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Isozymic forms of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase

(protein kinase C/autophosphorylation/polyclonal and monoclonal antibodies)

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ABSTRACT **Three forms of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase (EC 2.7.1.37) were separated by hydroxylapatite column chromatography. These enzymes, designated type I, II, and III protein kinase C, all have a molecular weight of 82,000, undergo autophosphorylation in the presence of Ca^{2+}, phosphatidylserine, and diolein, and bind [^{3}H]phorbol 12,13-dibutyrate. Autophosphorylation of these kinases resulted in an incorporation of 1–1.5 mol of ^{32}P per mol of enzyme. Two-dimensional peptide mapping analysis revealed that these kinases had different sites of autophosphorylation. Phosphoamino acid analysis showed that type I and type III protein kinase C primarily autophosphorylated at a serine residue, whereas type II kinase autophosphorylated at both serine and threonine residues. In addition, polyclonal antibodies raised against a mixture of three types of the kinase preferentially inhibited type I and type II enzymes. Monoclonal antibodies against type I and type II kinase only recognized their respective enzymes but not the type III enzyme. These results demonstrate the presence of isozymic forms of protein kinase C in rat brain.**

The Ca^{2+}-activated and phospholipid-dependent protein kinase (protein kinase C) was first found in rat brain as a proteolytically activated protein kinase (1, 2). This enzyme was later shown to require Ca^{2+} and phospholipid (3, 4) and to be further activated by diacylglycerol (DAG) (5), which markedly increased the affinity of the kinase for both Ca^{2+} and phospholipid. Protein kinase C has been purified to near homogeneity from many sources (6–12); however, the presence of isozymic forms of the enzyme has not been reported. This kinase is a monomeric polypeptide of molecular weight 82,000 that appears to be composed of two functionally different domains: one is a hydrophobic domain that may bind to membranes, and the other is a hydrophilic domain that carries the catalytically active center. These two domains are cleaved by Ca^{2+}-dependent neutral proteases to produce a Ca^{2+}- and phospholipid-independent active enzyme fragment (13). Intracellular stimulation of protein kinase C can be effected by the DAG generated from the signal-induced breakdown of inositol phospholipids or by the addition of synthetic DAG or tumor-promoting phorbol esters (14, 15).

Protein kinase C is an ubiquitous enzyme found in a variety of mammalian tissues (16, 17). This enzyme has attracted particular attention because it plays a pivotal role in controlling many cellular functions. In addition, this enzyme has been identified as a putative receptor for tumor-promoting phorbol esters (18, 19). Recently we have prepared polyclonal and monoclonal antibodies against rat brain protein kinase C. In the process of characterizing these antibodies we noticed that some purified kinase preparations were less sensitive to inhibition by these antibodies. Therefore, we refined our purification procedure to separate the different forms of protein kinase C. In this report, we provide evidence to demonstrate the existence of at least three types of protein kinase C in rat brain; these enzymes can be distinguished by immunological and chemical methods.

MATERIALS AND METHODS

Materials. The following materials were obtained from the indicated sources: histone IIIS and EGTA from Sigma; [γ-^{32}P]ATP, [^{3}H]phorbol-12, 13-dibutyrate (PBt_2), and [^{125}I]-protein A from New England Nuclear; (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin from Worthington; cellulose-coated thin-layer plate from Eastman Kodak; polyacrylamide gel electrophoresis, immunoblotting, and protein determination reagents and hydroxylapatite from Bio-Rad; phosphatidyl serine (PtdSer) and 1,2-dioleoylglycerol (diolein) from Avanti Polar Lipids; horseradish peroxidase-conjugated rabbit anti-goat IgG from Miles; rabbit anti-mouse IgG, goat anti-rabbit IgG, and peroxidase-conjugated rabbit anti-peroxidase antigen–antibody complex from Cooper Biochemical (Malvern, PA); and Pansorbin from Calbiochem.

Assay of Protein Kinase C Activity. The kinase activity was measured as previously described (21). The reaction mixture for autophosphorylation contained 10 mM Tris·HCl, pH 7.5/0.25 mM EDTA/0.25 mM EGTA/0.5 mM dithiothreitol/5% (vol/vol) glycerol/6.6 mM magnesium acetate/40 μM [γ-^{32}P]ATP/40 μg of PtdSer per ml/8 μg of diolein per ml/0.8 mM $CaCl_2$/protein kinase C. Reactions were carried out at 30°C, and the measurement of ^{32}P incorporation into proteins was done as described (20). One unit of protein kinase is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of ^{32}P into histone IIIS per min.

Purification of Rat Brain Protein Kinase C. Protein kinase C was purified from the cytosolic fraction of fresh rat brain by a modified procedure (21) of Kikkawa *et al.* (6). The purification procedure involved chromatography on DEAE-cellulose (4.0 × 12 cm, using a 0–0.3 M KCl gradient), phenyl-Sepharose (2.0 × 9 cm, using a 1.5–0 M KCl gradient), Sephacryl S-200 (2.5 × 95 cm), and polylysine-agarose (1.5 × 12 cm, using a 0–0.8 M KCl gradient). The pooled kinase fraction (5–7 mg of protein) was concentrated by ultrafiltration to approximately 10 ml and dialyzed against buffer A [0.02 M KPO_4, pH 7.5/0.5 mM EDTA/0.5 mM EGTA/1 mM dithiothreitol/10% (vol/vol) glycerol] before applying it to a hydroxylapatite column (1.5 × 5 cm) equilibrated with the same buffer. The column was washed with 60 ml of buffer A, and protein kinase C isozymes were eluted

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Abbreviations: Protein kinase C, Ca^{2+}-activated and phospholipid-dependent protein kinase; PtdSer, phosphatidylserine; DAG, diacylglycerol; diolein, 1,2-dioleoylglycerol; PBt_2, phorbol-12, 13-dibutyrate; U, unit(s).

with a gradient (Fig. 1) composed of buffer A and buffer A with 0.28 M KPO_4. The gradient was delivered by using an LKB Ultrograd gradient maker at a flow rate of 10 ml/hr for 16 hr.

Preparation of Antibodies Against Rat Brain Protein Kinase C. Polyclonal antibodies were raised in a female goat by injection of the purified heterogeneous protein kinase C that had been prepared without resolution into isozyme fractions (34). Monoclonal antibody was prepared by injection of the heterogeneous protein kinase C mentioned earlier into mice. The isolation and screening of hybridomas were done according to the standard procedure (22). Four clones of hybridomas secreting high levels of antibody against the heterogeneous protein kinase C were used to produce ascites fluid. The antibodies were tested for inhibition of each kinase separated from the hydroxylapatite column and for reactivity against these kinases by immunoblotting. Three hybridoma clones produced antibody against type II kinase and one clone, against type I enzyme. After screening more than 10 potentially positive hybridoma cell lines, we have not yet detected a positive one against the type III kinase.

Peptide Mapping of the Autophosphorylated Protein Kinase C. The autophosphorylated kinase was digested with trypsin (protein/trypsin, 5:1) at room temperature in 0.1 M NH_4HCO_3, pH 7.8, for 48 hr. Peptide mapping on thin-layer plate was performed by initial electrophoresis at 1000 V for 30–40 min at pH 6.5 (pyridine/acetic acid/water, 25:1:225) to allow the tracking dye, bromophenol blue, to migrate toward the anode for 6 cm. Thin-layer chromatography for the second dimension was done with freshly prepared solvent (*n*-butanol/acetic acid/water, 4:1:5). The ^{32}P-labeled peptide spots were visualized after autoradiography.

Immunoblot Analysis of Protein Kinase C. Immunoblotting of the kinase using polyclonal antibodies was done according to Towbin *et al.* (23) with reagents and procedures provided by Bio-Rad. The immunoreactive protein band was detected with ^{125}I-labeled protein A. Immunoblotting of the kinase by monoclonal antibody was done by using a peroxidase–anti-peroxidase reaction. The nitrocellulose membrane was incubated successively with 3% gelatin in Tris buffer (20 mM Tris·HCl, pH 7.5/500 mM NaCl) for 30 min to block nonspecific binding sites, with monoclonal antibody (IgG; 7.5 μg/ml) in the Tris buffer containing 1% gelatin for 2 hr, with rabbit anti-mouse IgG (1 μg/ml) in 1% gelatin for 1 hr, with goat anti-rabbit IgG (1 μg/ml) in 1% gelatin for 1 hr, and with peroxidase-conjugated rabbit anti-peroxidase antigen–antibody complex in 1% gelatin for 1 hr. Between incubations, the membrane was washed extensively with the Tris buffer containing 0.05% Tween-20. Immunoreactive bands were visualized by treatment with 4-chloro-1-naphthol. The molecular weight marker proteins were localized by brief staining with 0.1% amido black in 25% (vol/vol) isopropanol containing 10% (vol/vol) acetic acid.

Other Methods. Protein concentrations were determined by the method of Bradford (24) using bovine plasma albumin as standard. $NaDodSO_4$/PAGE was done according to the method of Laemmli (25).

RESULTS

Purification of Three Isozymic Forms of Protein Kinase C. Rat brain protein kinase C isolated by polylysine-agarose column chromatography was separated into three isozymic forms by hydroxylapatite column chromatography. The three kinase activity peaks that eluted at KPO_4 concentrations of 0.07 M, 0.08 M, and 0.15 M were designated as type I, II, and III protein kinase C, respectively. These kinase activity peaks coincided with those of the binding of $[^3H]PBt_2$ (Fig. 1). The kinase activities inherent in these purified enzyme preparations were stimulated to a different degree by Ca^{2+}, PtdSer, and diolein (Table 1): The stimulation we observed was ≈4- to 8-fold for type I and >15-fold for type II and type III kinases. The variable high basal Ca^{2+}/PtdSer-independent kinase activity in the type I enzyme preparations may be due to different quantities of that protease-degraded kinase. Both Ca^{2+} and PtdSer are required for the stimulation of all three isozymes, and diolein further enhances the kinase activity. Neither Ca^{2+}, PtdSer, diolein alone nor the combination of Ca^{2+} with diolein produces any obvious stimulation of the kinase over basal levels. Significant stimulation of the kinase was observed in the presence of PtdSer and diolein without added Ca^{2+}, indicating that the Ca^{2+} requirement is significantly reduced by the diolein.

The three types of protein kinase C were copurified by chromatography on DEAE-cellulose, phenyl-Sepharose, Sephacryl S-200, and polylysine-agarose, suggesting that all three isozymes have similar charges, sizes, and affinity characteristics toward hydrophobic and polycationic matrices. These three forms of protein kinase C separated by hydroxylapatite column chromatography appear to be highly purified. The specific activity of these enzymes ranged from 2000 to 3000 units/mg in several preparations. All three enzymes underwent autophosphorylation in the presence of Ca^{2+}, PtdSer, and diolein (Fig. 2). Autophosphorylation of the kinase incorporates the ^{32}P from [γ-^{32}P]ATP into the protein kinase C molecule (Fig. 2*B*). The autophosphorylated kinase primarily displayed a Coomassie blue-stained protein

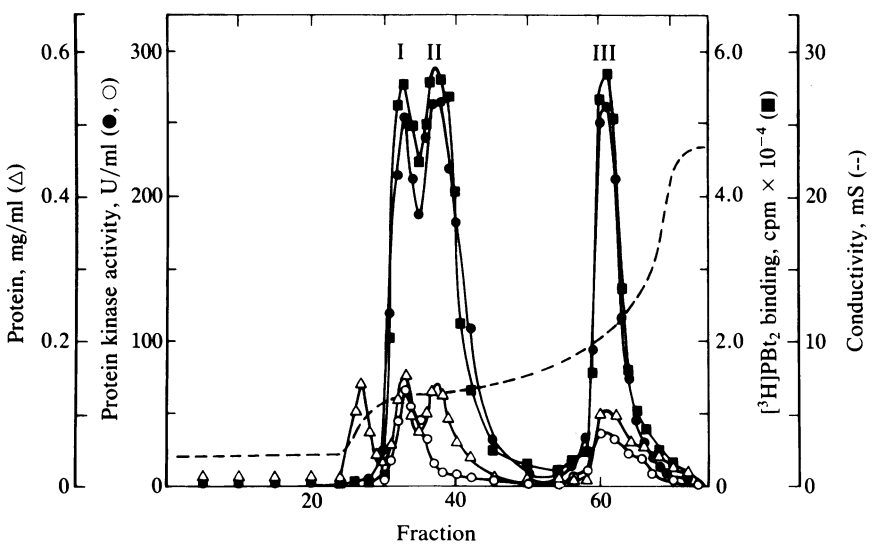

FIG. 1. Separation of three isozymic forms of protein kinase C by hydroxylapatite column chromatography. Fractions of 3 ml were collected for the measurements of protein kinase activity with Ca^{2+}, PtdSer, and diolein (●) and with EGTA without lipid (○). ■, Binding of $[^3H]PBt_2$ (11), Δ, protein concentration; and --, conductivity. The three activity peaks were designated type I, II, and III protein kinase C.

Table 1. Stimulation of three types of protein kinase C activity by Ca^{2+}, PtdSer, and diolein

Condition	Protein kinase activity, U/mg					
	Type I		Type II		Type III	
$+Ca^{2+}$, +PtdSer, +diolein	1810	(100%)	2504	(100%)	3090	(100%)
$-Ca^{2+}$, −PtdSer, −diolein, +EGTA	415	(23%)	133	(5.3%)	80	(2.6%)
$+Ca^{2+}$, −PtdSer, −diolein	409	(23%)	123	(4.9%)	129	(4.2%)
$-Ca^{2+}$, +PtdSer, −diolein, +EGTA	459	(25%)	188	(7.5%)	77	(2.5%)
$-Ca^{2+}$, −PtdSer, +diolein, +EGTA	443	(25%)	128	(5.1%)	111	(3.6%)
$+Ca^{2+}$, −PtdSer, +diolein	494	(28%)	168	(6.7%)	188	(6.1%)
$+Ca^{2+}$, +PtdSer, −diolein	1299	(72%)	1552	(62%)	2163	(70%)
$-Ca^{2+}$, +PtdSer, +diolein, +EGTA	828	(46%)	1578	(63%)	835	(27%)

Purified type I, II, and III protein kinase C were used to measure protein kinase activity in a basic reaction mixture (30 mM Tris·HCl buffer, pH 7.5/6 mM magnesium acetate/0.12 mM [γ-^{32}P]ATP/1 mg of histone IIIS per ml) plus the indicated ingredients in the following concentrations: Ca^{2+} (0.4 mM), PtdSer (20 μg/ml), diolein (4 μg/ml), and EGTA (1 mM). U, unit.

band of molecular weight from 82,000 to 88,000 after NaDodSO$_4$/PAGE (Fig. 2*A*).

Immunological Characterization of Protein Kinase C Isozymes. Immunoblot analysis of the various kinase fractions isolated from hydroxylapatite column chromatography with polyclonal antibodies that had been raised against a mixture of all three types of protein kinase C revealed an immunoreactive polypeptide with a molecular weight of 82,000 (Fig. 3*A*). These antibodies immunoprecipitated all three kinases (Fig. 4); however, the antibody titer against type III enzyme appeared lower than those of type I and type II kinase. Immunoblot analysis of the same kinase fractions using monoclonal antibody against type I (Fig. 3*B*) or type II (Fig. 3*C*) enzyme showed a selective immunoreactivity against each respective enzyme. As shown in Fig. 1, the separation of type I and type II kinase by hydroxylapatite column chromatography was not absolute, and, consequently, we found that certain fractions (nos. 35 and 36) were cross-reactive with both monoclonal antibodies. By careful screening of the effluent fractions we were able to pool the type I and type II kinase free from cross contamination. Type III kinase separated quite well from the two other enzyme forms; however, occasionally we found some minor cross-reactive protein against type II antibody in the higher-salt eluate of the type III activity peak (e.g., fraction 65). The major type III activity peak (from fraction 59 to 63) is free from immunoreactive material against either type I or type II monoclonal antibody. Monoclonal antibodies against type I or type II kinase only inhibit the respective enzyme, and neither inhibits the type III kinase (data not shown).

Autophosphorylation of Three Types of Protein Kinase C. All three types of protein kinase C undergo autophosphorylation in the presence of Ca^{2+}, PtdSer, and diolein (Fig. 5*A*). In the presence of EGTA without lipid the autophosphorylation rate of these kinases was low, indicating that the autocatalytic activity also depends on Ca^{2+} and PtdSer. We also found that the addition of diolein reduced the K_a for Ca^{2+} from ≈20 to 0.1 μM for the three enzymes. The stoichiometries of autophosphorylation for type I, II, and III enzymes were 1.5, 1.0, and 1.0 (mol/mol) of enzyme, respectively, when measured in the presence of 40 μM ATP, 40 μg of PtdSer per ml, and 8 μg of diolein per ml. Autophosphorylation of these kinases incorporates ^{32}P from [γ-^{32}P]ATP into the enzyme molecule. The autophosphorylated type I and type II kinases have respective apparent molecular weights of 88,000 and 84,000, which are greater than the nonphosphorylated en-

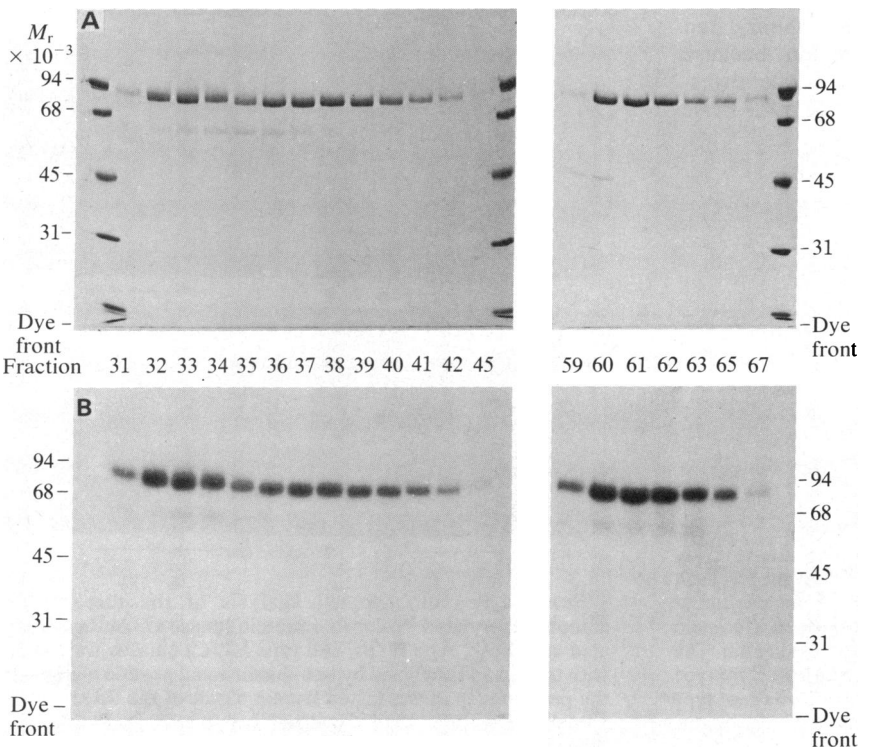

FIG. 2. Identification of protein kinase C isolated from hydroxylapatite column chromatography by autophosphorylation. Fractions from hydroxylapatite column chromatography (Fig. 1) were incubated under standard autophosphorylation assay conditions in the presence of [γ-^{32}P]ATP; the reaction was done at 30°C for 30 min, and the reaction mixtures were analyzed by NaDodSO$_4$/PAGE (10% gel) after heating at 95°C for 5 min in the presence of electrophoresis sample buffer. (*A*) Coomassie blue-stained gel. (*B*) Autoradiograph. Ordinates indicate position of molecular weight markers.

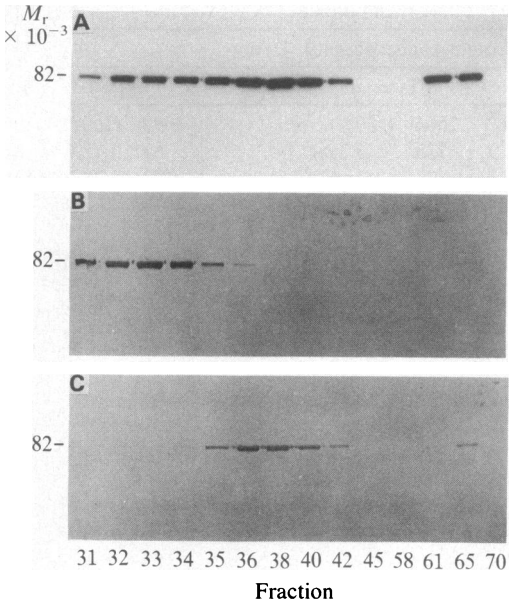

FIG. 3. Identification of protein kinase C isozymes by immunoblotting. Fractions from hydroxylapatite column chromatography were separated by NaDodSO$_4$/PAGE (10% gel) and transferred to nitrocellulose membrane. Immunoblot analysis was done with polyclonal antibodies against the heterogeneous protein kinase C (*A*), monoclonal antibody against type I enzyme (*B*), or monoclonal antibody against type II enzyme (*C*).

zyme of 82,000 (Fig. 5*B*). In contrast, autophosphorylation of the type III kinase caused no significant change in the apparent molecular weight.

Peptide Mapping Analysis of the Autophosphorylation Sites. An analysis of the ^{32}P-labeled tryptic peptides derived from autophosphorylated protein kinase C showed distinct separation patterns for type I (Fig. 6*A*), type II (Fig. 6*B*), and type III (Fig. 6*C*) kinases. The nonoverlapping peptide maps of the three ^{32}P-labeled kinase preparations were clearly recognized in a map from a mixture of these tryptic digests (separated under the same conditions) (Fig. 6*D*). The presence of multiple ^{32}P-labeled peptides in each autophosphorylated kinase is not due to incomplete tryptic digestion, because similar separation patterns could be seen after further digestion with additional trypsin. The best explanation for these

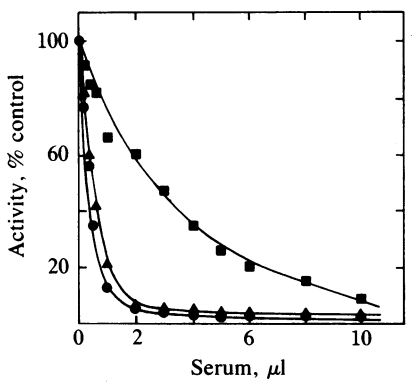

FIG. 4. Inhibition of type I, II, and III protein kinase C by polyclonal antibodies. Purified protein kinase C (50 ng for each isozyme) was incubated with various amounts of immune serum at room temperature for 30 min. Test samples were adjusted to contain equivalent amounts of protein by adding bovine serum albumin. The kinase activity (0.7 U for type I, 1.0 U for type II, and 0.96 U for type III) without immune serum was considered as 100%. Type I, ●; type II, ▲; and type III, ■. Addition of similar amounts of preimmune serum caused no inhibition of these kinases.

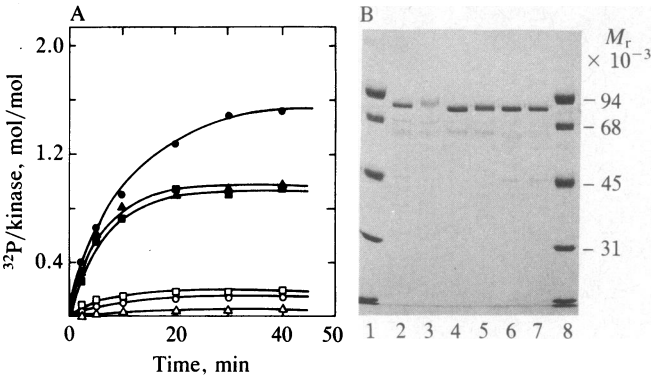

FIG. 5. Comparison of stoichiometry of autophosphorylation and resulting change in the electrophoretic mobility of the three types of protein kinase C. (*A*) Type I (●, ○), type II (▲, △), and type III (■, □) protein kinase C were autophosphorylated in the presence of Ca^{2+}, PtdSer, and diolein (●, ▲, ■), or in the presence of EGTA without lipid (○, △, □) for the measurement of stoichiometry. (*B*) After 40 min of incubation, aliquots were taken for analysis by NaDodSO$_4$/PAGE and staining with Coomassie blue. Lanes 1 and 8 are standard marker proteins; lanes 2, 4, and 6 are nonphosphorylated type I, II, and III protein kinase C, respectively; lanes 3, 5, and 7 are autophosphorylated type I, II, and III protein kinase C, respectively.

observations is that multiple sites can be phosphorylated; however, the reason for substoichiometric phosphorylation of such sites is unknown. Phosphoamino acid analysis of the autophosphorylated kinases revealed that both type I and type III kinases were phosphorylated at serine residues, whereas the type II enzyme was phosphorylated at both serine and threonine residues (Fig. 7). From these results the amino acid sequences surrounding the autophosphorylation sites of these three protein kinase C forms must be different.

DISCUSSION

These results demonstrate the presence of isozymic forms of rat brain protein kinase C. Based on the observations that (*i*) multiple enzyme forms can be separated by hydroxylapatite column chromatography, (*ii*) polyclonal antibodies have

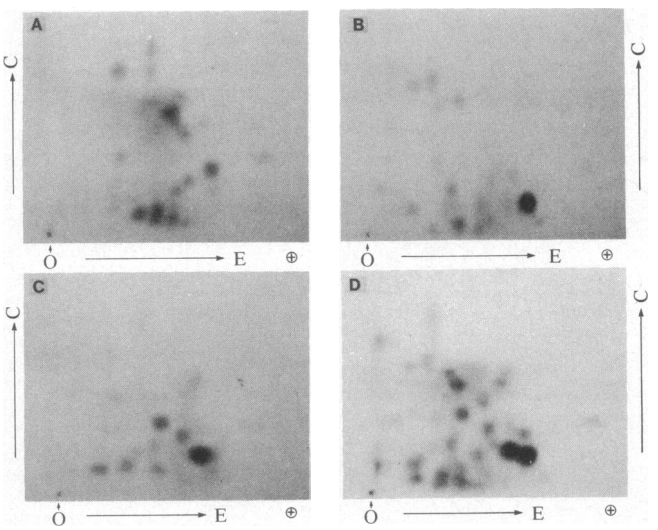

FIG. 6. Peptide mapping analysis of the three types of autophosphorylated ^{32}P-labeled protein kinase C. Autophosphorylated type I (*A*), type II (*B*), and type III (*C*) kinases were digested with trypsin and analyzed by two-dimensional peptide mapping; *D* is the peptide map of the mixed tryptic digest of the three isozymes. ^{32}P-labeled peptides were visualized by autoradiography. Directions of electrophoresis (→ E) and chromatography (→ C) are as indicated.

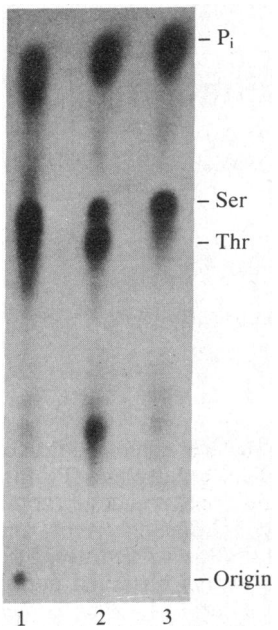

FIG. 7. Phosphoamino acid analysis of the three types of autophosphorylated protein kinase C. The ^{32}P-labeled tryptic peptides derived from the three types of autophosphorylated kinase were hydrolyzed in 6 M HCl at 110°C for 2 hr. Phosphoamino acids from type I (lane 1), II (lane 2), and III kinase (lane 3) were analyzed by thin-layer electrophoresis at pH 3.5 and then by autoradiography. P_i, inorganic phosphate; Ser, phosphoserine; Thr, phosphothreonine.

differential potency against these enzymes, (*iii*) there are specific monoclonal antibodies against the type I or type II enzyme, and (*iv*) these kinases have different sites of autophosphorylation, we have distinguished among these three isozymes. However, these enzymes also have similarities: (*i*) an apparent molecular weight of 82,000 when analyzed by $NaDodSO_4$/PAGE; (*ii*) copurification by ion exchange, hydrophobic, gel filtration, and affinity chromatography; (*iii*) binding of [^{3}H]PBt_2 in a Ca^{2+}- and PtdSer-dependent manner; and (*iv*) a kinase activity dependent on Ca^{2+} and PtdSer. Thus, the three types of protein kinase C represent a structurally and functionally related enzyme family in rat brain. By using polyclonal antibodies against these rat brain enzymes we have detected cross-reactivity against the same enzyme from various rat tissues as well as from cells of different origin including human platelets, neutrophils, and T lymphocytes. The immunoreactive determinants for protein kinase C from these mammalian origins must be similar. However, we have not detected an immunoreactive protein in yeast or *Escherichia coli* by using antibodies against rat brain protein kinase C. To establish the evolutionary phylogeny of this enzyme by determining the distribution of protein kinase C isozymes in different animals would be interesting.

Isozymic forms of protein kinases are a well-known phenomenon. The two major classes of cAMP-dependent protein kinase, designated type I and type II, are defined on the basis of their elution from DEAE-cellulose chromatographic columns (26). The differences between these two enzymes in molecular weights, sedimentation coefficients, autophosphorylation potential, subunit dissociation–association characteristics, cAMP-binding properties, and immunological properties have been attributed to changes in the primary structure of the regulatory subunits (27–29). Alteration in one subunit structure of the enzyme has also been reported to produce the different phosphorylase kinase isozymes in muscle (30, 31). In contrast, the isozymic forms of Ca^{2+}/calmodulin-dependent protein kinase II from different regions of the brain are due to differences in the ratios of their multiple subunits (32, 33). For the monomeric protein kinase C isozymes, the immunological differences and differences in autophosphorylation sites are clear indicators of their differences in primary structure; these isozymes are probably products of related but distinct genes.

Protein kinase C has been implicated in the transmembrane signaling that regulates many cellular functions and is involved in cellular proliferation (14). In addition, this enzyme has been identified as a putative receptor for the tumor-promoting phorbol esters (18, 19). The precise role of protein kinase C in regulating normal cellular function and pathological development is not clear. Because most of the isozymes are known to be localized in different tissues and subcellular sites, the various protein kinase C isozymes may be responsible for different regulatory roles within the cell. The identification of various protein kinase C isozymes should be useful in determining the association of these kinases with different cellular processes.

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