

Isolation and Characterization of Protein Kinase C from Y-1 Adrenal Cell Cytoskeleton

Vassilios Papadopoulos and Peter F. Hall

Department of Endocrinology, The Prince of Wales Hospital, Randwick, N.S.W. 2031 Australia

Abstract. The cytoskeletons of Y-1 mouse adrenal tumor cells contain a calcium and phospholipid-dependent protein kinase (protein kinase C) that is bound sufficiently tight to resist extraction by 0.5% Triton but not by 1.0% Triton. The enzyme has been purified to near homogeneity from cytoskeleton and cytosol. It shows features typical of this type of kinase, namely a requirement for Ca^{2+} and phospholipid, stimulation by tumor promoters but not by nontumor-promoting phorbol esters, and inhibition by trifluoperazine. The enzyme shows specificity for four substrates found in the cytoskeleton, namely 80, 33, 20, and 18 kD. The first three substrates are phosphorylated by the enzyme; the fourth is dephosphorylated and is therefore affected by the kinase indirectly. The 80-kD protein is the kinase enzyme itself which is autophosphorylated in vitro and in the cytoskeleton. The 20-kD protein is myosin light chain. The 33- and

18-kD proteins are unidentified. The same substrates were phosphorylated when Y-1 cells were permeabilized with digitonin and incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and phorbol-12-myristate-13-acetate. Partly purified protein kinase C changes the extent of phosphorylation of the same substrates when added to cytoskeletons previously extracted to remove endogenous protein kinase C. Addition of Ca^{2+} , phosphatidylserine, and phorbol-12-myristate-13-acetate to cytoskeletons, and addition of these three agents plus protein kinase C to extracted cytoskeletons, causes these structures to undergo a rapid and extensive rounding. A similar change is induced in intact cells by addition of phorbol ester. It is concluded that protein kinase C is capable of changing the shape of adrenal cells by an action that involves autophosphorylation and phosphorylation of myosin light chain. This response may in turn be related to the steroidogenic responses to ACTH and cyclic AMP.

PROTEIN kinase C is known to occur in at least three types of adrenocortical cells (35, 36). Attempts to determine whether or not this enzyme is involved in the regulation of steroid synthesis in these cells have given equivocal results (36). Since the cytoskeleton of adrenal cells is involved in the steroidogenic responses to ACTH and cyclic AMP (8, 22, 27), it was decided to determine whether protein kinase C is present in the cytoskeletons of adrenocortical cells. For this purpose we have used Y-1 mouse adrenal tumor cells which serve as a useful system for studies of the regulation of adrenal steroidogenesis (16). We show here that protein kinase C is present in the cytoskeletons of these cells.

Materials and Methods

Preparation and Culture of Cells

Methods for the culture of Y-1 cells in Ham's F12/DMEM (1:1 [vol/vol]) supplemented with 10% FCS have been given previously (21, 22). 1 d before each experiment, the medium was replaced by serum-free medium. Steroidogenic responses of the cells to ACTH were determined by measuring the production of 20α -dihydroprogesterone (21, 22).

Preparation of Cytoskeleton

Cells were scraped from the dishes into buffer A: NaCl (100 mM), sucrose (300 mM), MgCl_2 (3 mM), Pipes (10 mM, pH 6.8), Triton X-100 (0.5%),

PMSF (1.2 mM), and aprotinin (Trasyol; 100 U/ml). Pancreatic deoxyribonuclease I (400 $\mu\text{g}/\text{ml}$) and pancreatic ribonuclease A (400 $\mu\text{g}/\text{ml}$) were then added for 15 min at room temperature. $(\text{NH}_4)_2\text{SO}_4$ was added to a final concentration of 0.1 M and incubation continued for 5 min at room temperature. The "soluble" and "chromatin" fractions were then removed as a supernatant after 10 min centrifugation at 10,000 g leaving the cytoskeletons as a pellet. In some experiments the cytoskeletons were extracted with buffer A containing 1% Triton X-100 (vol/vol) or 0.5 M KOH after preparation by the above method. In some cases cytoskeletons were washed twice with buffer A. In other experiments the detergent Triton X-100 was replaced by *n*-octyl- β -D-glucopyranoside (octylglycoside) in the preparation of the cytoskeleton.

The above extraction procedures consisted of stirring the sample for 5 min on ice. To examine the shapes of cytoskeletons, these structures must be prepared from cells in situ; i.e., on culture dishes. Such preparations are not biochemically comparable with the structures prepared as described above, for example nuclei remain. The method used to prepare such cytoskeletons in situ is given elsewhere (28). The more stringent procedure used in most of the present studies requires that the cells be removed from culture dishes and that nuclear material is digested (see above).

Measurement of Protein Kinase C

Protein kinase C was measured by determining the incorporation of $[\text{}^{32}\text{P}]$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into exogenous histone H-1 (33). Before use in these experiments, the cytoskeleton was washed twice for 5 min on ice with buffer B: Pipes (50 mM), MgCl_2 (10 mM), EDTA (2 mM), EGTA (2 mM), PMSF (1.2 mM), and aprotinin (100 U/ml) final pH 7.0. These washes removed calmodulin to undetectable levels, which was desirable to avoid activation of Ca^{2+} -calmodulin-dependent protein kinase (37). The cytoskeletons were homogenized with five passes of a tight-fitting glass-glass Dounce

homogenizer and incubated in a total volume of 100 μ L using a protein concentration of 1.0 mg/ml with various additions (including 20 μ g of histone H-1) where indicated. The reaction was started by addition of [γ - 32 P]ATP (10 nmols, 10^6 cpm per tube).

This concentration of ATP was found to produce maximal incorporation of 32 P into cytoskeletal substrates since greater concentrations of the same specific activity did not influence the degree of phosphorylation of the various substrates (not shown). If the [γ - 32 P]ATP was diluted with further addition of ATP, 32 P incorporation into substrates was decreased. The reaction was continued for 1 min at 37°C followed by 3 min at room temperature and stopped by addition of ice-cold TCA (1 ml of 12.5% [wt/vol]). The mixture was stood on ice for 3 h and the precipitate was washed three times with TCA (12.5%) and once with ethyl ether. The precipitate was solubilized in aquasol-2 (1 ml). Triton-toluene scintillation fluid (5 ml) was added and the samples were examined by liquid scintillation spectrometry to measure 32 P. Values for unincubated (zero time) samples were found to be in the range of 300 to 500 cpm/tube. These values were subtracted from the experimental values.

For the phosphorylation of endogenous proteins, the method described above was followed except that exogenous histone was omitted and the reaction was stopped by addition of hot sample buffer in preparation for one-dimensional gel electrophoresis by the method of Laemmli (17) or two-dimensional gels (focusing \rightarrow electrophoresis) by the method of O'Farrell (26). Cytoskeletons were solubilized for one-dimensional gels by repeated pipetting in sample buffer followed by boiling for 5 min.

Samples (50 μ g) were applied to a 5–15% linear gradient of polyacrylamide with a 4% stacking gel. For the method of O'Farrell (26), 100 μ g protein was applied in the first dimension and the acrylamide gradient in the second dimension was as described above. Autoradiography was performed for 12–24 h at -70°C on X-OMAT AR film (Eastman Kodak Co., Rochester, NY) in the presence of Cronex Lighting-Plus (DuPont Co., Wilmington, DE) intensifying screens. Bands were cut from the gels and incubated with 0.5 ml of H_2O_2 (30% [vol/vol]) for 12 h at 60°C. 32 P was measured by liquid scintillation spectrometry as described above.

Purification of Protein Kinase C

Cytoskeleton. Cytoskeletons were prepared from 45×10^8 Y-1 cells. Cytoskeletons were washed with buffer I: Tris-HCl (20 mM), EDTA (2 mM), EGTA (5 mM), PMSF (50 μ g), and aprotinin (100 U/ml), final pH 7.4, and containing 1.0% Triton X-100 (vol/vol) and homogenized in the same buffer. The homogenate was centrifuged at 20,000 g for 10 min at 4°C. The supernate was diluted 10 times with buffer I and applied to a column (1 \times 5 cm) of DEAE-Sephacel equilibrated with buffer II: Triton X-100 (0.1% [vol/vol]), Tris-HCl (20 mM), EDTA (2 mM), EGTA (2 mM), final pH 7.4). The column was washed with 40 ml of buffer II followed by 40 ml of a linear gradient 0–400 mM NaCl in Buffer II. Fractions containing protein kinase C were pooled and dialyzed against the following buffer: MES (10 mM), CaCl_2 (2 mM), leupeptin (4 μ g/ml), final pH 6.5. The sample was applied to a column (1 \times 10 cm) composed of phosphatidylserine immobilized in polyacrylamide (33, 34) equilibrated in buffer III: MES (10 mM), NaCl (200 mM), 2-mercaptoethanol (0.1% [wt/vol]), and leupeptin (4 μ g/ml) containing CaCl_2 (5 mM), final pH 6.5. The column was washed with 20 ml of buffer III containing 0.1 mM CaCl_2 followed by buffer III containing 2 mM EGTA. Fractions (1 ml) containing protein kinase C were pooled and concentrated–dialyzed with an Amicon micropartition system (MPS-1) against buffer III containing 100 mM NaCl and 10% (wt/vol), glycerol. The highly purified protein kinase C was stored at -20°C .

Cytosol. The procedure used for purification of the cytosolic enzyme was similar to that just described except as follows. Cells from 10 150-mm plates (2×10^8 cells) were washed three times with ice-cold Tris-HCl (20 mM, pH 7.4) and homogenized in buffer I containing sucrose (0.25 M) using a glass/glass tight-fitting homogenizer (Dounce, Vineland, NJ). The homogenate was centrifuged (105,000 g/60 min per 4°C) and the supernate was directly applied to the DEAE-Sephacel column. The column was washed with buffer II without Triton X-100 and fractions containing the protein kinase were pooled and desalted on a column of PD10 Sephadex G25. The sample was divided into two parts one of which was used as partly purified (crude) protein kinase C in studies with the cytoskeleton and the remainder was purified as described above. For the latter purpose the sample was taken to MES (10 mM), CaCl_2 (2 mM), and leupeptin (4 μ g/ml), final pH 6.5, and applied to the affinity column described above.

Fractions collected during purification were subjected to an assay for protein kinase C as follows. Fractions were incubated at 37°C with 200 μ g/ml histone H-1, 10 U/ml protein kinase inhibitor, [γ - 32 P]ATP (10 nmols; 10^6 cpm) with and without 2 mM CaCl_2 , 10 μ g/ml phosphatidylserine, and

100 nM PMA. Incubation was started by addition of ATP and continued as described above. Incorporation of 32 P into protein was measured as described above. Activity of protein kinase C was expressed as pmols of 32 P incorporated/min per mg protein calculated by subtracting zero time values from those obtained with incubated samples.

Reconstitution of Cytoskeleton plus Protein Kinase C

Cytoskeletons were extracted with buffer A containing Triton X-100 (1% [vol/vol]). To the extracted cytoskeletons (50 μ g protein) partly purified protein kinase C (50 μ g/ml) and [γ - 32 P]ATP (10 nmol, 10^6 cpm per tube) in buffer B were added. The reaction was stopped by addition of 0.5 ml of the following solution: 0.1 mM NaF, 20 mM EDTA, 2 mM EGTA, 2 mM sodium pyrophosphate, pH 7.4, at 4°C. The mixture was centrifuged at 12,000 g for 15 min at 4°C to remove any soluble phosphoprotein in the partly purified protein kinase C. The resulting pellet was resuspended in Laemmli buffer and subjected to one-dimensional gel electrophoresis followed by autoradiography.

Partial Proteolytic Mapping

Cytoskeletal protein (100 μ g) and highly purified protein kinase C from cytoskeleton and cytosol (2 μ g each), were incubated with [γ - 32 P]ATP and cofactors as described above. The samples were subjected to electrophoresis on polyacrylamide gels (15%) with sodium dodecyl SO_4 . Bands corresponding to molecular mass 80 kD were excised and incubated separately with *Staphylococcus aureus* V₈ protease as described elsewhere (5). Samples were then subjected to one-dimensional gel electrophoresis a second time followed by autoradiography.

Permeabilization of Cells with Digitonin

Intracellular phosphorylation in adrenal cells was measured by permeabilization of cells with digitonin as described by Lee and Holz (18). Cells were treated for 30 min with or without phorbol-12-myristate-13-acetate (100 nM) followed by incubation with 20 μ M digitonin and [γ - 32 P]ATP (50 μ Ci/dish) for 10 min in the presence and absence of phorbol-12-myristate-13-acetate (100 nM). The experiment was terminated by preparation of the cytoskeleton as described above. In some experiments, cells were first permeabilized by incubation with 20 μ M digitonin and then subsequently incubated with [γ - 32 P]ATP in presence or absence of the phorbol ester.

Miscellaneous

Protein was measured by the method of Bradford (3) with BSA as standard. ATP was measured on cytoskeletons by precipitating protein with 10% TCA (wt/vol) followed by extraction with ether and column chromatography as described by Dzandu and Johnson (7). ATP was measured on cells by freeze-thawing followed by sonication for 20 s at 0°C. Protein was precipitated and assays were performed as described for cytoskeletons. One-dimensional electrophoresis on polyacrylamide gels with sodium dodecyl SO_4 was performed as described by Laemmli (17) and two-dimensional gels (isoelectric focusing \rightarrow electrophoresis) by the method of O'Farrell (26). Gels were stained either with Coomassie Blue or with silver nitrate according to Morrissey (20). Cells and cytoskeletons were examined under phase contrast using an inverted Nikon Photomicroscope. The following standard proteins were used for gel electrophoresis: myosin (200 kD), β -galactosidase (116 and 25 kD), phosphorylase B (92.5 kD), BSA (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soyabean trypsin inhibitor (21.5 kD), lysozyme (14.4 kD), and bovine insulin monocomponent (6 kD). The isoelectric focusing standards used were: phycocyanin (Pi, 4.65), β -lactoglobulin (Pi, 5.10), bovine carbonic anhydrase (Pi, 6.00), human carbonic anhydrase (Pi, 6.50), equine myoglobin (Pi, 7.00), whale myoglobin (Pi, 8.05), a chymotrypsin (Pi, 8.80), and cytochrome C (Pi, 9.60).

Materials

The following substances were purchased from Sigma Chemical Company (St. Louis, MO): octylglycoside, PMSF, deoxyribonuclease I, ribonuclease A, protein kinase inhibitor, *Staphylococcus aureus* V₈ protease, phosphatidylserine, phorbol 12-myristate-13-acetate, 4 α -phorbol-12,13-didecanoate, mezerein, porcine ACTH (71 U/mg), lysine-rich histone (type VS corresponding to histone H-1), trifluoperazine hydrochloride, myosin light chain from bovine muscle, and reagents for silver staining of gels. The following materials were purchased from various companies: culture media (Filtron),

FCS (Flow Laboratories, Inc., McLean, VA), Triton X-100 and electrophoresis reagents (Bio Rad Laboratories, Richmond, CA), acrylamide, bis-acrylamide (LKB Instruments, Gaithersburg, MD), [γ - 32 P]ATP (3,000 Ci/mmol, lot No. 8732, 8803, and 8809 from Amersham Corp. [Arlington Heights, IL], lot No. 2526-294 from New England Nuclear, Boston, MA), DEAE-Sephacel and PD10-Sephadex G25M (Pharmacia Fine Chemicals, Piscataway, NJ). Water was filtered with milli Q (Milipore Continental Water Systems, Bedford, MA). All buffers were filtered through filters (model No. 1; Whatman Inc., Clifton, NJ).

Results

Properties of Y1 Cell Protein Kinase C

Assay for protein kinase C performed on Y1 cell cytoskeleton showed the presence of a typical Ca^{2+} -phospholipid-dependent kinase in these structures (Table I). The cofactor requirements for the cytoskeletal enzyme are those of a typical protein kinase C in that it requires Ca^{2+} and phospholipid and is stimulated by phorbol-12-myristate-13-acetate. One phorbol ester that does not promote tumors (4 α -phorbol-12,13-didecanoate) does not increase protein kinase C activity and trifluoperazine inhibits the response of the enzyme to phorbol ester. The nonphorbol tumor promoter mezerein stimulates the kinase. It should be added that treatment of cells with ACTH before preparing cytoskeletons was without effect on the activity of this cytoskeletal enzyme (data not shown). It can be seen from Table II that protein kinase C is readily removed from the cytoskeleton by washing twice with buffer A (which contains 0.5% [vol/vol] Triton X-100) or by Triton X-100 (1% [vol/vol]) or by high salt. It is important to notice that the milder detergent octylglycoside does not remove the kinase from the cytoskeleton (Table II). None of the extracting agents shown in Table II inhibits protein kinase C when added in the concentrations shown to either cell homogenate or cytoskeleton (not shown). Experiments identical to those shown in Tables I and II were repeated and the values closely resembled those shown in the tables. In numerous experiments we have seen no change in the total amount of the enzyme on treating adrenal cells with phorbol ester.

Measurement of ATP in Cytoskeletons of Y1 Cells

When cytoskeletons were prepared by the modification that permits light microscopy of these structures on the culture

Table I. Properties of Protein Kinase C from Y1 Cell Cytoskeletons

Addition	Enzyme activity <i>pmol/min per mg protein</i>
None	5.5 \pm 0.5
Ca^{2+}	8.5 \pm 0.5
Ca^{2+} , PS	14.0 \pm 1.0
Ca^{2+} , PS, PMA	22.0 \pm 2.0
Ca^{2+} , PS, PDA	11.5 \pm 0.7
Ca^{2+} , PS, PMA, TFP	12.5 \pm 1.3
Ca^{2+} , PS, MEZ	20.0 \pm 1.8

Samples of cytoskeleton from Y-1 cells were subjected to an assay for protein kinase C based on phosphorylation of histone (Materials and Methods). Ca^{2+} , CaCl_2 (2 mM); PS, phosphatidylserine (10 $\mu\text{g/ml}$); PMA, phorbol-12-myristate-13-acetate (100 nM); PDA, 4 α -phorbol-12,13-didecanoate (100 nM); and MEZ, Mezerein (100 nM).

Table II. Extraction of Protein Kinase C from Cytoskeletons of Y-1 Cells

Extraction medium	[32 P]Incorporated <i>pmol/min per mg protein</i>
None (no cofactors)	8.0 \pm 1.0
None	21.0 \pm 3.0
Octylglycoside (0.5% [wt/vol])	22.0 \pm 2.5
Octylglycoside (1%)	18.0 \pm 1.5
Extraction buffer (2 \times)	11.5 \pm 0.5
Triton X-100 (1% [vol/vol])	6.5 \pm 0.5
KCl (0.5 mM)	6.5 \pm 1.5

The experiment was performed as described in Table I. All tubes (except *no cofactors*) contained CaCl_2 (2 mM), phosphatidylserine (10 $\mu\text{g/ml}$), and phorbol-12-myristate-13-acetate (100 nM). Where indicated, cytoskeletons were treated with the extraction buffers shown. 2 \times refers to two successive extractions (5 min on ice) before centrifugation and measurement of protein kinase C. The detergents were added in buffer A (Materials and Methods).

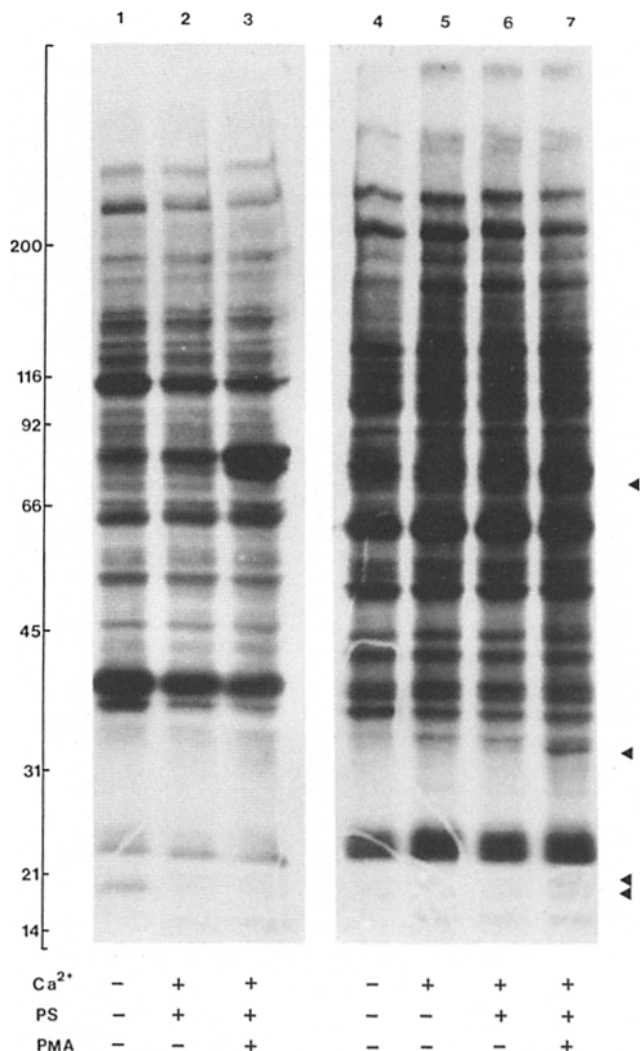


Figure 1. Autoradiograms of one-dimensional gel electrophoresis of phosphorylated cytoskeletons from Y-1 cells. Cytoskeletons of Y-1 cells were prepared with octylglycoside (0.5% [wt/vol]; lanes 1-3) or Triton X-100 (0.5% [vol/vol]; lanes 4-7; Materials and Methods). The washed cytoskeletons were subjected to conditions used to determine phosphorylation of endogenous substrates for PKC (Materials and Methods). After incubation proteins were separated by gel electrophoresis followed by autoradiography.

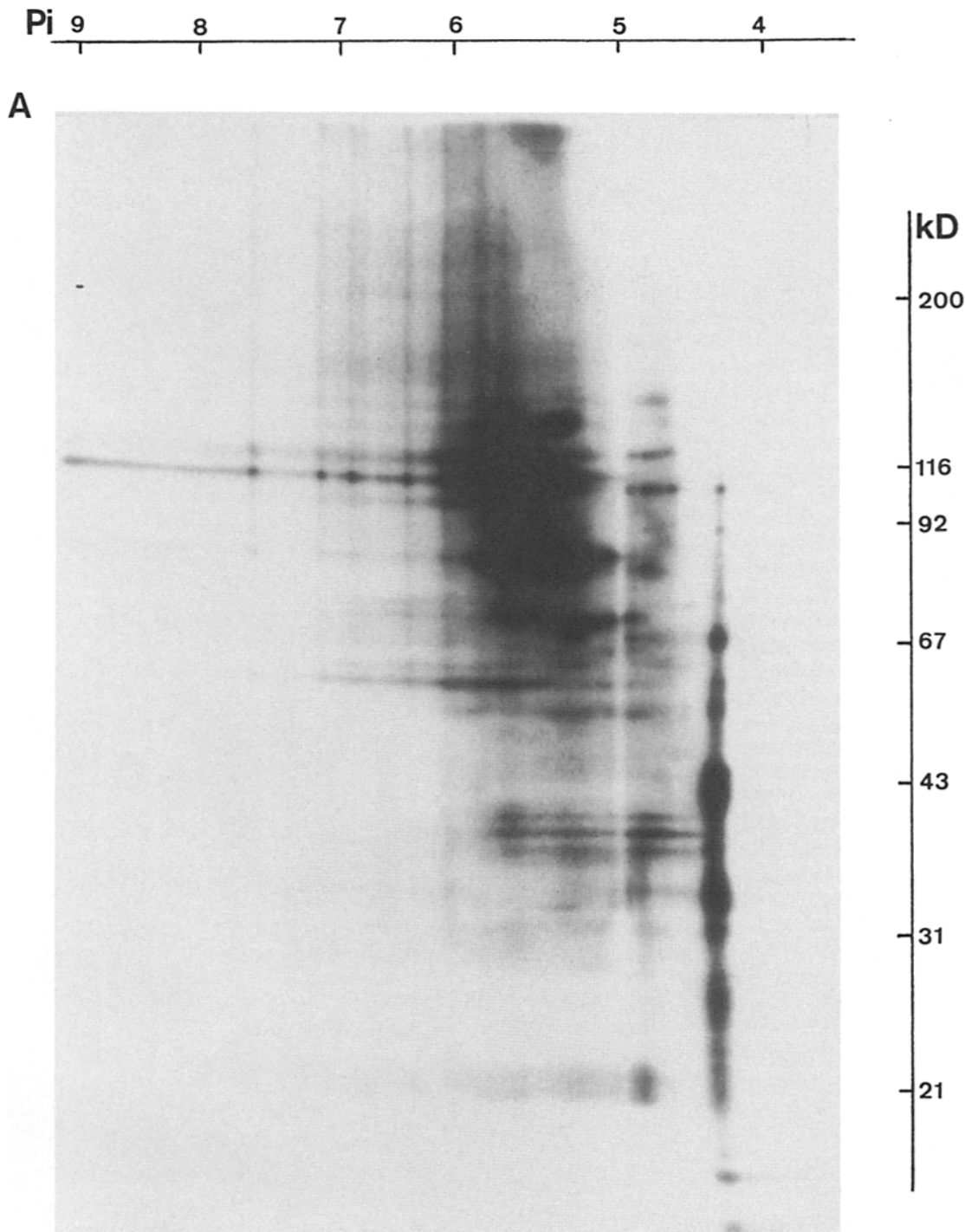
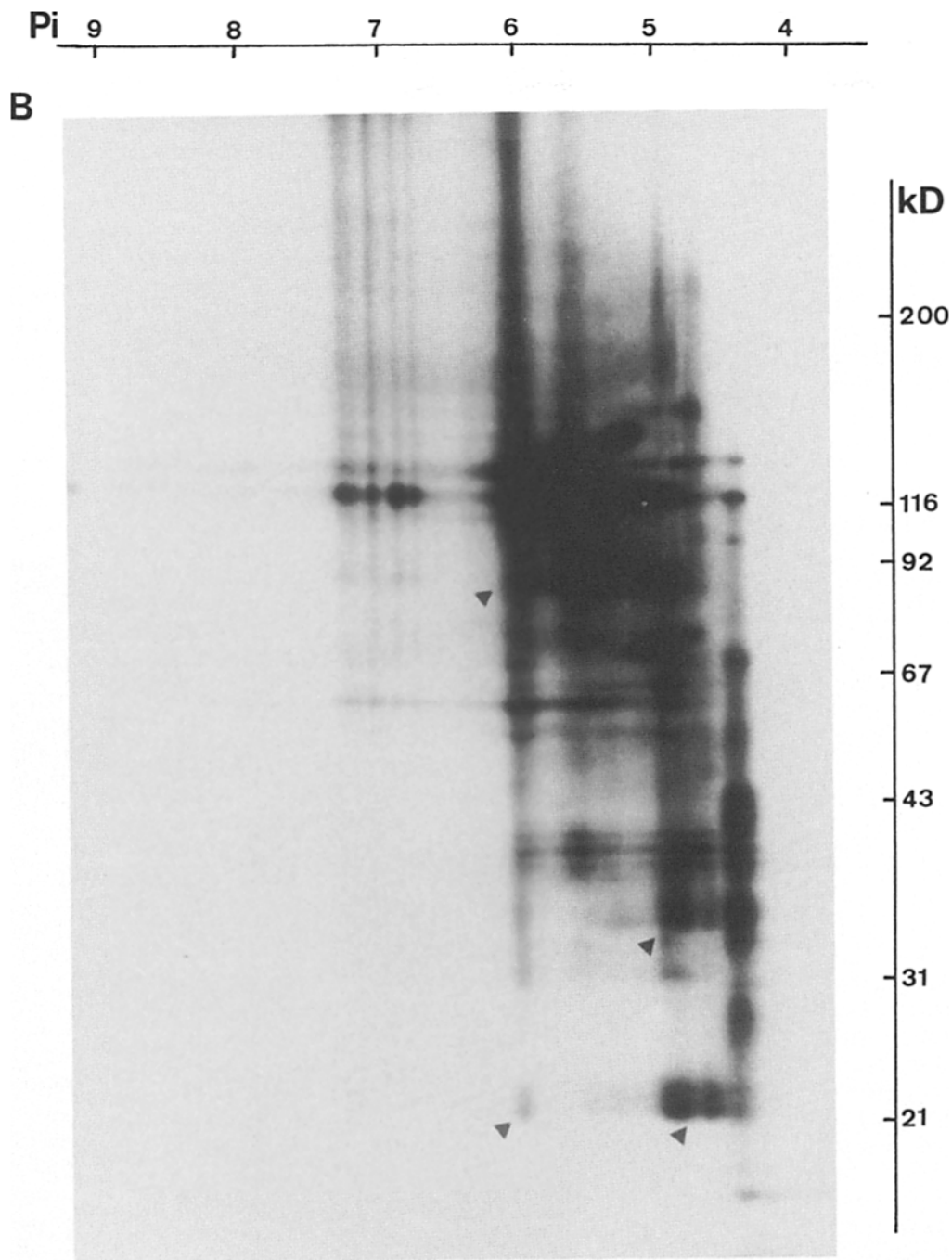


Figure 2. Autoradiograms of two-dimensional gel electrophoresis of phosphorylated cytoskeletons from Y-1 cells. Cytoskeletons of Y-1 cells were prepared with Triton X-100 (0.5% [vol/vol]; Materials and Methods). The washed cytoskeletons were subjected to conditions used to determine phosphorylation of endogenous substrates for PKC (Materials and Methods) without (A) and with (B) cofactors.

dishes, ATP is found when measured as described in Materials and Methods. These cytoskeletons contain 50 pmol of ATP per 10^6 cytoskeletons compared with 440 pmoles per 10^6 whole cells. Cytoskeletons prepared by the routine procedure contain <1 pmole in cytoskeletal material from 10^6 cells.

Distribution of Protein Kinase C in Y-1 Cells

When the amount of protein kinase C activity was determined by enzyme assay with histone H-1 in the cytoskeletons and whole cell extracts of Y-1 cells, it was found that cytoskeletons contained 0.4 ± 0.09 U of activity (pmoles of ^{32}P incorporated into histone H-1/min per 10^6 cells) and whole



cell extract contained 5.8 ± 1.1 U (means and ranges of three determinations); i.e., the cytoskeleton contains at least 10% of total cell protein kinase C. To obtain these values, extracts were purified by DEAE-Sephacel to remove inhibitors before assay.

Phosphorylation of Cytoskeletal Proteins by Endogenous Protein Kinase C

Fig. 1 shows an autoradiogram of Y-1 cytoskeletal proteins after incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ followed by gel electrophoresis in one dimension. Lanes 1-3 show the phosphorylation of proteins when the cytoskeleton is first prepared with

octylglycoside. Three proteins are more phosphorylated with all three cofactors than in the control (no cofactors), namely 80, 33, and 20 kD. One protein (18 kD) is less phosphorylated with the cofactors present. Phosphorylation of the three proteins and dephosphorylation of 18 kD are seen but less clearly with Ca^{2+} and phosphatidylserine but without phorbol ester than with all three agents. When the cytoskeleton is prepared with Triton X-100 (0.5%, [vol/vol]; Lanes 4-7), changes in phosphorylation of the four proteins just described were somewhat reduced (Fig. 1). When an identical study was performed with cytoskeletons prepared from cells treated with ACTH, the results were indistinguishable from those shown in Fig. 1 (not shown).

To examine the phosphorylation of these proteins in greater detail, we have used two-dimensional gels of cytoskeletons from cells incubated with and without phorbol ester (Fig. 2). It will be seen that the following proteins are more phosphorylated with phorbol ester than without, namely 80 (Pi, 5.9), 35 (Pi, 4.9), and three proteins of 20 kD (Pi's, 5.9, 4.9, and 4.4). Two of the 20-kD proteins were not phosphorylated without phorbol ester (\blacktriangle in Fig. 2 B).

Purification of Protein Kinase C from Y-1 Cell Cytoskeletons

Protein kinase C was purified from cytoskeletons of Y-1 cells (Table III). It will be seen that purification of 2,400-fold was achieved if values for the assay used (Materials and Methods) are taken at face value. It is, however, well-known that inhibitors of protein kinase C are found in various cells (23, 31). Insufficient material was available to purify the enzyme (e.g., by gel chromatography) before assay at each step. However, specific activity increased throughout purification while yield decreased. Moreover, assay conditions provided saturating levels of Ca^{2+} , phospholipid, and phorbol ester at all steps. In the first step of the purification procedure, protein kinase C was eluted from DEAE-Sephacel (Fig. 3, left; Materials and Methods). Fig. 3, right shows purification of this enzyme by the second step (affinity chromatography). The highly purified enzyme was eluted from the phospholipid affinity column as a single peak after addition of EGTA. The purity of the enzyme is shown by gel electrophoresis using a highly sensitive silver stain (Fig. 4). Fig. 4 A shows a minor contaminant (77 kD) with trace contaminants at 60–62 kD and at 33–35 kD. The 77 kD protein could be an isoform of the protein kinase C (39). Moreover, the highly purified protein is phosphorylated by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as shown in the accompanying autoradiogram (Fig. 4 B, lane +). Some phosphorylation is seen without added cofactors (Fig. 4 B, lane -). Presumably some tightly bound divalent metal ion may be responsible for this activity or a small degree of activity may be possible without cofactors. Such a phenomenon has been previously reported with protein kinase C eluted from this type of affinity column (34). Faint phosphorylation was also seen at 60–62 kD consistent with the presence of a band of this molecular mass on silver staining of polyacrylamide gels (see above). The amount of purified protein was too small for further purification to remove the trace contaminants that may be proteolytic fragments of the

80-kD kinase (19) or possibly small amounts of phosphatidylserine-binding proteins removed by the affinity column (34). Fig. 4, C and D shows autoradiograms of two-dimensional gel electrophoresis of the pure cytoskeletal enzyme incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. As with one-dimensional gels (Fig. 4 B), some phosphorylation is seen without added cofactors. With cofactors (Fig. 4 D) more intense phosphorylation is seen showing two spots of the same molecular mass (80 kD) $\sim\text{pH}$ 6.0. In some of our two-dimensional gels trace phosphoprotein was seen at 80 kD and pH 4.2 (Fig. 4 D). This may represent small amounts of an 80K substrate of PKC (2) or more acidic form of PKC (38). Similar observations were made with the corresponding enzyme from cytosol which showed the same mobility in two-dimensional gels as the cytoskeletal form of the enzyme as judged by superimposing the stained gels (not shown).

Protein Kinase C from Cytosol of Y-1 Cells

To provide sufficient protein kinase C from Y-1 cells to use in studies requiring addition of the exogenous enzyme to cytoskeletons, the cytosolic protein kinase C, which is present in relatively large amounts, was purified as described under Materials and Methods (Table IV). The cytosolic protein kinase C behaves in essentially the same way as the cytoskeletal enzyme. One interesting feature of the cytosolic enzyme was the elution from DEAE-Sephacel of small amounts of a kinase that was active without cofactors (not shown). This may be a proteolytically activated kinase C (so-called protein kinase M) (12, 14, 32). Table IV shows an increase in yield of enzyme at the last step, no doubt as the result of removal of one or more inhibitors. Assays of protein kinase C were performed with all combinations of Ca^{2+} , phosphatidylserine, and PMA (not shown).

Phosphorylation of Cytoskeletal Proteins by Exogenous Protein Kinase C

Fig. 5 shows an autoradiogram of cytoskeletal proteins after incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ without and with various additions. Once again it can be seen that with two cofactors (Ca^{2+} and phosphatidylserine) and PMA, phosphorylation of three proteins (80, 33, and 20 kD) and dephosphorylation of a fourth (18 kD) are increased. Mezerein also produces similar increases but the effect on 80 kD is not obvious in this autoradiogram. Moreover, extraction of protein kinase C by means of Triton X-100 (1.0% [vol/vol]), completely abolishes all these changes (Fig. 5, lane 4). When exogenous crude protein kinase C prepared from Y-1 cells is added to the extracted cytoskeleton, these changes in phosphorylation of 33, 20, and 18 kD are all restored provided cofactors are present (Fig. 4 and Fig. 5, lanes 6 and 7). In addition, a band of phosphorylation corresponding to a molecular mass of 12 kD appears greatly intensified when cofactors are present (lane 3) and when exogenous protein kinase C and cofactors are present (lane 7). The experiment presented in Fig. 6 shows the influence of the concentration of the three cofactors (Ca^{2+} , phosphatidylserine, and phorbol ester) on the phosphorylation of cytoskeletal proteins by exogenous, partly purified protein kinase C prepared from Y-1 cell cytosol. To avoid an unmanageable number of conditions, increasing concentrations of the cofactors in a fixed proportion have been added to the mixture of extracted cytoskeleton plus pro-

Table III. Purification of Protein Kinase C from Y-1 Cell Cytoskeletons

Step	Protein	Total activity	Specific activity	Yield purification	
				(in percent)	(fold)
	mg	nmol/min	nmol/min per mg protein		
Cytoskeleton	180.0	3.78	0.02	100	1
Triton extract	9.3	2.25	0.24	99	11
DEAE-Sephacel	2.06	1.82	0.88	48	42
Affinity column	0.023	1.16	50.4	30	2,400

Protein kinase C was measured by an assay based upon phosphorylation of histone with and without cofactors (Materials and Methods).

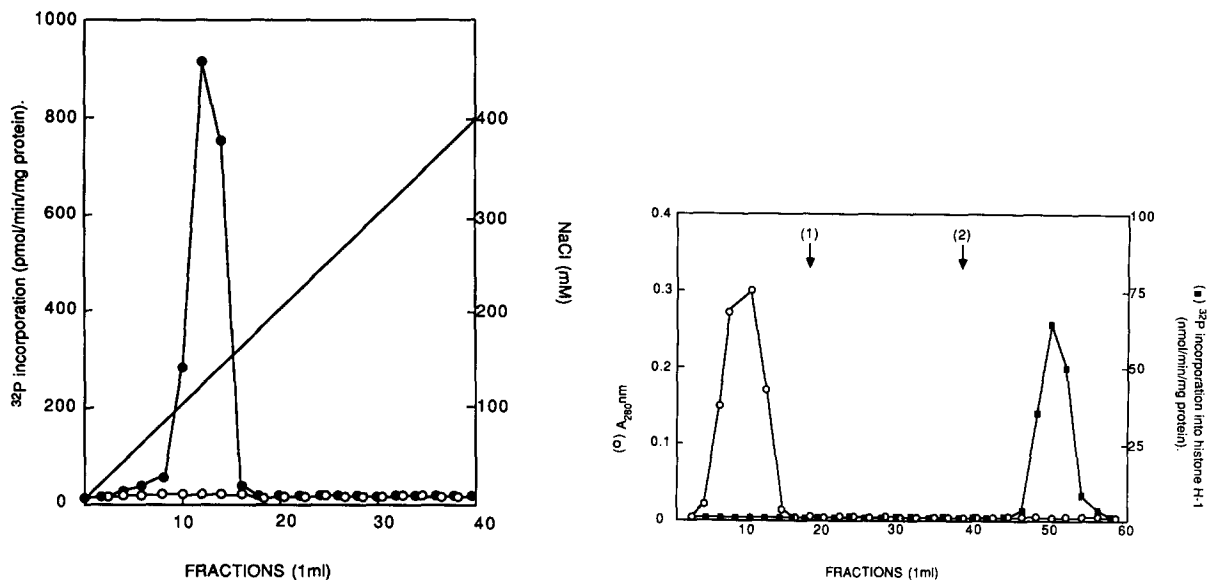


Figure 3. Chromatography of cytoskeletal protein kinase C. (*Left*) DEAE-Sephacel: Conditions for column chromatography are given in Materials and Methods. Activity of protein kinase C is shown as a function of elution volume. The column was eluted with a gradient of NaCl (0–400 mM) in buffer II. ○, no additions; ●, Ca²⁺ (2 mM); phosphatidylserine (10 μg/ml); phorbol-12-myristate-13-acetate (100 nM). (*Right*) Affinity chromatography: The conditions for affinity chromatography of the pooled fractions from DEAE-Sephacel are given in Materials and Methods. 1 indicates elution with CaCl₂ (0.1 mM) and 2 indicates elution with EDTA (2 mM). The figure shows A₂₈₀ and activity of protein kinase C as a function of elution volume.

tein kinase C. Protein kinase C activity with histone H-1 is shown on the left ordinate. Phosphorylation of 80 (○) and 33 kD (Δ) increase in parallel with increase in activity of protein kinase with histone H-1 (◆). The degree of dephosphorylation of 18 kD (●) also shows a parallel relationship to total activity (presented as the inverse relationship, i.e., extent of phosphorylation). By contrast, the 20-kD protein (▲) is maximally phosphorylated at a value of 6.2% whereas kinase activity, phosphorylation of 33 kD, and dephosphorylation of 18 kD reach plateaux at higher concentrations. Values for EC₅₀ for the latter three activities are not significantly different (*p* > 0.1) and are approximately 6% of maximal values.

Identification of 20-kD Protein

The partly purified protein kinase C from Y-1 cells is capable of catalyzing phosphorylation of exogenous myosin light chain

Table IV. Purification of Cytosolic Protein Kinase C from Y-1 Cells

Step	Protein mg	Total activity nmol/min nmol/min per mg protein	Specific activity nmol/min per mg protein	Yield Purification	
				(in percent)	(fold)
Crude cell extract	30.0	1.65	0.055	100	1
DEAE-Sephacel	2.6	1.14	0.44	63	8
Affinity chromatography	0.014	2.7	155.0	131	2,823

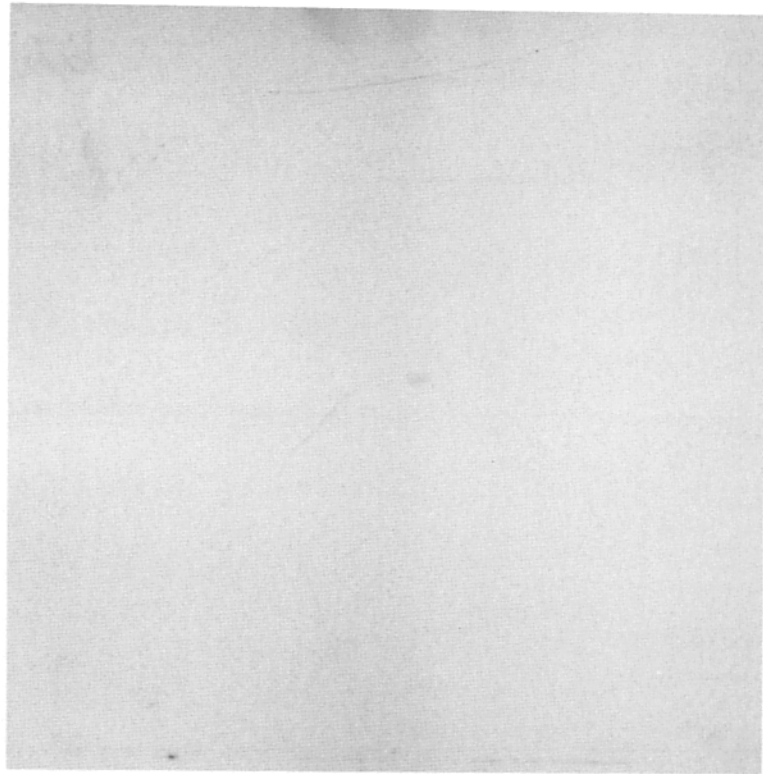
Fractions shown in the first column were subjected to measurement of protein kinase C (Materials and Methods) and determination of protein content.

from skeletal muscle (Fig. 7). The exogenous protein is seen in autoradiograms of two-dimensional gels (*lower autoradiogram*); no phosphorylation above background is seen with endogenous proteins only (*arrow in upper autoradiogram*). The identity of 20 kD as endogenous myosin light chain has been established directly by Western blot and peptide mapping (Papadopoulos, V., and P. F. Hall, manuscript in preparation).

Effect of Various Agents on Shape of Y-1 Cell Cytoskeleton

The shapes of cytoskeletons prepared from Y-1 cells in situ, i.e., on cultured cells, can be seen when the dishes are viewed under phase microscopy using an inverted microscope (Fig. 8). In viewing the figure it is necessary to examine the contour of the cytoskeleton carefully to avoid confusion with the strongly defined shape of the nucleus. Flat cytoskeletons are seen when PBS is added to keep the cytoskeletons moist (Fig. 8 A). When the three cofactors (Ca²⁺, phosphatidylserine, and phorbol-12-myristate-13-acetate) are added together, intense and rapid (<30 s) rounding of the cytoskeletons is seen (Fig. 8 B). Mezerein (100 nM) also stimulates the cytoskeletons to round up (Fig. 8 C). Rounding does not occur when the cytoskeletons are extracted with Triton X-100 (1.0% [vol/vol]) with or without cofactors (Fig. 8, D and E). However, intense rounding is seen when partly purified protein kinase C is added to the extracted cytoskeleton with but not without the other two cofactors (Fig. 8, F and G). Ca²⁺ alone, phosphatidylserine alone, and phorbol-12-myristate-13-acetate alone are all without effect (not shown). Some rounding occurs with Ca²⁺ plus phosphatidylserine (not shown). Intact cells show similar rounding on addition of phorbol 12-myristate-13-acetate (not shown). Rounding of

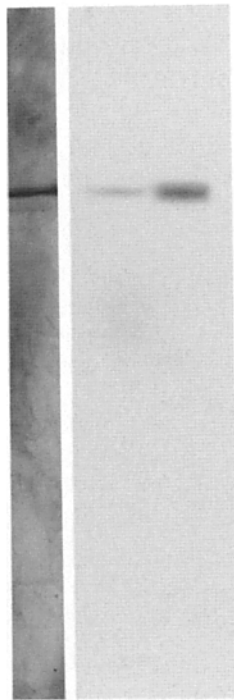
PI - 4 5 6 7 8+



80 kD

C

PI - 4 5 6 7 8 +



80 kD

A

- +
B



80 kD

D

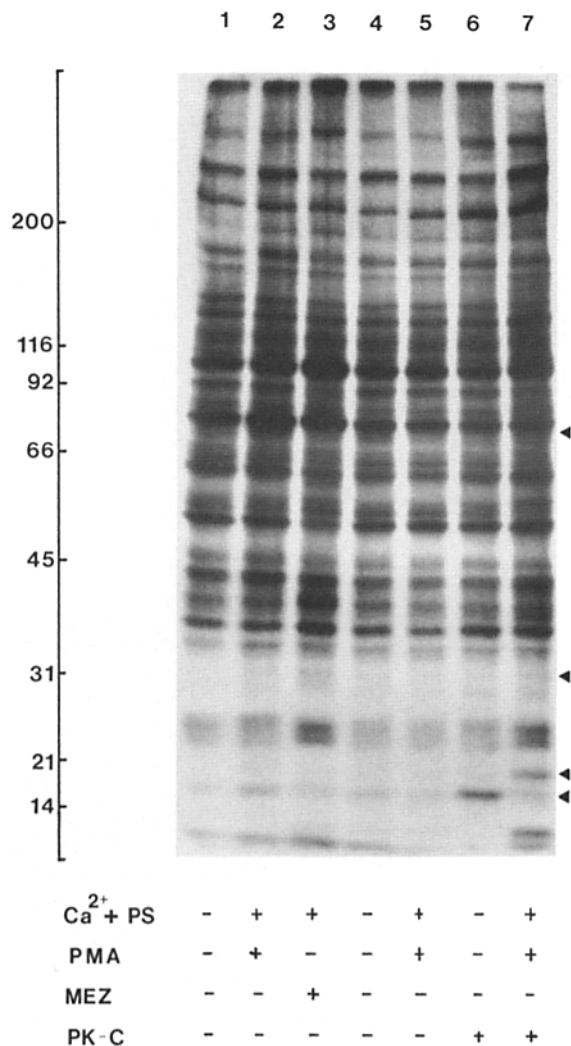


Figure 5. Phosphorylation of cytoskeletal proteins by endogenous and exogenous protein kinase C. Cytoskeletons were prepared from Y-1 cells and were incubated with [γ -³²P]ATP (10 nmol, 10⁶ cpm per tube) and the additions shown. After incubation proteins were separated by gel electrophoresis followed by autoradiography. Lanes 1-3 were from cytoskeletons prepared in the standard way. Lanes 4-7 were from cytoskeletons that were extracted with Triton X-100 (1.0% [vol/vol]) before incubation with the additions shown. The following concentrations were used: Ca²⁺, 2 mM; phosphatidyserine (PS), 10 μ g/ml; phorbol-12-myristate-13-acetate (PMA), 100 nm; mezerein (MEZ), 100 nM; and exogenous protein kinase C prepared from cytosol (PK-C), 50 μ g/ml (lanes 6 and 7).

cells is considerably slower (>1 h) than that of the cytoskeleton.

Partial Proteolysis

Samples of purified protein kinase C (2 μ g each) and of

cytoskeletal proteins (100 μ g) were incubated under conditions that promoted phosphorylation (Materials and Methods). Samples were then subjected to one-dimensional electrophoresis on polyacrylamide followed by autoradiography. Bands corresponding to a molecular mass of 80 kD were cut from the slab gels and were incubated with *S. aureus* V₈ protease and were again examined by one-dimensional electrophoresis on polyacrylamide followed by autoradiography (Materials and Methods). Fig. 9 shows that the same five peptides (molecular masses: 80, 67, 50, 38, and 14 kD) were formed with the same protease under the same conditions with highly purified cytoskeletal protein kinase C (lane A), highly purified cytosolic protein kinase C (lane B), and cytoskeletal proteins (lane C). In Fig. 9, lane C (cytoskeletal proteins) a band corresponding to 44 kD is seen. The nature of this material is unknown.

Action of Protein Kinase C in Permeabilized Cells

When Y-1 cells were treated with digitonin (20 μ M) followed by [γ -³²P]ATP in the presence and absence of phorbol-12-myristate-13-acetate (100 nM), phosphorylated proteins were identified by electrophoresis in polyacrylamide gels of the cytoskeletons of the cells prepared as described in Materials and Methods followed by autoradiography (Fig. 10). Phosphoproteins of various molecular masses can be seen. Bands corresponding to 80, 67, 40, 33, and 20 kD on the autoradiographs were more intense when the cells were treated with phorbol ester (PMA, 100 nM) after treatment with digitonin (lane 2) or both before and after digitonin (lane 3). No phosphoproteins were observed when cells were incubated with [γ -³²P]ATP with or without PMA, but without digitonin (not shown); evidently permeabilization was necessary. The phosphorylation of these proteins was also seen when the cells were incubated with [γ -³²P]ATP and digitonin with and without PMA all added at the same time. Again, increase in the intensity of the bands mentioned above was observed with PMA. The presence of CaCl₂ in the incubation medium was without demonstrable effect on phosphorylation of proteins in the presence or absence of PMA (not shown).

Discussion

The observations reported here establish that a typical protein kinase C is present in the cytoskeletons of Y-1 cells. This enzyme is also seen in cytosol and plasma membranes of these cells, as reported previously (36). The enzyme appears to be rather loosely associated with the plasma membrane in keeping with possible redistribution of the protein within the cell as a feature of its functional activity (15, 29, 36). The principal interest in the present study concerns the protein kinase C of cytoskeleton. As with other examples of protein

Figure 4. Purification and autophosphorylation of protein kinase C. (A) One-dimensional electrophoresis of highly purified protein kinase C on polyacrylamide (silver stain). (B) Phosphorylation of highly purified protein kinase C. Protein kinase C from Y-1 cells was incubated with [γ -³²P]ATP (10 nmol, 10⁶ cpm) without (-) and with (+) the three cofactors (Fig. 1) for 1 min at 37°C followed by 3 min at room temperature. The sample was subjected to gel electrophoresis followed by autoradiography. (C and D) Highly purified cytoskeletal protein kinase C (2 μ g) was incubated with [γ -³²P]ATP under phosphorylating conditions (Materials and Methods) and then subjected to two-dimensional electrophoresis on polyacrylamide followed by autoradiography. Phosphorylation was performed without (C) and with (D) cofactors (Materials and Methods).

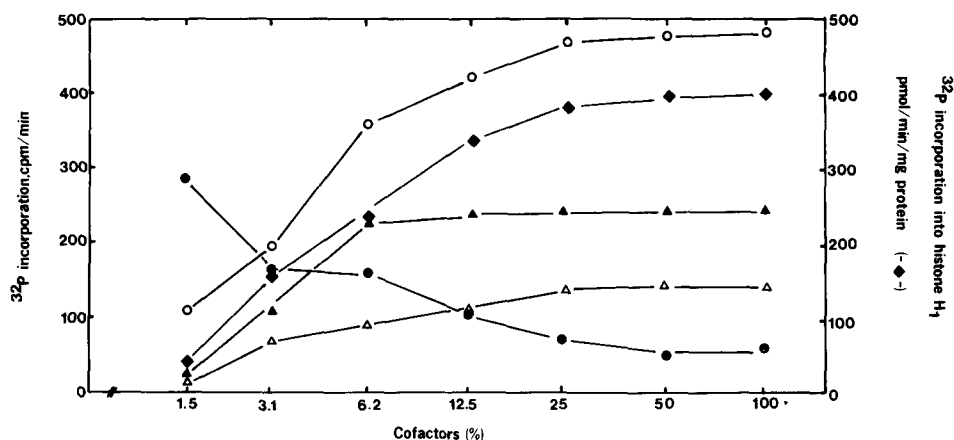


Figure 6. Effects of Y-1 cell protein kinase C and cofactors on specific cytoskeletal substrates. Cytoskeletons from Y-1 cells were incubated with [γ - 32 P]ATP (10 nmol; 10^6 cpm/flask) as described under Materials and Methods. Various volumes of the three agents (Ca^{2+} , phosphatidylserine, and phorbol-12-myristate-13-acetate) were added as shown so that 100% represents the following concentrations: 1 mM, 10 $\mu\text{g}/\text{ml}$, and 20 nM, respectively. The relative concentrations of these factors are shown as percentages

of the maximal concentrations and are presented on a log scale. After incubation, one fraction of each sample was used to measure protein kinase C and a second fraction was boiled in Laemmli buffer and subjected to one-dimensional gel electrophoresis. Bands corresponding to 80 (○), 33 (△), 20 (▲), and 18 (●) kD were cut from the gels and ^{32}P was measured by liquid scintillation spectrometry.

kinase C, the enzyme associated with the cytoskeletons of adrenal cells requires Ca^{2+} and phospholipid for activity; phorbol-12-myristate-13-acetate causes further stimulation of activity beyond that seen with Ca^{2+} and phospholipid. A phorbol ester that does not produce tumors, namely 4 α -phorbol-12,13-didecanoate, is without effect on the enzyme, whereas mezerein, a nonphorbol tumor-promoting agent, is a powerful stimulus to the kinase. Again, inhibition of the Y-1 cell enzyme by trifluoperazine is consistent with observations made with protein kinase C from other tissues (6, 38). It is possible that protein kinase C may associate reversibly with the cytoskeleton (15, 29). However, if this association is only established during disruption of the cells, it is sufficiently intense to resist treatment with Triton X-100 (0.5%) and octylglycoside (0.5%). Again, the cytoskeleton possesses distinctive substrates for the enzyme that differ from those found in the plasma membranes of Y-1 cells (36). Moreover, protein kinase C added to cytoskeletons alters the shapes of these structures (Fig. 8) and hence the shape of the whole cell, so that the association of the protein kinase with the cytoskeleton has functional consequences. Rounding of the cells appears to be related to the main function of adrenal cells; i.e., the production of steroids (1, 36). It may be that attachment of protein kinase C to the cytoskeleton is regulated in the whole cell. These considerations make it very likely that protein kinase C acts on the cytoskeleton; whether it is a true component of the cytoskeleton could turn out to be a problem of definition since the composition of the cytoskeleton is to some extent determined operationally. Presumably the association of the enzyme with the cytoskeleton is sufficiently tight to allow it to act on this structure and yet sufficiently loose to permit association and disassociation.

The cytoskeleton prepared as described here has been characterized previously and is known to be free of contaminating plasma membranes and organelles (28). It proved important to our studies that protein kinase C can be readily and completely removed from the cytoskeleton by extraction with Triton X-100 (1% [vol/vol]). This meant that the enzyme could be added back to the extracted cytoskeleton directly to study its effects on cytoskeletal proteins.

This appears to be the first report of the association of this

protein kinase with the cytoskeleton, although other protein kinase enzymes have been reported in the cytoskeletons of adrenal and other cells (28, 30). Our studies also show that the protein kinase enzymes from the cytosol and the cytoskeleton are indistinguishable on gel electrophoresis, auto-phosphorylation, and limited proteolysis. It should be pointed out that the specific activity of the cytoskeletal protein kinase C is considerably less than that reported for the brain enzyme (39). The reason for this difference is not clear but may relate to some loss of activity under the relatively harsh conditions used for the preparation of the cytoskeleton.

The purification of protein kinase C from Y-1 cells in two major steps also proved useful in these studies. In the first place, the highly purified enzyme established the identity of the kinase from cytoskeletons of Y-1 cells. In the second place, when the highly purified enzyme was incubated with [γ - 32 P]ATP, it showed intense auto-phosphorylation. This observation enabled us to demonstrate that the enzyme undergoes phosphorylation *in situ* because extracting the cytoskeleton with Triton (1%) removed not only all Ca^{2+} /phospholipid-dependent kinase activity but also removed the 80-kD substrate of protein kinase C which was not seen in extracted cytoskeletons either by silver staining of polyacrylamide gels (not shown) or by autoradiography after incubation with [γ - 32 P]ATP (Fig. 5). Phosphorylation of the other substrates of protein kinase C (33 and 20 kD) was restored by addition of partly purified protein kinase C to the extracted cytoskeleton. However, under these conditions the exogenous kinase C was not seen in the polyacrylamide gels because this protein is soluble and therefore removed during preparation of cytoskeleton for electrophoresis (Fig. 5, lane 7) in contrast to stably bound endogenous protein kinase C (Fig. 5, lane 3). This observation incidentally supports the idea that the protein kinase is not merely associated with the cytoskeleton as the result of homogenization of cells.

Limited proteolysis followed by electrophoresis and autoradiography and two-dimensional polyacrylamide gels both showed that the phosphorylated 80 kD protein is protein kinase C and not a substrate of similar molecular mass that has been found in other cells (2). The proteolytic fragments are characteristic of protein kinase C and the isoelectric point of

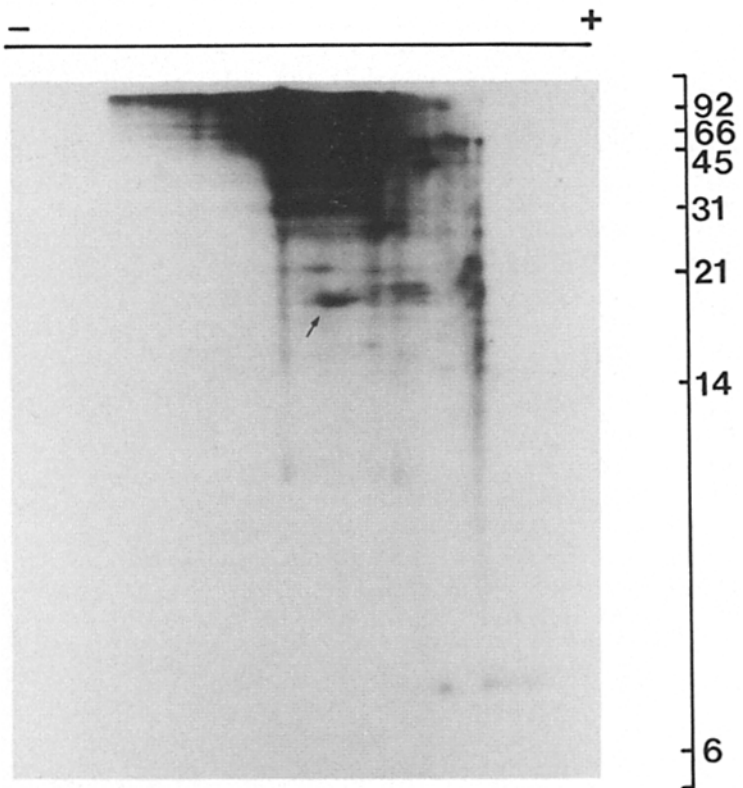
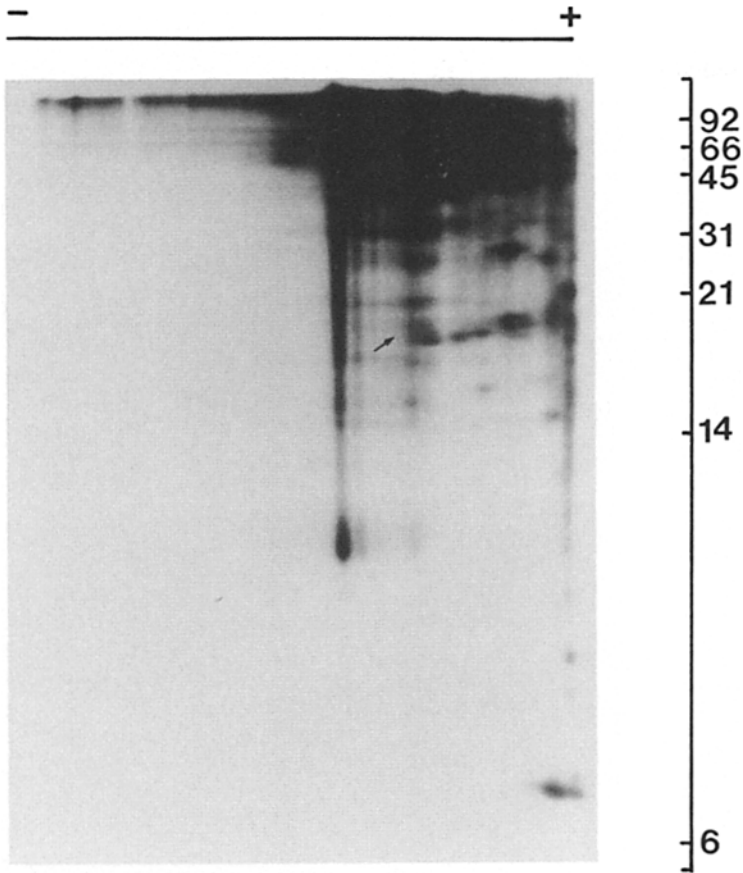


Figure 7. Phosphorylation of myosin light chain. Cytoskeletons from Y-1 cells without (*top*) and with (*bottom*) exogenous myosin light chain from skeletal muscle (10 μ g) were incubated with [γ - 32 P]ATP (10 nmol; 10^6 cpm) and the three cofactors (see Fig. 1). Proteins were separated by two-dimensional electrophoresis on polyacrylamide gels (isofocusing \rightarrow electrophoresis) using 15% polyacrylamide in the second dimension followed by autoradiography. *Arrow*, myosin light chain.

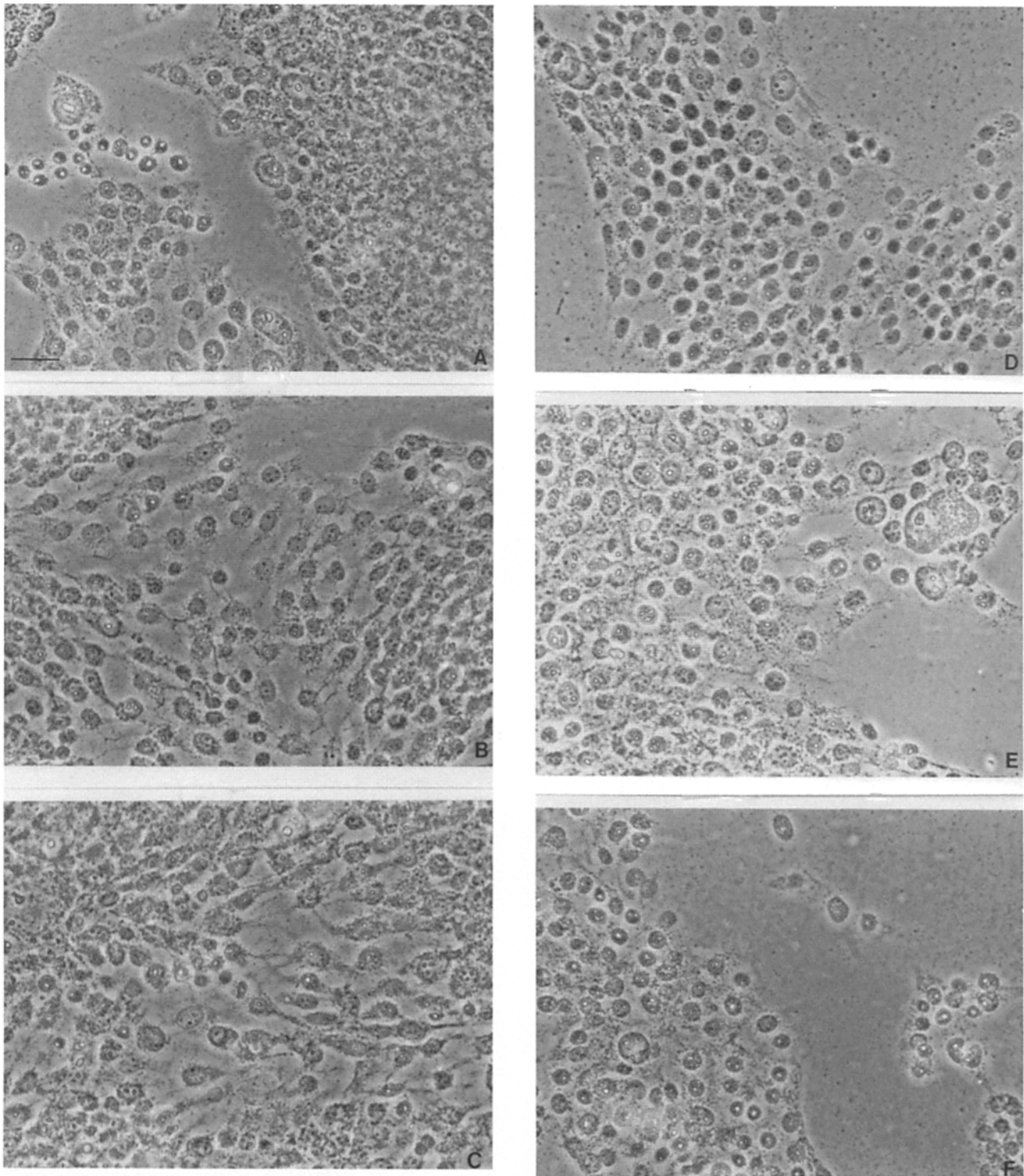
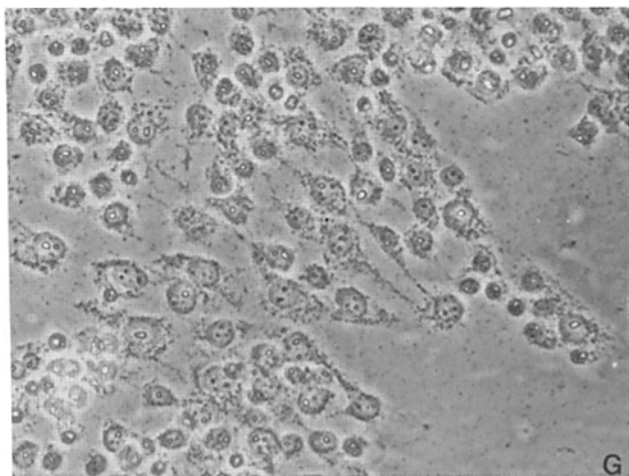


Figure 8 Effect of protein kinase C on shape of cytoskeleton. Cytoskeletons were prepared from Y-1 cells in culture dishes and were examined by phase microscopy immediately after the following additions: (A) PBS; (B) Ca^{2+} (2 mM), PS (10 $\mu\text{g}/\text{ml}$), and PMA (100 nM); (C) Ca^{2+} (2 mM), PS (10 $\mu\text{g}/\text{ml}$), and mezerein (100 nM); (D) cytoskeleton extracted with Triton X-100 (1% [vol/vol]); (E) cytoskeleton extracted with Triton X-100 + Ca^{2+} + PS + PMA (100 nM); (F) cytoskeleton extracted with Triton X-100 + protein kinase C; (G) cytoskeleton extracted with Triton X-100 + protein kinase C + Ca^{2+} (2 mM) + PS (10 $\mu\text{g}/\text{ml}$) + PMA (100 nM). PS, phosphatidylserine. Bar, 25 μm .



the phosphorylated protein is quite different from that of the 80 kD substrate (2). Our preparations may contain small amounts of a substrate for protein kinase C of 80 kD; alternatively the protein kinase C may exist as a more acidic form in addition to the usual form, as described in brain (39). In this connection it is interesting that similar peptides were seen when we examined protein kinase C in homogenate of

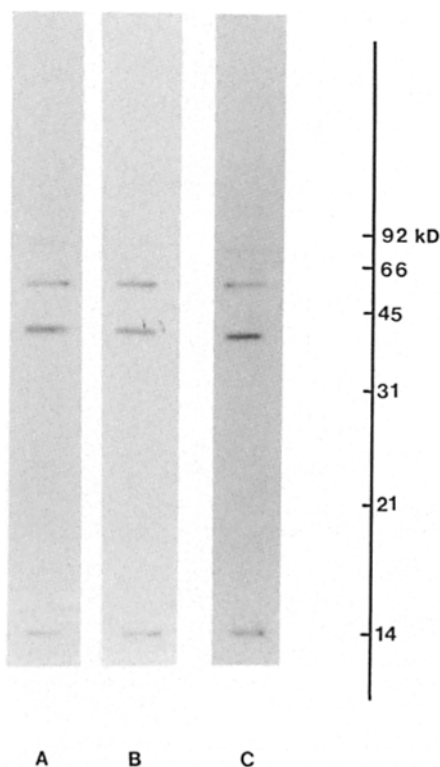


Figure 9. Partial proteolysis of protein kinase C. Protein kinase C from cytoskeleton (lane *A*), cytosol (2 μ g each; lane *B*), and cytoskeletal proteins (100 μ g; lane *C*) were incubated with [γ - 32 P]ATP under phosphorylating conditions (Materials and Methods) and applied to one-dimensional gel electrophoresis. Bands corresponding to 80 kD were cut from each gel, incubated with *S. aureus* V_8 protease and again run on one-dimensional electrophoresis in polyacrylamide gels. Autoradiography was then performed to reveal bands labeled with 32 P.

Y-1 cells (36). In this case fewer inhibitors of proteolysis were used than in the present studies and since 80 kD protein was not seen, it is likely that the enzyme was degraded by endogenous proteolytic enzymes (36). Moreover, Fig. 9 shows the same peptides as those reported for a tryptic digest of protein kinase C from brain (10). Clearly the 80 kD protein substrate is the enzyme itself which catalyzes classical autophosphorylation; this is a well-recognized property of protein kinase C (13, 18, 39). In our studies this phenomenon was observed not only with the purified enzyme but also with the enzyme in situ in cytoskeletons. In this connection the similarity between the two curves (and values for EC_{50}) for protein kinase C activity and autophosphorylation of the enzyme as a function of the concentration of cofactors (Fig. 6), suggests that the activity of the enzyme with histone H-1 is closely related to the state of phosphorylation of the enzyme itself.

A second substrate for the cytoskeletal protein kinase C has been identified in these studies, namely myosin light chain (20 kD). Phosphorylation of myosin light chain by protein kinase C has been reported for smooth muscle myosin (9, 24, 25). This is the first report of phosphorylation of endogenous myosin light chain in the cytoskeleton of a nonmuscle cell. It is worth noticing that myosin light chain phosphorylation by Y-1 cell protein kinase C is saturated with cofactors at a concentration that is submaximal for autophosphorylation of the enzyme (Fig. 6). This suggests an important difference which could be of functional significance if local concentrations of Ca^{2+} were to reach the gap between micromolar and fully saturating levels.

It is important that phosphorylation of the same cytoskeletal proteins was observed in cells permeabilized by digitonin (Fig. 10) as those seen when phosphorylation was performed in the isolated cytoskeletons. This strongly suggests that the three substrates (80, 33, and 20 kD) are likely to be phosphorylated by protein kinase C in the intact cell and that such phosphorylation cannot be dismissed as the result of artificial

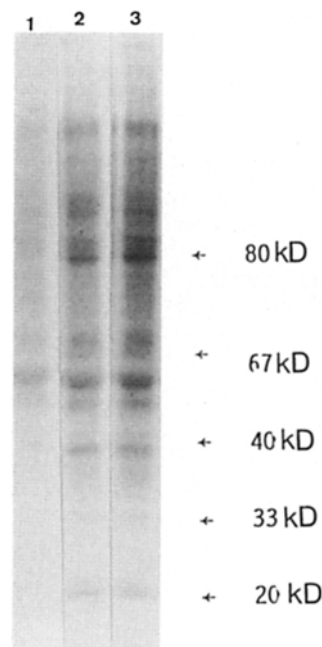


Figure 10. Phosphorylation of cytoskeletal proteins in permeabilized cells. Y-1 cells were incubated without (lanes 1 and 2) or with (lane 3) PMA (100 nM) for 30 min. All cells were then treated with digitonin (20 μ M) (see Materials and Methods) and [γ - 32 P]ATP (50 μ Ci; 16 pmol/dish) for 10 min without (lane 1) or with (lanes 2 and 3) PMA (100 nM). Cytoskeletons were prepared and submitted to electrophoresis in polyacrylamide gels followed by autoradiography.

exposure of these substrates to the enzyme as the result of extracting the cells to prepare cytoskeletons.

The dephosphorylation of 18K is of interest. Such a phenomenon does not appear to have been reported in other cells. One possible explanation for this dephosphorylation would be protein kinase C-dependent phosphorylation of a specific phosphatase. Again this response appears to be closely related to the degree of phosphorylation of the kinase as judged by EC_{50} for cofactors (Fig. 6). At this time the 18- and 33-kD proteins remain unidentified although the 33-kD protein may be a proteolytic product of protein kinase C itself (19).

The functional significance of adrenal cell protein kinase C remains unclear. One significant finding in this connection is the observation that protein kinase C causes rounding of the cytoskeletons of Y-1 cells when the kinase is added to the extracted cytoskeleton in the presence of cofactors. When cytoskeletons were prepared by a less severe procedure than the routine method, they remained adherent to culture dishes so that they could be used for light microscopy and they contained sufficient ATP to support protein kinase C activity. Moreover, phorbol ester produces rounding of the intact cells. The rounding of the cytoskeleton is very rapid compared to that of cells; presumably the response of the cytoskeleton to the added enzyme is more direct than that of the cytoskeleton within the cell. No doubt the change in shape of the cytoskeleton is, in turn, responsible for rounding of cells. In this connection it has been reported that phosphorylation of myosin light chain by protein kinase C involves different amino acid residues from those phosphorylated by the light chain kinase (25), and that phosphorylation by protein kinase C causes relaxation of smooth muscle myosin and decreased myosin ATPase (11). Furthermore, it has been proposed that the myosin of stress fibers produces isometric tension, which helps to bind the cell to the substratum (4). Relaxation of the light chain, under the influence of protein kinase C, would decrease this isometric tension and this, in turn, would weaken the attachment of the cell to the substratum thereby allowing it to round up.

There is much evidence that rounding of Y-1 cells is accompanied by increased steroid synthesis (1). Moreover, ACTH and cyclic AMP increase the total activity of protein kinase C in Y-1 cells (reference 35 and present data) and it is well known that ACTH and cyclic AMP cause intense rounding of Y-1 cells (40). Presumably the phenomenon of rounding up is at least a component in the steroidogenic response to these two agents, although the exact relationship between protein kinase C and the response to ACTH remains to be determined.

The authors are grateful to Barbara Law for typing the manuscript and to the Department of Medical Illustration at The Prince of Wales Hospital for the photography.

This work was supported by National Health and Medical Research Council grant No. M255390 and The Prince Henry Hospital Centenary Research Fund.

Received for publication 3 November 1987, and in revised form 3 October 1988.

References

1. Betz, G., and P. F. Hall. 1987. Steroidogenesis in adrenal tumor cells: influence of cell shape. *Endocrinology*. 120:2547-2554.
2. Blackshear, P. J., L. Wen, B. P. Glynn, and L. A. Witters. 1986. Protein kinase C-stimulated phosphorylation in vitro of a Mr 80,000 protein phosphorylated in response to phorbol esters and growth factors in intact fibroblasts. *J. Biol. Chem.* 120:2547-2554.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
4. Burridge, K. 1981. Stress fibres contractile. *Nature (Lond.)*. 294:691-692.
5. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102-1106.
6. Davis, J. S., and M. R. Clark. 1983. Activation of protein kinase in the bovine corpus luteum by phospholipid and Ca^{2+} . *Biochem. J.* 214:569-574.
7. Dzandu, J. K., and R. M. Johnson. 1980. Membrane protein phosphorylation in intact normal and sickle cell erythrocytes. *J. Biol. Chem.* 255:6382-6386.
8. Hall, P. F., C. Charbonnier, M. Nakamura, and G. Gabbiani. 1979. The role of microfilaments in the response of adrenal tumor cells to adrenocorticotrophic hormone. *J. Biol. Chem.* 254:9080-9084.
9. Hassell, T. C., B. E. Kemp, and R. A. Masaracchia. 1986. Nonmuscle myosin phosphorylation sites for calcium-dependent and calcium-independent protein kinases. *Biochem. Biophys. Res. Commun.* 134:240-247.
10. Huang, K.-P., K.-F. J. Chan, T. J. Singh, H. Nakabayashi, and F. L. Huang. 1986. Autophosphorylation of rat brain Ca^{2+} -activated and phospholipid-dependent protein kinase. *J. Biol. Chem.* 261:12134-12140.
11. Inagaki, M., H. Yokokura, T. Itoh, Y. Kanmura, H. Kuriyama, and H. Hidaka. 1987. Purified rabbit brain protein kinase C relaxes skinned vascular smooth muscle and phosphorylates myosin light chain. *Arch. Biochem. Biophys.* 254:136-141.
12. Inoue, M., A. Kishimoto, Y. Takai, and Y. Nishizuka. 1977. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. *J. Biol. Chem.* 252:7610-7616.
13. Kikkawa, U., Y. Takai, R. Minakuchi, S. Inohara, and Y. Nishizuka. 1982. Calcium-activated, phospholipid-dependent protein kinase from rat brain. Subcellular distribution, purification, and properties. *J. Biol. Chem.* 257:13341-13348.
14. Kishimoto, A., N. Kajiakawa, M. Shiota, and Y. Nishizuka. 1983. Proteolytic activation of calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease. *J. Biol. Chem.* 258:1156-1164.
15. Kraft, A. S., and W. B. Anderson. 1983. Phorbol esters increase the amount of Ca^{2+} , phospholipid-dependent protein kinase associated with plasma membrane. *Nature (Lond.)*. 301:621-623.
16. Kowal, J. 1970. ACTH and the metabolism of adrenal cell cultures. *Recent Prog. Horm. Res.* 26:623-687.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
18. Lee, S.-A., and R. W. Holz. 1986. Protein phosphorylation and secretion in digitonin-permeabilized adrenal chromaffin cells. *J. Biol. Chem.* 261:17089-17098.
19. Mochly-Rosen, D., and D. E. Koshland, Jr. 1987. Domain structure and phosphorylation of protein kinase C. *J. Biol. Chem.* 262:2291-2297.
20. Morrissey, J. H. 1981. Silver stain for protein in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117:307-310.
21. Mrotek, J. J., and P. F. Hall. 1975. The influence of cytochalasin B on the response of adrenal tumor cells to ACTH and cyclic AMP. *Biochem. Biophys. Res. Commun.* 4:891-894.
22. Mrotek, J. J., and P. F. Hall. 1977. Response of adrenal tumor cells to adrenocorticotropin: site of inhibition of cytochalasin B. *Biochemistry*. 16:3177-3181.
23. Niedel, J. E., L. J. Kuhn, and G. R. Vandenberg. 1983. Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. USA*. 80:36-40.
24. Nishikawa, M., H. Hidaka, and R. S. Adelstein. 1983. Phosphorylation of smooth muscle heavy meromyosin by calcium-activated, phospholipid-dependent protein kinase. The effect on actin-activated MgATPase activity. *J. Biol. Chem.* 258:14069-14072.
25. Nishikawa, M., J. R. Sellar, R. S. Adelstein, and H. Hidaka. 1984. Protein kinase C modulates in vitro phosphorylation of the smooth muscle heavy meromyosin by myosin light chain kinase. *J. Biol. Chem.* 259:8808-8814.
26. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
27. Osawa, S., G. Betz, and P. F. Hall. 1984. The role of actin in the responses of adrenal cells to ACTH and cyclic AMP: inhibition of DNase I. *J. Cell Biol.* 99:1335-1342.
28. Osawa, S., and P. F. Hall. 1985. Adenosine 3',5'-monophosphate-dependent protein kinase associated with the cytoskeleton of adrenal tumor cells. *Endocrinology*. 117:2347-2356.
29. Pelech, S. L., K. E. Meier, and E. G. Krebs. 1986. Rapid microassay for protein kinase C translocation in Swiss 3T3 cells. *Biochemistry*. 25:8348-8353.
30. Sahyoun, N., H. LeVine, III, D. Bronson, F. Siegel-Greenstein, and P. Cuatrecasas. 1985. Cytoskeletal calmodulin-dependent protein kinase. Characterization, solubilization and purification from rat brain. *J. Biol.*

- Chem.* 260:1230-1237.
31. Schwantke, N., and C. J. Le Peuch. 1984. A protein kinase C inhibitory activity is present in rat brain homogenate. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 177:36-40.
 32. Takai, Y., A. Kishimoto, M. Inoue, and Y. Nishizuka. 1977. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. *J. Biol. Chem.* 252:7603-7609.
 33. Takai, Y., A. Kishimoto, Y. Iwasa, Y. Kawahara, T. Mori, and Y. Nishizuka. 1979. Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.* 254:3692-3695.
 34. Uchida, T., and C. R. Filburn. 1984. Affinity chromatography of protein kinase C-phorbol ester receptor on polyacrylamide-immobilized phosphatidylserine. *J. Biol. Chem.* 259:12311-12314.
 35. Vilgrain, I., C. Cochet, and E. M. Chambaz. 1984. Hormonal regulation of a calcium-activated, phospholipid-dependent protein kinase in bovine adrenal cortex. *J. Biol. Chem.* 259:3403-3406.
 36. Widmaier, E., and P. F. Hall. 1985. Protein kinase C in adrenal cells: possible role in regulation of steroid synthesis. *Mol. Cell. Endocrinol.* 43: 181-188.
 37. Widmaier, E., and P. F. Hall. 1987. Calmodulin-binding proteins in plasma membranes from adrenocortical cells. *Endocrinology.* 121:914-923.
 38. Wise, B. C., R. L. Raynor, and J. F. Kuo. 1982. Phospholipid-sensitive Ca^{2+} -dependent protein kinase from heart. I. Purification and general properties. *J. Biol. Chem.* 257:8481-8488.
 39. Woodgett, J. R., and T. Hunter. 1987. Isolation and characterization of two distinct forms of protein kinase C. *J. Biol. Chem.* 262:4836-4843.
 40. Yasamura, Y. 1968. Shape changed in cultured adrenal cells induced by ACTH. *Am. Zool.* 8:285-290.