Isolation and Characterization of the Calcium- and Phospholipid-Dependent Protein Kinase (Protein Kinase C) Subtypes from Bovine Heart†

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ABSTRACT: Protein kinase C was isolated from bovine heart by chromatography on DEAE-Sephacel, phenyl-Sepharose, poly(L-lysine) agarose, and hydroxylapatite. Estimates based upon enzyme recovery indicate 10–20 nmol/min of protein kinase C activity per gram of bovine ventricular myocardium. Hydroxylapatite column chromatography resolved the preparation into two peaks of calcium- and phospholipid-dependent protein kinase activity. By Western blot analysis, peaks 1 and 2 contained subtypes II (β_2) and III (α), respectively. No cross-reactivity was observed, indicating that separation was complete. Type III, the major subtype detected, was subsequently purified to apparent homogeneity by chromatography on phosphatidylserine (PS) acrylamide. Type II activity could not be recovered following phosphatidylserine affinity chromatography. Phospho amino acid analysis showed that type III autophosphorylated at serine residues, whereas type II autophosphorylated at both serine and threonine residues. Among the various phospholipids tested for activity, PS was the most effective. Both subtypes were activated by 1-stearoyl-2-arachidonylglycerol (SAG) in the presence of phosphatidylserine and calcium. Activation of both subtypes occurred at calcium concentrations of less than 1 μM. In addition to several similarities, these two subtypes showed differences in activation and kinetic properties: type II was activated by cardiolipin, 1,2- and 1,3-dioleoylglycerol, and both cis- and trans-unsaturated fatty acids. Type III was activated to a lesser degree by cardiolipin and showed no response to 1,3-dioleoylglycerol. Type III was activated to a greater extent by 1,2-diacylglycerols and by cis-unsaturated fatty acids. In the presence of PS and SAG, type II exhibited substantial activity in the presence of 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) without added calcium. Activation of types II and III by unsaturated fatty acids was independent of phospholipid and showed a lower apparent calcium affinity than that observed for activation by phosphatidylserine. These results show that cardiac protein kinase C subtypes II and III were functionally distinguishable and may play unique roles in the regulation of cardiac function.

Protein kinase C (PKC) is a ubiquitous (Kuo et al., 1980) calcium- and phospholipid-dependent serine and threonine protein kinase that has been implicated in the regulation of many intracellular processes (Nishizuka, 1986). Although activated to a degree by acidic phospholipids, the most effective phospholipid for activation of protein kinase C is phosphatidylserine (Takai et al., 1979a; Kaibuchi et al., 1981). Unsaturated 1,2-diacylglycerols stimulate the activity of protein kinase C by increasing the apparent affinity of the enzyme for both phosphatidylserine and calcium (Takai et al., 1979b; Kishimoto et al., 1980; Kaibuchi et al., 1981) such that enzyme activation may occur without an increase in intracellular calcium (Nishizuka, 1986). 1,2-Diacylglycerols may be generated by the phospholipase C mediated hydrolysis of inositol phospholipids (Nishizuka, 1984; Berridge & Irvine, 1984) or possibly by the breakdown of other phospholipids such as phosphatidylcholine (Besterman et al., 1986). Tumor-promoting phorbol esters have been shown to mimic the effects of 1,2-diacylglycerols in the activation of protein kinase C. Upon activation, protein kinase C phosphorylates a range of cellular proteins including troponins T and I (Mazzei & Kuo, 1984; Katoh et al., 1983), the epidermal growth factor receptor (Cochet et al., 1984), myelin basic protein (Turner et al., 1984), the gap junction protein (Takeda et al., 1987), ribosomal protein S6 (LePeuch et al., 1983), microtubule-associated protein (Kikkawa et al., 1982), and phospholamban (Movesian et al., 1984).

Molecular cloning analysis of brain cDNA libraries has revealed that protein kinase C, once thought to be a single protein, exists as a family of at least seven subtypes (α, β_1, β_2, γ, δ, ϵ, and ζ) having similar structural characteristics (Huang et al., 1986b; Knopf et al., 1986; Ohno et al., 1987; Nishizuka, 1988) but different activators or kinetic properties (Sekiguchi et al., 1987; Huang et al., 1988; Sekiguchi et al., 1988; Marais & Parker, 1989). Protein kinase C isolated from rat (Huang et al., 1986b; Kosaka et al., 1988; Shearman et al., 1987; Yoshida et al., 1988), monkey (Huang et al., 1987a), and rabbit (Jaken & Kiley, 1987) brain has been separated into three subtypes by chromatography on hydroxylapatite columns. On the basis of the order and phosphate concentration required for elution, these subtypes have been named types I, II, and III (Huang et al., 1986b). The genetic origin of the subtypes has been determined by transient expression analysis in COS cells (Huang et al., 1987b; Kikkawa et al., 1987). Clones γ, β_1/β_2, and α encode subtypes I, II, and III, respectively. Examination of the distribution of various protein kinase C mRNAs indicates a tissue- and cell-specific, rather than a species-specific, pattern of expression (Knopf et al., 1986; Ohno et al., 1987). Protein kinase C type I is found only in tissues of the central nervous system, whereas types II and III are common in various tissues (Kosaka et al., 1988; Yoshida et al., 1988; Naor, 1990). Immunocytochemical studies have shown different regional localization (Huang et

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al., 1987a, 1989, 1990; Shearman et al., 1987; Hidaka et al., 1988; Yoshida et al., 1988; Hosoda et al., 1989; Saito et al., 1989) and subcellular distribution (Saito et al., 1989; Huang et al., 1990) of protein kinase C subtypes in mammalian brain. The specific expression patterns and intracellular localization of protein kinase C subtypes suggest that each subtype may have a defined function in the processing and modulation of tissue responses to external stimuli.

In the heart, activation of α_1-adrenergic, muscarinic cholinergic, adenosine, or angiotensin II receptors stimulate phosphatidylinositol turnover (Brown & Brown, 1984; Brown et al., 1985; Allen et al., 1988; Legssyer et al., 1988). Perfusion of isolated beating rat hearts with either membrane permeant 1,2-diacylglycerols or tumor-promoting phorbol esters produces dose-dependent negative inotropic and chronotropic changes (Yuan et al., 1987). These observations suggest that protein kinase C may be involved in the regulation of cardiac function. It has been found that protein kinase C from bovine heart differs from that in brain in selectivity for exogenous substrates (Wise et al., 1982). Rat heart contains two subtypes of protein kinase C, types II and III (Kosaka et al., 1988). The type II enzyme from rat heart appears immunochemically distinct from type II from other tissues and displays a lower requirement for calcium (Kosaka et al., 1988). This potentially novel subtype has not been characterized.

The present study was undertaken to determine the biochemical characteristics of the subtypes of protein kinase C from bovine heart. Two subtypes of cardiac protein kinase C were separated by hydroxylapatite column chromatography and identified as types II (β_2) and III (α) with the use of isozyme-specific antibodies. Type III was the major subtype present in this preparation. The kinase activities of these subtypes were distinguishable with respect to activation by calcium, phospholipids, neutral lipids, and fatty acids.

Materials and Methods

Materials. The following materials were obtained from the indicated sources: Tris–ATP, histone H1 type III-S, EGTA,[1] poly(L-lysine) agarose, DEAE-Sephacel, pepstatin A, aprotinin, soybean trypsin inhibitor, and HEPES were from Sigma Chemical Co.; dithiothreitol, Triton X-100, phenylmethanesulfonyl fluoride and leupeptin were from Boehringer Mannheim; [γ-^{32}P]ATP (10–30 Ci/mmol) was from Amersham; DNA grade hydroxylapatite (Bio-Gel HTP), phenyl-Sepharose, Chelex-100, and protein determination, immunoblotting, and polyacrylamide gel electrophoresis reagents were from Bio-Rad. All lipids were from Serdary Research Laboratories. Analytical grade calcium chloride and magnesium chloride were from BDH. Ultrapure grade ammonium sulfate was from Schwarz/Mann Biotech. P-81 ion-exchange paper was from Whatman. Human IgG adsorbed horseradish peroxidase conjugated affinity isolated goat F(ab')2 anti-mouse immunoglobulins and anti-rabbit immunoglobulins were purchased from TAGO Immunologicals Inc. Monoclonal antibodies to protein kinase C type I (γ) were from Seikagaku America, Inc. Antisera against protein kinase C types II (β_2) and III (α), provided by Dr. Christopher Wilson (Department of Pediatrics, University of Washington, Seattle, WA), were raised against synthetic peptides that appear in the amino acid sequences of the subspecies as deduced from their cDNA sequences (Kikkawa et al., 1987a). The polyclonal antibodies against type II (β_2) and type III (α) were prepared against peptides SFVNSEFLKPEVKS (β_2 sequence, amino acid residues 660–673) and AGNKVISPSEDRRQ (α sequence, amino acid residues 313–326), respectively. In the latter peptide, the conservative substitution of an arginine for lysine 325 was made (Makowski et al., 1988).

Enzyme Assay. Protein kinase C activity was determined as described by Kikkawa et al. (1982). The reaction mixture (60 μL) contained (final concentration) 20 mM HEPES/KOH buffer (pH 7.5 at 30 °C), 1 mg/mL histone III-S, 0.1 mM [γ-^{32}P]ATP (100–300 cpm/pmol), 5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 20 μg/mL leupeptin, 1 mM EGTA, 0.875 mM $CaCl_2$ (1 μM free), 80 μg/mL phosphatidylserine, 8 μg/mL 1-stearoyl-2-arachidonylglycerol, and protein kinase. The phospholipid-independent kinase activity was measured under the same conditions without the addition of calcium or phospholipid. Following a 3-min preincubation, the reaction was initiated by the addition of [^{32}P]ATP and quenched after 2 min by the transfer of a 40-μL aliquot of the mixture onto a 1.5 × 1.5 cm square of P-81 paper. Phospho cellulose squares were then washed with 75 mM phosphoric acid (Roskoski, 1983) and dried, and their ^{32}P content was determined by liquid scintillation counting. One unit of kinase activity was defined as the amount of enzyme incorporating 1 nmol of phosphate from ATP into histone type III-S in 1 min at 30 °C. All assays were performed in polypropylene tubes and were linear with respect to time and enzyme concentration. To autophosphorylate protein kinase C, the conditions were as described above. However, histone was omitted from the medium, and the reaction was terminated by adding SDS sample buffer. Lipids were stored at −20 °C in either chloroform/methanol (95:5) (phospholipids) or hexane (neutral lipids, fatty acids) containing 0.05% (w/v) butylated hydroxytoluene. The purity of neutral lipids and phospholipids used in these studies was checked by thin-layer chromatography (Kates, 1986). Lipid vesicles were prepared immediately prior to use by evaporation to dryness under a gentle stream of nitrogen gas followed by sonication into 20 mM HEPES/KOH (pH 7.5) in a bath-type sonicator (Branson 1200).

Preparation of Cardiac Protein Kinase C. Protein kinase C was purified by a modification of the procedure previously described by Huang and co-workers (1986b). Bovine hearts, obtained fresh from the slaughterhouse, were placed on ice and transported to the laboratory. All subsequent procedures were performed at 4 °C. Tissue (50 g) was minced and then homogenized in 500 mL of buffer A with a Waring blender. Buffer A consisted of 20 mM Tris/HCl buffer (pH 7.5 at 20 °C) containing 10 mM EGTA, 2 mM EDTA, 0.29 M sucrose, 2 mM DTT, 2 mM phenylmethanesulfonyl fluoride, 0.5% (v/v) Triton X-100, 10 μg/mL soybean trypsin inhibitor, 0.7 μg/mL pepstatin A, 1 μg/mL aprotinin, and 1 μg/mL leupeptin. The blender bottle assembly was filled so as to exclude air. The resulting homogenate was allowed to stir for 30 min, which was followed by centrifugation for 60 min at 13 500 rpm in a Beckman Type JA-14 rotor. The high-speed supernatant was passed through eight layers of cheesecloth and then adjusted to 1% (v/v) Triton X-100 and pH 7.5 prior to being loaded onto a DEAE-Sephacel column (2.6 × 38 cm, 125 mL/h) previously equilibrated with 20 mM Tris/HCl buffer (pH 7.5, 4 °C) containing 5 mM EGTA, 2 mM EDTA, 2 mM DTT, 1% Triton X-100, and 10% (v/v) glycerol. The column

[1] Abbreviations: Ca^{2+}, free calcium; DG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; Tris/HCl, tris(hydroxymethyl)aminomethane hydrochloride; SAG, 1-stearoyl-2-arachidonylglycerol; U, unit.

was washed with 1 bed vol of buffer A followed by 1 bed vol of 20 mM Tris/HCl (pH 7.5, 4 °C), 0.5 mM EGTA, 0.5 mM EDTA, 2 mM DTT, 10% (v/v) glycerol (buffer B). The absorbance (at 280 nm) of the column eluate was monitored to ensure that the detergent was removed by this wash step. PKC was subsequently eluted with 150 mM NaCl in buffer B. Powdered ammonium sulfate was added to the DEAE pool to achieve a 20% saturated solution (144 g/L), which was stirred for 30 min and then centrifuged at 10000 rpm in a Beckman Type JA-20 rotor for 15 min. The resulting supernatant was applied to a phenyl-Sepharose column (10 × 2.5 cm, 100 mL/h) equilibrated with buffer B containing 0.8 M $(NH_4)_2SO_4$, and the column was washed with 250 mL of this buffer. The column was eluted with a 300-mL linear gradient of 0.8–0.0 M $(NH_4)_2SO_4$ in buffer B at 30 mL/h. Fractions of 10 mL were collected. The gradient was followed by an additional wash with 150 mL of buffer B alone. Fractions containing calcium- and phospholipid-dependent protein kinase activity were pooled, concentrated by ultrafiltration to a volume of 10 mL, and dialyzed overnight against 1.0 L of buffer B. The dialyzed fraction was applied to a poly(L-lysine) agarose column (1.5 × 14 cm, 20 mL/h) that was eluted with a gradient of 0–0.8 M KCl in buffer B (180 mL, 20 mL/h). Fractions of 5 mL were collected. The active fractions were pooled, concentrated to 10 mL by ultrafiltration, and dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.5 at 4 °C) containing 0.5 mM EGTA, 0.5 mM EDTA, 10% (v/v) glycerol, 0.2 mM phenylmethanesulfonyl fluoride, 0.02% (w/v) sodium azide, and 2 mM DTT (buffer C). The concentrated fraction was then loaded onto a Bio-Gel HTP column (1.5 × 5 cm, 10 mL/h) previously equilibrated with buffer C. The column was developed by use of an exponential gradient (90 mL) of 0–70 mM KH_2PO_4 in buffer C followed by a linear gradient (70 mL) of 70–280 mM KH_2PO_4 in buffer C. Fractions of 2.5 mL were collected. Active fractions were pooled, concentrated to 2 mL by ultrafiltration, and dialyzed against 20 mM HEPES/KOH buffer (pH 7.5 at 4 °C) containing 0.5 mM EGTA, 0.5 mM EDTA, 20% (v/v) glycerol, 0.05% (v/v) Triton X-100, 2 mM DTT, and 0.02% sodium azide (storage buffer). For subsequent purification by chromatography on phosphatidylserine acrylamide, the pooled enzyme was adjusted to 7.5 mM $MgCl_2$, 0.2 M KCl, 20 μg/mL leupeptin, and 0.2 mM $CaCl_2$ as described by Jaken and Kiley (1987). Samples were loaded onto phosphatidylserine acrylamide columns (1.0 × 3.0 cm), prepared as described by Uchida and Filburn (1984), equilibrated with 10 mM 2-(*N*-morpholino)ethanesulfonic acid/KOH (pH 6.5 at 4 °C) containing 10% (v/v) glycerol, 0.2 M KCl, 1 μg/mL leupeptin, and 2 mM DTT (buffer D) plus 0.2 mM $CaCl_2$. Columns were washed with 25 mL of buffer D containing 0.2 mM $CaCl_2$ and eluted with 15 mL of buffer D containing 2 mM EGTA. The resulting pools were concentrated, dialyzed into storage buffer, and stored at −80 °C.

Immunoblotting. Immunoblotting was carried out according to the method of Dunn (1986) with reagents and procedures provided by Bio-Rad. Polypeptides from each sample were separated by SDS–polyacrylamide gel (5–20%) electrophoresis followed by electrophoretic transfer to Zeta Probe membranes. Nonspecific binding sites on the membrane were blocked by incubation overnight at 4 °C with 5% BLOTTO in Tris-buffered saline (TBS) (20 mM Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl). The membrane was then incubated with isozyme-specific antibodies in TBS containing 1% BLOTTO for 2 h followed by horseradish peroxidase conjugated anti-immunoglobulin antibodies (1:2000) for 1 h. In between incubations, the membrane was washed extensively with TBS containing 0.02% Tween-20. Immunoreactive bands were then visualized by reaction with 4-chloro-1-naphthol. The M_r of immunoreactive bands was determined with the use of prestained molecular weight markers (Bio-Rad).

Table I: Purification of Protein Kinase C from Bovine Heart

fraction	volume (mL)	protein (mg/mL)	specific activity[a] (U/mg)	total activity (U)
crude supernatant	550	10.9	ND	
DEAE-Sephacel	190	4.5	ND	
phenyl-Sepharose	79	1.2	3.7	350
poly(L-lysine) agarose	37	0.75	26	730
hydroxylapatite 1	12	0.22	8.0	39
hydroxylapatite 2	38	0.048	330	590
PS acrylamide	15	0.0045	4300	290

[a] Protein kinase C was assayed as described in Materials and Methods. One unit of protein kinase was defined as the amount of enzyme catalyzing the incorporation of 1 nmol of ^{32}P into histone III-S per minute at 30 °C. ND indicates that no calcium- and phospholipid-dependent protein kinase activity was detected. Aliquots with volumes of 5, 10, 15, and 20 μL were assayed, and protein kinase activity was determined by linear regression.

Miscellaneous Methods. SDS–polyacrylamide gradient (5–20%) gel electrophoresis was carried out according to the method of Laemmli (1970). Protein concentrations were determined by the method of Bradford (1976) with bovine γ-globulin as standard. Free calcium concentrations were determined as described previously (Allen et al., 1987) by use of the computer program CATIONS (Goldstein, 1979).

RESULTS

Purification of Cardiac Protein Kinase C. Various procedures for the extraction and purification of protein kinase C were investigated in order to optimize recovery and allow determination of the subtypes present in cardiac tissue. Results from the most efficient and reproducible of these are detailed in Table I and involve sequential chromatography on DEAE-Sephacel, phenyl-Sepharose, poly(L-lysine) agarose, and hydroxylapatite (see Materials and Methods). No calcium- and phospholipid-dependent protein kinase activity was detected in either the supernatant from the homogenate or the DEAE-Sephacel pool. In the supernatant, this may have been due to the presence of Triton X-100. However, steps were taken to ensure that the fractions from the DEAE-Sephacel column were essentially free of detergent. Modifications to the assay such as an increase in the reaction time, an alteration of the volume of enzyme solution added to the assay, or the addition of phospho protein phosphatase inhibitors did not improve the detection of PKC activity in the fractions eluted from DEAE-Sephacel column. One possible explanation is that endogenous inhibitors of protein kinase C (Schwantke & LePeuch, 1984; McDonald & Walsh, 1985, 1986; Pripilla et al., 1988; Pearson et al., 1990) may have been present and thus prevented the detection of calcium- and phospholipid-dependent protein kinase activity during these earlier stages of purification. This latter possibility appears likely, as during purification the recovery of protein kinase C activity increased, rather than decreased, up to the poly(L-lysine) agarose step (Table I).

The protein kinase C activity pooled from the poly(L-lysine) agarose column was separated into two peaks of calcium- and phospholipid-dependent protein kinase activity on a hydroxylapatite column (Figure 1). Peak 1 eluted at 25–40 mL of the gradient; peak 2 eluted at 50–100 mL. The most abundant subtype, peak 2, could be isolated in large amounts with a

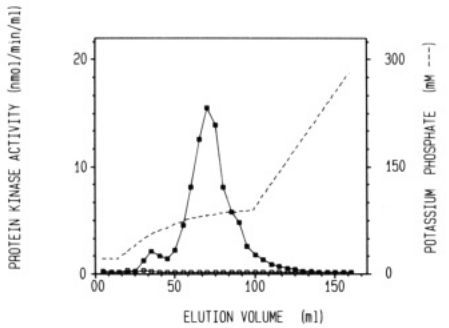

FIGURE 1: Separation of subtypes of cardiac protein kinase C by hydroxylapatite column chromatography. Fractions of 2.5 mL were collected for the measurement of protein kinase activity in the presence of calcium, PS, and SAG (■) or 1 mM EGTA without lipid (□). The phosphate concentration for each fraction was derived from direct determinations of conductivity. The active fractions within the two activity peaks were pooled and designated pools 1 and 2.

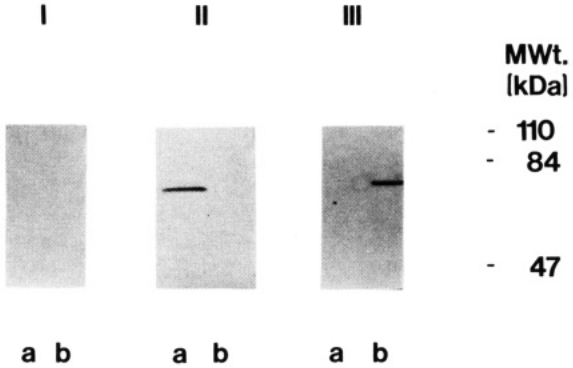

FIGURE 2: Immunoblot of pools 1 and 2 from the hydroxylapatite column. The two peaks of activity from the hydroxylapatite column were pooled and concentrated by ultrafiltration. Eighty milliunits per lane of pool 1 (a) or pool 2 (b) was electrophoresed and transferred to Zeta Probe membranes, and immunoblot analysis was performed as described. Antibodies used were anti-PKC I (γ, I), anti-PKC II (β_2, II), and anti-PKC III (α, III). The standard molecular weights indicated are for the prestained markers phosphorylase B (110), bovine serum albumin (84), and ovalbumin (47).

specific activity of 300–350 U/mg. Fractions lying between the two peaks were discarded to avoid cross-contamination of the two activities. The isozyme content of the separate pools was determined by protein immunoblot analysis using isozyme-selective antisera (Figure 2). It was found that peaks 1 and 2 contained types II (β_2) and III (α), respectively. No cross-reactivity was observed between pools 1 and 2, indicating that separation of the two subtypes was complete. With phosphatidylserine (PS) affinity chromatography as a final step, type III could be obtained in an electrophoretically homogeneous form with a high specific activity (4200–4400 U/mg). Protein kinase C type II activity, though, could not be recovered following application to a PS affinity column and became very labile upon further purification. Although protein kinase C type II could not be purified to homogeneity, no calcium/calmodulin- or cAMP-dependent protein kinase activities were detected in this fraction. As type II did not contain kinase activities other than protein kinase C and separation of types II and III was complete following chromatography on a hydroxylapatite column, all subsequent biochemical characterizations of type II were performed on this fraction. The protein kinase C type III employed in these studies was purified by PS affinity chromatography. During storage (see Materials and Methods), type III retained its full activity and calcium/lipid dependency for over a year, whereas the activity of type II decreased markedly ($t_{1/2}$ = 4–6 mo).

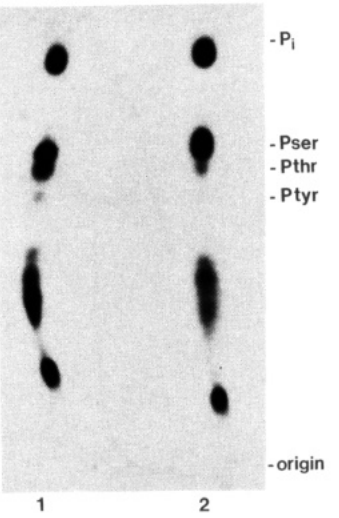

FIGURE 3: Phospho amino acid analysis of autophosphorylated protein kinase C types II and III. Autophosphorylated (see Materials and Methods) protein kinase C subtypes (1 U/lane) were localized on gels by autoradiography and excised. Gel fragments were rehydrated and incubated overnight at 30 °C in 0.5 mL NH_4HCO_3 buffer containing 0.15 mg/mL of TPCK-treated trypsin. ^{32}P-Labeled tryptic peptides were hydrolyzed in 6 M HCl at 110 °C for 1 h. The acid hydrolysate was lyophilized and resuspended in deionized water containing phospho amino acid standards (1 mg/mL). Phospho amino acids from type II (lane 1) and type III (lane 2) kinase were analyzed by electrophoresis on cellulose TLC plates at pH 2.8 in pyridine/acetic acid/water (0.125:5:95) for 30 min at 1 kV. ^{32}P-Phospho amino acids and phospho amino acid standards were visualized by autoradiography and ninhydrin staining, respectively. Key: P_i, inorganic phosphate; Pser, phosphoserine; Pthr, phosphothreonine; Ptyr, phosphotyrosine.

Similar results have been obtained for protein kinase isolated from canine hearts.

Autophosphorylation of Protein Kinase C Isozymes. In the presence of calcium, phosphatidylserine, and 1-stearoyl-2-arachidonylglycerol, subtypes II and III encorporated ^{32}P from [γ-^{32}P]ATP into the kinase molecule. In the presence of EGTA without lipid, ^{32}P encorporation was low, indicating that, as with catalytic activity, autophosphorylation was calcium- and lipid-dependent. The molecular masses of the autophosphorylated forms of types II and III were 81 and 83 kDa, respectively. Phospho amino acid analysis of the autophosphorylated kinase (Figure 3) revealed that type III was phosphorylated at serine residues, whereas type II was strongly phosphorylated at both serine and threonine residues as previously demonstrated for types II and III from rat brain (Huang et al., 1986b).

Activation of Protein Kinase C Isozymes by Calcium. The effect of calcium concentration on both the phospholipid-dependent and the fatty acid dependent activation of cardiac protein kinase C subtypes was examined. When assayed in the presence of phosphatidylserine and concentrations of EGTA less than 0.5 mM, no calcium dependency was observed (not shown). Pretreatment of all buffers with Chelex-100, inclusion of 1 mM EGTA, and titration with $CaCl_2$ (see Materials and Methods), to achieve the desired free calcium concentrations, resulted in kinase activity that, in the presence of either phosphatidylserine or phosphatidylserine plus 1-stearoyl-2-arachidonylglycerol, was dependent on the presence of calcium (Figure 4). This concentration of EGTA did not affect the maximum activity of the enzyme. In the presence of 80 μg/mL PS, both type II and type III were activated within a similar range of calcium concentrations. Under these assay conditions, 1-stearoyl-2-arachidonylglycerol (8 μg/mL) produced a small decrease in the calcium requirement and an increase in the activity of type III. The effect of 1-stearoyl-

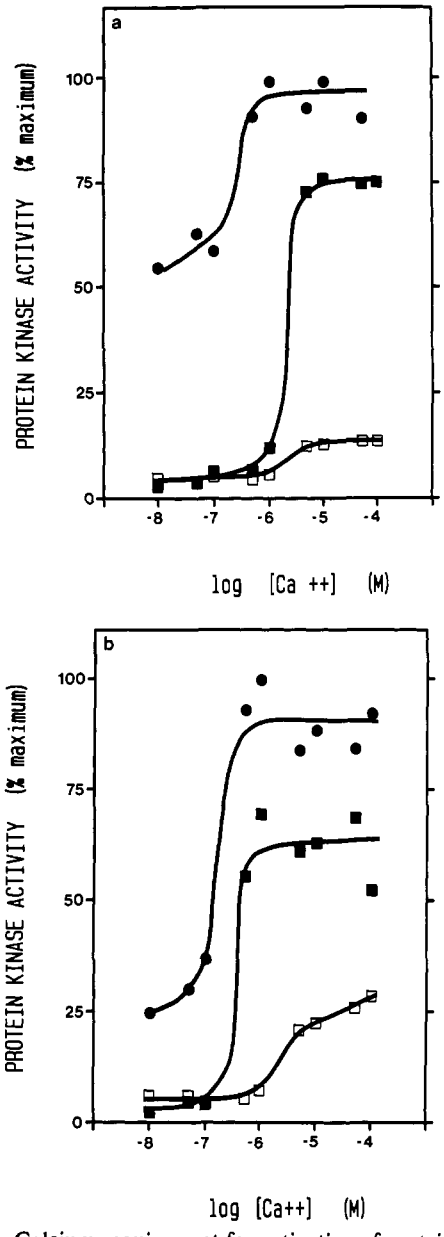

FIGURE 4: Calcium requirement for activation of protein kinase C types II and III. Protein kinase activity of types II (panel a) and III (panel b) was measured under the standard assay condition containing 0.05 U/mL protein kinase C and either 80 μg/mL PS alone (■), 8 μg/mL SAG alone (□), or PS plus SAG (●) and increasing concentrations of free calcium. Enzyme activities in the presence of 1 mM EGTA without lipid were subtracted from the measured activities.

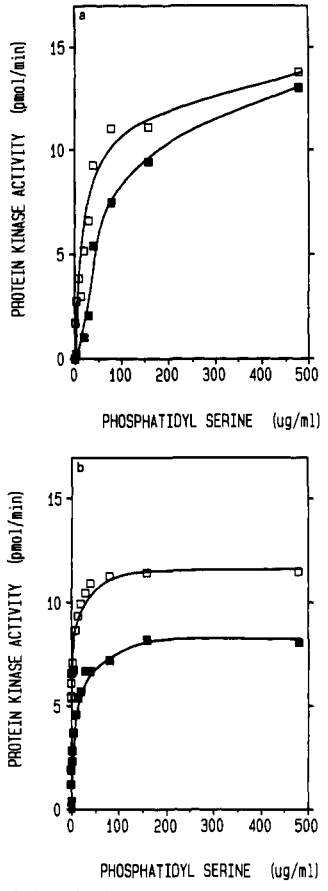

FIGURE 5: Effect of phosphatidylserine on the activity of protein kinase C type III. Protein kinase activity of type III was measured under the standard assay conditions with increasing concentration of PS in the presence (□) or absence (■) of 8 μg/mL SAG and at concentrations of 1 μM (panel a) or 10 μM (panel b) free calcium. Enzyme activities in the presence of 1 mM EGTA without lipid were subtracted from the measured activities.

2-arachidonylglycerol on type II was more pronounced, producing a component of activation that appeared to be calcium-independent. At calcium concentrations greater than 5 μM, both enzymes were partially activated by 1-stearoyl-2-arachidonylglycerol alone. Pretreatment of the lipid suspensions with Chelex-100 had no effect on the apparent K_{Ca}. These results indicate that types II and III have similar affinities for calcium in a pure PS system. However, in the presence of both PS and diacylglycerol, type II appears less calcium-dependent than type III.

Activation of Protein Kinase C Isozymes by Phospholipids. The specificity of types II and III for activating phospholipids was examined in the presence and absence of 1-stearoyl-2-arachidonylglycerol. In order to establish reaction conditions to enable this comparison, activation of type III by phosphatidylserine alone and phosphatidylserine plus 1-stearoyl-2-arachidonylglycerol was examined at calcium concentrations of 1 and 10 μM (Figure 5). Although PS produced a greater maximum activation of kinase activity at 1 μM calcium, the apparent affinity of the enzyme for phosphatidylserine was greater at 10 μM calcium ($K_{0.5}$ = 10 μg/mL compared to $K_{0.5}$ = 40 μg/mL at 1 μM). At the lower calcium concentration, diacylglycerol increased both the apparent affinity for phosphatidylserine and the maximum velocity. At 10 μM calcium, due to a high degree of activation of the enzyme by diacylglycerol and calcium alone, an effect upon the affinity for phosphatidylserine could not be discerned. On the basis of these observations, activation of types II and III by phospholipids was determined in the presence of 80 μg/mL phospholipid and 1 μM free calcium. In the absence of 1-stearoyl-2-arachidonylglycerol, types II and III were activated by PS and cardiolipin (not shown). Cardiolipin activated type II to the same extent as did PS, whereas type III was 50% less responsive to this phospholipid as compared to PS. In the presence of 1-stearoyl-2-arachidonylglycerol, the two kinases were maximally stimulated by PS (Table II). Other phospholipids, such as lysophosphatidylserine, phosphatidylinositol, phosphatidic acid, and cardiolipin were less effective. Phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin were ineffective. Of the phospholipids able to produce partial activation, type II was activated to a greater extent than type III, suggesting that this subtype may be less selective with respect to activation by various phospholipids.

Stimulation of Protein Kinase C Isozymes by Neutral Lipids and Phorbol Esters. Activation of protein kinase C

Table II: Activation of Cardiac Protein Kinase C Types II and III by Various Phospholipids in the Presence of 1-Stearoyl-2-arachidonylglycerol

phospholipid (80 μg/mL)	PKC activity[a] (%) type II	type III
phosphatidylserine	100	100
phosphatidylethanolamine	8	8
phosphatidylcholine	0	2
phosphatidylinositol	23	11
phosphatidic acid	59	34
cardiolipin	42	24
phosphatidylglycerol	9	7
lysophosphatidylserine	22	12
lysophosphatidylethanolamine	19	11
lysophosphatidylcholine	9	3
lysophosphatidylglycerol	3	2
cerebroside	3	4
sphingomyelin	6	3

[a] Protein kinase C (0.05 U/mL) was assayed in the presence of 1 μM free calcium, 8 μg/mL SAG, and the phospholipids indicated. Results are normalized to the activity obtained with PS. Other conditions were as described in Materials and Methods.

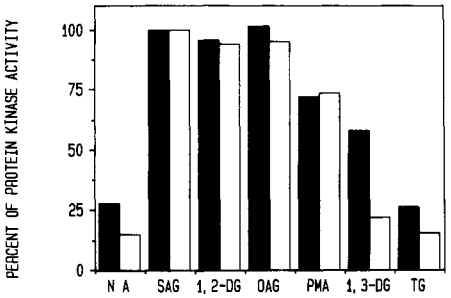

FIGURE 6: Effects of different neutral lipids and phorbol 12-myristate 13-acetate on protein kinase C types II and III enzyme activity. Protein kinase activities (0.05 U/mL) of types II (solid bar) and III (open bar) were measured in the presence of 80 μg/mL PS and 1 μM free calcium alone (NA) or with 8 μg/mL 1-stearoyl-2-arachidonylglycerol (SAG), 1,2-dioleoylglycerol (1,2-DG), 1-oleoyl-2-acetylglycerol (OAG), 1,3-dioleoylglycerol (1,3-DG), trioleoylglycerol (TG), or 1 μM phorbol 12-myristate 13-acetate (PMA). Kinase activity in the presence of 80 μg/mL PS and 8 μg/mL SAG was taken as 100%. Enzyme activities in the presence of 1 mM EGTA without lipid were substracted from the measured activities.

by diacylglycerols, triacylglycerols, and phorbol 12-myristate 13-acetate (PMA) was determined in the presence of PS (80 μg/mL) and calcium (1 μM, free). The results obtained were normalized to the value obtained with 1-stearoyl-2-arachidonylglycerol, the major species of diacylglycerol resulting from inositol phospholipid hydrolysis. In the presence of PS and calcium alone, type II was activated to a greater extent than type III (Figure 6). 1,2-Dioleoylglycerol and 1-oleoyl-2-acetylglycerol (OAG) activated both enzymes to a level comparable to that of 1-stearoyl-2-arachidonylglycerol. The tumor promoter PMA (1 μM) produced partial activation of both subtypes. Type II was also partially activated by 1,3-dioleoylglycerol. Trioleoylglycerol was ineffective at activating either subtype. These results indicate that, although both are stimulated by diacylglycerols, the subtypes may be differentially activated depending on the nature of the activating lipid.

Activation of Protein Kinase C Isozymes by Fatty Acids. Activation of both type II and type III by oleic acid was calcium-dependent but required much higher calcium concentrations than activation by phospholipids. Increasing the oleate concentration augmented the activation of type III at all calcium concentrations examined (Figure 7b). A similar effect of calcium was observed upon the activation of type III by oleic acid (Figure 8). This synergy between oleic acid and calcium was not observed for type II (Figure 7a). Maximum

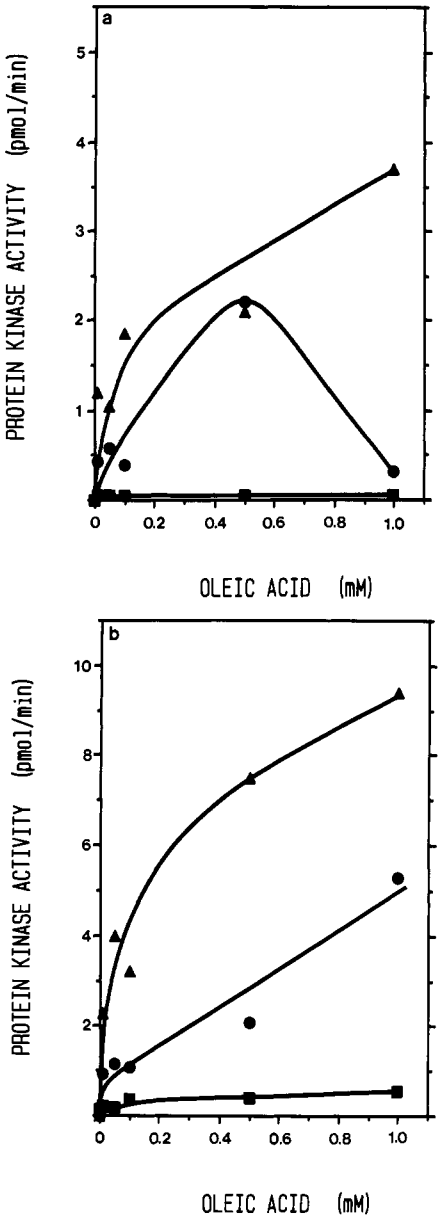

FIGURE 7: Effect of oleic acid concentration on protein kinase C types II and III enzyme activity. Protein kinase C types II (0.35 U/mL, panel a) and III (0.16 U/mL, panel b) were measured under the standard assay conditions with increasing concentrations of oleic acid in the presence of 1 (■), 10 (●), or 100 (▲) μM free calcium. Enzyme activities in the presence of 1 mM EGTA without lipid were subtracted from the measured activities.

activation of both enzymes required oleic acid concentrations of 400–600 μM; however, under the reaction conditions employed, type II was not activated to the same degree by oleate as was type III. Increasing the chain length of a saturated fatty acid from C14 to C20 resulted in decreased kinase activity (Table III). A single cis unsaturation increased kinase activity markedly. Type II was also activated by a fatty acid containing a single trans unsaturation. Fatty acids containing multiple sites of cis unsaturation were less effective activators particularly of type II, which was only marginally activated by arachidonic acid. These results indicate that, although both subtypes are activated by fatty acids, they possess different structural requirements for either binding to or activation by these lipids.

Discussion

This paper reports on the isolation, identification, and

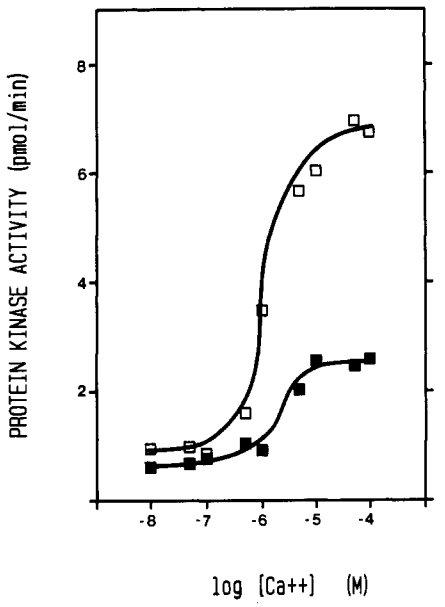

FIGURE 8: Effect of calcium on activation of protein kinase C type III by oleic acid. Protein kinase C type III (0.16 U/mL) enzyme activity was measured under the standard assay conditions with increasing concentrations of free calcium in the presence of either 0.04 (■) or 0.4 (□) mM oleic acid. Enzyme activities in the presence of 1 mM EGTA without lipid were subtracted from the measured activities.

Table III: Activation of Cardiac Protein Kinase C Types II and III by Fatty Acids

fatty acid (0.5 mM)	PKC activity[a] (pmol/min) type II	type III
myristic	2.5	2.3
palmitic	2.4	1.8
stearic	1.9	1.9
arachidic	0.6	0.4
palmitoleic	3.5	2.5
oleic	4.6	7.5
elaidic	3.4	2.4
linoleic	1.8	3.2
γ-linolenic	0.4	2.6
arachidonic	1.8	5.2

[a] Protein kinase C (0.35 U/mL type II, 0.16 U/mL type III) was assayed in the presence of 0.1 mM free calcium and the fatty acids indicated. Results are expressed as picomoles of phosphate transfered per minute. Other conditions were as described in Materials and Methods.

biochemical characterization of two subtypes of protein kinase C from bovine heart. In previous studies, the amount of protein kinase C either measured in or isolated from cardiac muscle ranged from 0.006 to 2 nmol/min per gram of tissue (Kuo et al., 1980; Wise et al., 1982; Katoh & Kuo, 1982; Yuan & Sen, 1986; Yuan et al., 1987). Wise and co-workers (1982) obtained 12 units of kinase from 2 kg of bovine heart. The amount of activity recovered by the purification procedure described here (10–20 U/gram of tissue) indicates that previous determinations underestimated the amount of protein kinase C present in cardiac tissue. Although the reason for these differences in activity are not apparent, it may include a more complete extraction of protein kinase C from the tissue during homogenization. Alternatively, endogenous inhibitors of protein kinase C are known to exist (Schwantke & LePeuch, 1984; McDonald & Walsh, 1985, 1986; Pripilla et al., 1988; Pearson et al., 1990). Failure to remove these inhibitors prior to assay in previous studies may have contributed to an underestimation of the amount of protein kinase C activity present.

Previous workers found no protein kinase C type II immunoreactivity in homogenates from heart (Yoshida et al., 1988). Chromatography on hydroxylapatite revealed that protein kinase C in heart consists of a mixture of types II and III (Kosaka et al., 1988). However, the kinase from heart that was identified as type II, on the basis of its elution from hydroxylapatite, was immunologically and biochemically distinct from the type II from brain (Kosaka et al., 1988). In the present study, protein kinase C has been isolated from bovine heart and subsequently fractionated into two subtypes by chromatography on a hydroxylapatite column. The major peak of kinase activity eluted at 75–90 mM phosphate and was identified as type III by Western blot analysis using isozyme-specific antibodies. A minor peak eluted at a slightly lower phosphate concentration and contained type II (β_2). The ratio of type III to type II in bovine heart was 15:1. Although type III could be further purified to apparent homogeneity by chromatography on phosphatidylserine acrylamide, no type II activity could be recovered from this column. Marais and Parker (1989) found that type II (β_1) from bovine brain is not retained on a PS affinity column. In the present study, no activity was detected in the flow-through fraction, suggesting that type II (β_2) was in fact binding to PS acrylamide. Loss of all type II activity and 45–55% of type III activity could have resulted from nonspecific binding of the enzyme to the column packing. Alternatively, the kinases may have been inactivated while bound to the column (Huang & Huang, 1990). As attempts to further purify type II resulted in complete loss of activity, it was decided to employ this fraction for biochemical characterization of the cardiac type II enzyme. This was not unreasonable, as type II was found to be free of other calcium-dependent histone kinase activities and completely separated from type III following chromatography on hydroxylapatite.

As with protein kinase C subtypes from brain, cardiac types II and III were maximally activated by a 1,2-diacylglycerol in the presence of phosphatidylserine and at physiological calcium concentrations. The most effective phospholipid activator of both heart protein kinase C subtypes was PS. Huang and co-workers have shown that either PS or cardiolipin will maximally activate all three subtypes from brain (Huang et al., 1988). However, these latter results were obtained by use of a mixed micelle assay system containing Nonidet P-40 and activating lipids, which may account for the observed difference. Activation of cardiac protein kinase C by fatty acids required higher calcium concentrations than activation by PS. Although similarities were observed between cardiac types II and III, species differences in activation were apparent. The most marked difference was the high degree of activity displayed by type II in the absence of calcium upon addition of 1,2-DG. Type II was also activated to a greater degree by cardiolipin and by 1,3-dioleoylglycerol. Cis-unsaturated fatty acids such as arachidonic and oleic acid have been shown to activate the various protein kinase C subtypes from rat brain (Sekiguchi et al., 1987, 1988; Murakami & Routtenberg, 1985; Murakami et al., 1986). For types I and II, the maximal degree of activation produced by fatty acids was less than that produced by phosphatidylserine and diacylglycerol (Sekiguchi et al., 1987, 1988). As with the various subtypes from rat brain, cardiac subtypes II and III displayed differences in activation by fatty acids. Although both subtypes were activated by cis-unsaturated fatty acids, only type III showed high activity in the presence of either oleic or arachidonic acid. Type II was also activated to a greater degree in the presence of a trans-unsaturated fatty acid. Fatty acids containing more

than four double bonds fail to activate protein kinase C (Sekiguchi et al., 1988). The biological significance of this effect of fatty acids is not known; however, it is of interest that arachidonic acid, a lipid that is released during certain cellular responses, is capable of preferentially activating subtypes. The observed variations in activation by calcium and lipids suggest that differences exist in the physical and structural requirements of these two subtypes for binding to or activation by lipids.

Recent studies have shown autophosphorylation to affect the kinetics of activation (Huang et al., 1986), association with membranes (Wolf et al., 1985), affinity for target proteins (Rosen & Koshland, 1987), and, possibly, the down-regulation of PKC (Ohno et al., 1990). Huang et al. (1986b) have demonstrated that PKC types I, II, and III from rat brain autophosphorylate at serine residues, while type II also phosphorylates at threonine residues. However, Woodgett and Hunter (1987) found that two unidentified PKC subtypes from rat brain appeared to be phosphorylated at multiple sites on both serine and threonine residues. In addition, a PKC subtype from bovine neutrophils, tentatively identified as type I, autophosphorylates at both serine and threonine residues (Dianoux et al., 1989). There appears to be some uncertainty as to the exact identity of the PKC subtypes analyzed in some of these studies. In the present study, where the identity of the PKC subtypes was confirmed with the use of subtype-specific antibodies, the results of phospho amino acid determination are consistent with those reported by Huang and co-workers (1986b), where the identity of the subtypes was determined on the basis of hydroxylapatite chromatography. Two of the threonine residues that are labeled during the autophosphorylation of PKC type II are also present in the C-terminal region of types I and III (Flint et al., 1990). As autophosphorylation occurs as an intramolecular process (Newton & Koshland, 1987), other characteristic physical properties of the PKC subtypes, in addition to the presence of a phosphorylatible residue, appear to be involved in determining the extent and localization of autophosphorylation.

Although protein kinase C type II from rat heart has been reported to be an immunochemically distinct subtype (Kosaka et al., 1988), the type II isolated from bovine heart was recognized by antibodies to the brain type II enzyme. As previously reported, the heart type II was found to be less dependent upon calcium for activation. A similar calcium-independent type II activity has been isolated from human platelets (Tsukuda et al., 1988). The reasons for these differences are not apparent. However, a new member of the protein kinase C family, PKC-ϵ, has been found in both lung (Ohno et al., 1988; Ono et al., 1988; Schaap et al., 1989) and heart (Housey et al., 1987; Schaap et al., 1989). This subtype autophosphorylates at both serine and threonine residues, as do types II from brain (Huang et al., 1986b) and heart (this paper), and copurifies with type II during hydroxylapatite column chromatography (Konno et al., 1989). PKC-ϵ is activated by phospholipids and diacylglycerols; however, the activation of this kinase by phospholipids is not calcium-dependent (Konno et al., 1989; Schaap et al., 1989). It is possible that the lower calcium dependency of protein kinase C type II reported here and elsewhere (Kosaka et al., 1988; Sekiguchi et al., 1988) is in fact due to copurification of type II and PKC-ϵ. Alternatively, when PS and diacylglycerol are incorporated into detergent micelles (Triton X-100 or Nonidet P-40), type II does not exhibit this calcium-independent behavior (Huang et al., 1988; Marais & Parker, 1989). PKC-ϵ is active in a mixed micellar assay (Schaap et al., 1989), suggesting that the calcium-independent activation of type II is related to the structure of micelles containing PS and diacylglycerol. These findings indicate that, in addition to differing in lipid content, protein kinase C types II and III may differ in the structure of the lipid vesicle by which they are activated.

In summary, we have isolated protein kinase C from bovine heart and found it to contain two subtypes, types II and III. Type III was found to be the most abundant and was purified to homogeneity. These enzymes differed with respect to activation by phospholipids, fatty acids, and diacylglycerols. In the presence of phosphatidylserine plus 1-stearoyl-2-arachidonylglycerol, type II was less calcium-dependent than type III. These results, along with those from other groups, indicate that there are functional differences between cardiac protein kinase C subtypes and suggest that these enzymes may each play a unique role in signal transduction in the heart. Further studies concerning the substrate specificity and functional significance of cardiac protein kinase C subtypes are currently underway.

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Enzymological Evidence for Separate Pathways for Aflatoxin B_1 and B_2 Biosynthesis

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ABSTRACT: Aflatoxins B_1 (AFB_1) and B_2 (AFB_2) are biologically active secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*. These toxins are synthesized by the fungi from pathway precursors: sterigmatocystin (ST) → *O*-methylsterigmatocystin (OMST) → AFB_1; dihydrosterigmatocystin (DHST) → dihydro-*O*-methylsterigmatocystin (DHOMST) → AFB_2. The late stages of AFB_1 synthesis are carried out by two enzyme activities, a methyltransferase (MT) (ST → OMST), and an oxidoreductase (OR) (OMST → AFB_1). Properties of the purified MT have been identified in a previous investigation [Bhatnagar et al. (1988) *Prep. Biochem. 18*, 321]. In the current study, the OR was partially purified (150-fold of specific activity) from fungal cell-free extracts and characterized with extended investigation of the late stages of AFB_1 and AFB_2 synthesis. Whole cells of an isolate of *A. flavus* (SRRC 141), which produce only AFB_2, were able to produce AFB_1 in ST and OMST feeding studies; the results suggested that the enzymes involved in AFB_2 biosynthesis also carry out AFB_1 synthesis. Substrate competition experiments carried out with the OR showed that an increasing concentration of either OMST or DHOMST in the presence of a fixed, nonsaturating concentration of either DHOMST or OMST, respectively, resulted in a decline in production of one aflatoxin (B_1 or B_2) with a corresponding increase in the synthesis of the other toxin (B_2 or B_1). OMST was a preferred substrate (K_m, 1.2 μM) for the oxidoreductase as compared to DHOMST (K_m, 13.4 μM). Similar, substrate competition experiments showed that ST (K_m, 2.0 μM) was a preferred substrate over DHST (K_m, 22.5 μM) for a homogeneous MT. The results suggest that AFB_1 and AFB_2 synthesis is catalyzed by common enzymes that use separate precursors as substrates for the synthesis of each toxin. A biosynthetic grid for the AFB_1 and AFB_2 synthesis is presented on the basis of enzyme substrate specificity studies and cellular conversions of pertinent metabolites.

Aflatoxins B_1 and B_2 (AFB_1 and AFB_2)[1] are toxic secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. The mode of action, metabolism, and chemical biosynthesis of AFB_1 have been extensively studied (Malik, 1982; Bennett & Christensen, 1983; Townsend, 1986). Biotransformation experiments with mutants of *A. parasiticus* blocked in toxin formation and ^{14}C-radiolabeled precursor studies (Bhatnagar et al., 1987; McCormick et al., 1987) have resulted in the following scheme of precursors in aflatoxin B_1 biosynthesis: norsolorinic acid → averantin → averufanin → averufin → versiconal hemiacetal acetate → versicolorin A → sterigmatocystin (ST) → *O*-methylsterigmatocystin (OMST) → aflatoxin B_1 (Figure 1). Several analogues of aflatoxin B_1 and its precursors (Figure 2) have been identified from extracts of *Aspergillus* mycelia (Cole & Cox, 1981).

Fractionation of subcellular *A. parasiticus* mycelial extracts resolved two pertinent enzyme activities (Cleveland et al., 1987b): (1) a methyltransferase (MT) catalyzing the conversion of ST to OMST and (2) an oxidoreductase (OR) catalyzing the OMST to AFB_1 conversion (Figure 1). The methyltransferase has recently been purified to homogeneity in our laboratory (Bhatnager et al., 1988); this enzyme is the first catalyst specific for aflatoxin biosynthesis (Cleveland & Bhatnagar, 1989) that has been purified to homogeneity.

There have been many conflicting hypotheses concerning the biosynthetic pathways of AFB_2. Several reports (Maggon & Venkitasubramanian, 1973; Biollaz et al., 1970; Heathcote et al., 1976; Thomas, 1965) suggested that AFB_2 may be metabolically related to AFB_1 by a direct interconversion process, whereas others (Dutton et al., 1985; Floyd et al., 1987; Dutton, 1988) had postulated that AFB_1 and AFB_2 may be synthesized in fungal mycelia by separate pathways, the branch

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[1] Abbreviations: AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFB_{2a}, aflatoxin B_{2a}; ST, sterigmatocystin; OMST, *O*-methylsterigmatocystin; DHST, dihydrosterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin; HST, sterigmatocystin hemiacetal; HOMST, *O*-methylsterigmatocystin hemiacetal; MST, 5-methoxysterigmatocystin (7-hydroxy-6,10-dimethoxydifuroxanthone); HMST, 5-methoxysterigmatocystin hemiacetal; EST, sterigmatocystin ethoxy acetal; MT, methyltransferase; OR, oxidoreductase.