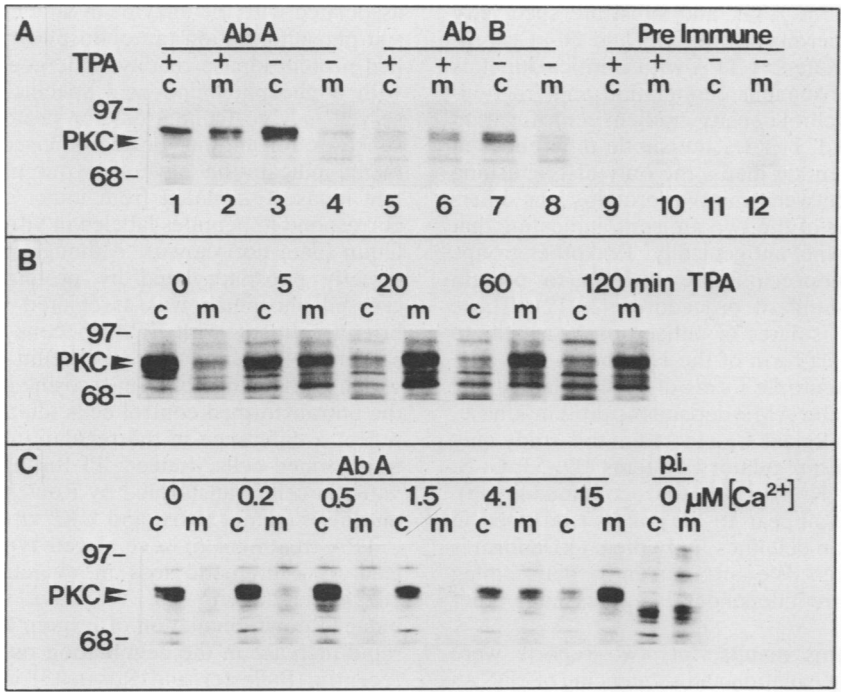

FIG. 8. Translocation of protein kinase C. (A) [^{35}S]methionine-labeled mouse B82 cells were treated with either 50 ng of TPA per ml (lanes 1, 2, 5, 6, 9, and 10) or 4-β-phorbol (lanes, 3, 4, 7, 8, 11, and 12) for 5 min. Cells were incubated in hypotonic swelling buffer in the presence of 0.5 mM EGTA and lysed as described in Materials and Methods. Lysates were partitioned into cytosol (c) and membrane (m) fractions by centrifugation and immunoprecipitated with antiserum A (lanes 1 to 4), antiserum B (lanes 5 to 8) or preimmune serum (lanes 9 to 12). (B) [^{35}S]methionine-labeled B82 cells were treated with 50 ng of TPA per ml for the times indicated and lysed and fractionated as described for panel A. The cytosol (c) and membrane (m) fractions were immunoprecipitated with antiserum A. (C) [^{35}S]methionine-labeled mouse B82 cells were lysed in the presence of Ca^{2+}-EGTA buffers as detailed in Materials and Methods and fractionated into cytosol (c) and membrane (m) components as described for panel A. Immunoprecipitation was performed with antiserum A, except for the two right-hand lanes, for which preimmune serum was used. The free Ca^{2+} concentration of each lysate as determined with a Ca^{2+}-selective electrode is given above each pair of lanes. All immunoprecipitates were resolved in 12.5% SDS-polyacrylamide gels, and radioactivity was detected by autoradiography. Exposure times of the enhanced gels were 18 h for panel A, 30 h for panel B, and 18 h for panel C. To simplify presentation, only the portions of the gels containing the protein kinase C bands are shown.

prepared in the absence of phosphatase inhibitors but recognizes neither the phosphorylated nor the dephosphorylated form of the 78-kDa protein. This demonstrates that the difference in antigenicity between the two forms is not due to phosphorylation (data not shown).

It is possible that the two forms are related by a posttranslational modification other than phosphorylation. However, by two-dimensional gel analysis, we were unable to detect any charge differences between the two species, which both exhibit multiple charge species (submitted). We were also unable to detect myristylation of protein kinase C. Both forms can be detected in a 15-min pulse-label (data not shown), which suggests either that the putative posttranslational modification occurs very rapidly or that the two forms are distinct primary translation products. This could be tested directly by immunoprecipitation of protein kinase C from in vitro translation products of RNA derived from sources expressing both forms of the enzyme.

Previous enzymological and structural studies showed that the 78/80-kDa doublet of the purified protein was not generated by either partial proteolysis or differential phosphorylation (submitted). Furthermore, like the immunoprecipitated protein, the purified enzyme generated a faster-migrating doublet of 74/76 kDa upon treatment with potato acid phosphatase, indicating that it too contained phosphate (submitted). In this paper, we show that the 80- and 78-kDa species of protein kinase C purified from rat brain are highly related, if not identical, to two forms of protein kinase C that can be immunoprecipitated from a variety of cell lines. Together, these data provide a strong argument for the physiological presence in mammalian cells of two distinct forms of protein kinase C, which we suggest may be true isozymes.

While most previous reports have described only one form of this enzyme in mammalian tissues, Turgeon et al. (60) noted two peaks of enzyme activity upon DEAE-cellulose chromatography of rat and sheep anterior pituitary gland. Although it is presently unclear why certain purification schemes yield only one polypeptide, these preparations either require 4 to 7 days for completion or involve exposure of the enzyme to Ca^{2+} (and hence Ca^{2+}-dependent proteases) at early stages (30, 38, 51, 62). In contrast, the scheme used here to purify protein kinase C is completed within 20 h, and the 78/80-kDa doublet is apparent prior to exposure to Ca^{2+}, which occurs during the last purification step (submitted).

The possible function of multiple forms of this enzyme is as yet unknown. While not separable by nondenaturing techniques, the purified 78- and 80-kDa proteins did not exhibit any significant differences in enzymatic properties when jointly assayed compared with preparations of protein kinase C purified by the methods that yield only one polypeptide upon SDS-polyacrylamide gel electrophoresis (30, 38, 51, 62). Such parameters include K_a for Ca^{2+} and

phosphatidylserine, K_m for ATP, and substrate specificity (data not shown). Furthermore, both 78- and 80-kDa proteins were similarly regulated by TPA with identical kinetics of translocation and subsequent degradation and fractionated identically upon glycerol density gradient centrifugation (data not shown). Indeed, besides the subtle differences in molecular weight and peptide maps, the only distinguishing property we observed between the two proteins was differential recognition by one of the two antisera, indicating that the proteins differ somewhat antigenically. Two other groups have generated immunoprecipitating antisera to protein kinase C purified by standard procedures (3, 17). These antisera, like the initial isolates of antiserum A, appear to recognize only the 80 kDa-form of the enzyme.

By analogy with the multiple forms of the regulatory and catalytic subunits of cyclic AMP-dependent protein kinase, the two forms of protein kinase C detected in this study may interact differentially with regulatory systems (39, 53; G. S. McKnight and E. G. Krebs, personal communication). While both components appear to be jointly expressed in several mouse and human cell lines, only the 80-kDa form is present in ANN-1 cells. We are currently determining whether the two forms are independently expressed in other cell lines and tissues.

During revision of this manuscript, two reports were published concerning the isolation and sequencing of cDNAs of protein kinase C (12, 33). These reports support our assertion of the existence of multiple forms of protein kinase C, since the two groups found that in bovine, human, and rat brain cDNA libraries, there were at least three distinct representations of protein kinase C cDNA. The autonomy of the three human forms was confirmed by their distinct chromosomal locations (12). The different forms were very highly related, exhibiting >90% identity, and differences were clustered in discrete "variable" regions. Preliminary data revealed that the forms were differentially expressed in regions of the brain and other tissues, and genomic DNA analysis suggested the existence of additional genes (12, 33). We are currently determining the relationship between the two forms of protein kinase C reported here and the forms predicted to exist from the cDNA analysis.

Girard et al. (18, 19) have recently described antisera that detect protein kinase C by Western blotting analysis. These antisera recognize a minor band of 80 kDa and major bands of approximately 67 and 50 kDa in various cell extracts. The authors argued that the lower-molecular-weight components may represent proteolyzed forms of protein kinase C, the smallest of which might correspond to a 50-kDa form of the enzyme that can be generated in vitro, termed M-kinase (31). Indeed, a physiological role for M-kinase, the activity of which is Ca^{2+} and phospholipid independent in vitro (31), has been proposed in the mediation of signals caused by PI turnover or TPA from the plasma membrane to intracellular compartments (22, 23, 45, 46, 56–58). Our antisera recognize proteolytic fragments of protein kinase C of 50 to 75 kDa generated in vitro from the purified protein (data not shown). Although we have detected putative degradation products of the enzyme by pulse-labeling after TPA treatment, these polypeptides do not accumulate and are rapidly degraded to fragments not recognized by our antisera. Thus if M-kinase has a physiological role in vivo, it would appear to be of extremely transient nature. Furthermore, other groups have failed to detect changes in the amount of M-kinase activity after TPA or hormonal activation of protein kinase C in vivo (10, 61).

Protein kinase C is a phosphoprotein in vivo. Phosphate associated with the enzyme in intact cells may be a result of self-phosphorylation (autophosphorylation), since the purified protein kinase readily undergoes this reaction in vitro with a phosphoamino acid specificity similar to that observed in vivo (submitted), or protein kinase C may be a substrate for another protein kinase(s). Preliminary experiments indicate that several tryptic phosphopeptides of protein kinase C isolated from intact cells treated with TPA correspond to peptides labeled in vitro upon autophosphorylation (data not shown). Although protein kinase C is not directly phosphorylated by protein-tyrosine kinases, increased phosphate was associated with the enzyme after brief treatment with TPA in cells transformed by Rous sarcoma virus and Snyder-Theilin feline sarcoma virus, which both encode protein-tyrosine kinases, compared with the untransformed control cells (data not shown). This may reflect a difference in the regulation of protein kinase C in transformed cells. Indeed, PI turnover appears to be activated in cells transformed by Rous sarcoma virus, Abelson murine leukemia virus, and UR2 virus (13, 17, 40).

TPA treatment of several cell types has been shown to lead to a dramatic loss in detectable protein kinase C enzymatic activity (2). We have shown that this TPA-induced down-regulation of protein kinase C is caused by a rapid increase in the degradation rate of the enzyme. Very recently, Ballester and Rosen (3) also reported that TPA causes the loss of immunoprecipitable protein kinase C in a time- and dose-dependent manner. In this case, however, pituitary GH_3 cells were pulse-labeled with [^{35}S]methionine for 6 h and then chased during treatment with TPA. Thus, only the fate of presynthesized protein kinase C molecules was determined. In contrast, we labeled cells before and during TPA treatment and thus monitored all molecules of protein kinase C synthesized during the labeling and treatment period. After 18 h of TPA treatment, immunoprecipitable protein kinase C was virtually undetectable, suggesting that the effect of TPA on the degradation rate is persistent and is probably reversed only upon removal of this activator. TPA did not significantly affect the rate of synthesis of protein kinase C, however, even though the rates of synthesis of several other gene products are affected by this agent (52).

Several reports have recently documented that TPA and various agonists of PI turnover cause translocation of protein kinase C from the cytosol to the membrane fraction. All of these studies determined the levels of protein kinase C in fractions from cells lysed in the presence of EGTA (0.5 to 10 mM) by assaying protein kinase activity (15, 16, 35, 47, 48, 61), except in one case, when immunoprecipitation was used (3). Because of the problems inherent in measuring protein kinase C activity accurately in crude cell fractions, we determined the distribution of protein kinase C by immunoprecipitating [^{35}S]methionine-labeled cell fractions in the presence or absence of Ca^{2+}. We found that the subcellular localization of protein kinase C in unstimulated cells is dependent on the intracellular Ca^{2+} concentration, with half-maximal association with membranes observed at 4 μM Ca^{2+}. Although this is higher than the Ca^{2+} concentration in resting cells, such levels may be achieved during elevated Ca^{2+} flux, particularly from extracellular sources (see below). A Ca^{2+}-dependent translocation of protein kinase C would have not have been detected in earlier studies owing to the use of EGTA, which disrupts this association. The affinity of the enzyme for phospholipids and the presence of low but finite concentrations of Ca^{2+} may be important in maintaining the protein in close proximity to

membranes for maximum rapidity of response to agents which stimulate PI turnover.

Presumably, DAG or phorbol esters enhance the Ca^{2+}-induced binding such that the binding becomes resistant to Ca^{2+} chelators. Since the protein kinase is extractable by detergents only in the presence of these activators, the enhanced binding is probably hydrophobic in nature. Our results on the localization of protein kinase C, as determined by direct detection of the protein, are in basic concordance with those of Wolf et al. (63, 64), who showed that Ca^{2+} induced purified protein kinase C to associate reversibly with erythrocyte ghost membranes in the absence of activators, although the membrane-bound enzyme was in an inactive state. The Ca^{2+} concentrations required for this association, however, were 10-fold lower than those observed in this study. Because the Ca^{2+} requirement of purified protein kinase C is dependent on phospholipid levels (32), our determination of the Ca^{2+} requirement for translocation is possibly an overestimate, since the effective phospholipid concentration in our experiments was very low. One role for Ca^{2+} might be to potentiate activation by DAG. Indeed, Wolf et al. (63) reported that subsequent addition of TPA synergized with Ca^{2+} to promote binding and cause activation of the protein kinase. A similar synergism between Ca^{2+} and phorbol esters was noted for platelet activation and mitogenic response of lymphocytes (27, 28). Very recently, Dougherty and Niedel (14) showed that elevation of intracellular Ca^{2+} increases the affinity of protein kinase C for phorbol esters. Therefore, Ca^{2+} released from intracellular stores by one of the products of PI turnover, inositol trisphosphate, acts to sensitize the protein kinase for rapid response to the transient and limited production of DAG, the second product of this signal transduction system. Similarly, influx of extracellular Ca^{2+} might ensure restriction of protein kinase C molecules to the membrane environment, thus priming the enzyme for activation by other agonists.

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