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Calcium-Activated, Phospholipid-Dependent Protein Kinases from Rat Liver: Subcellular Distribution, Purification, and Characterization of Multiple Forms†

Salman Azhar,* John Butte, and Eve Reaven
Department of Medicine, Stanford University School of Medicine, and Geriatric Research, Education and Clinical Center, Veterans Administration Medical Center, Palo Alto, California 94304
Received April 3, 1987; Revised Manuscript Received June 11, 1987

ABSTRACT: Three forms of Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) were extensively purified from rat liver homogenate. Subcellular fractionation analysis indicated that the majority (~85%) of the activity was associated with particulate fractions of the liver. Among these, the microsomal and nuclear fractions accounted for ~63% and ~10% of total activity. The remaining 15% of protein kinase C was recovered in the soluble fraction following differential centrifugation. It was also found that most of the membrane-associated protein kinase C was latent, with 4–6-fold stimulation with detergents such as 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate, octyl β-glucoside, or Triton X-100. The activity of both the bound form and the soluble enzyme was enhanced by the addition of Ca^{2+} and phosphatidylserine, when histone H_1 was used as substrate. The bound protein kinase C activity was dissociated by homogenization of liver in buffer containing ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, ethylenediaminetetraacetic acid, and various proteolytic inhibitors, and the solubilized extract was used to purify multiple forms of the enzyme. The purification procedure sequentially utilized $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography on DEAE-cellulose, gel permeation chromatography on Fractogel TSK HW-55 (F), ion-exchange chromatography on hydroxylapatite, gel permeation chromatography on Ultrogel AcA34, and affinity chromatography on polyacrylamide-immobilized phosphatidylserine. On hydroxylapatite columns, protein kinase C activity was resolved into three isoenzymic forms designated C-I, C-II, and C-III. The molecular weights of the three isoenzymic forms were in the range of 208 000–225 000 as shown by chromatography on calibrated Ultrogel AcA34 columns and sucrose density gradient centrifugation. Furthermore, all three isoenzymes demonstrated a single peak with a sedimentation coefficient ($s_{20,w}$) in the range of 9.0–9.2. However, with polyacrylamide gel electrophoresis, all the forms showed a single protein component with average molecular weight of 64K, suggesting that the native isoenzymes may be composed by subunits. Finally, all three isoenzymes exhibited nearly identical enzymatic properties.

The unique feature of protein kinase C[1] is its dependence on phospholipids (especially phosphatidylserine) as a cofactor (Takai et al., 1979; Kaibuchi et al., 1981). This characteristic as well as the enzyme's requirement for Ca^{2+} (Inoue et al., 1977; Takai et al., 1979; Kaibuchi et al., 1981), its ubiquitous presence in eukaryotes (Kuo et al., 1980; Minakuchi et al., 1981), and its reported dual membrane and cytosolic distribution in cells (Kraft & Anderson, 1983a; Farrar et al., 1985; Wooten & Wrenn, 1985; Tanabe et al., 1985; Naor et al., 1985) suggests a potential role for protein kinase C in membrane events during cell secretion (Nishizuka et al., 1984; Nishizuka, 1984; Berridge & Irvine, 1984). With the recent information that protein kinase C acts as the intracellular receptor site for agents such as phorbol esters (Costagna et al., 1982; Ashendel, 1985), which have been shown to stimulate protein and steroid secretion (Drust & Martin, 1984; Berridge & Irvine, 1984; Gunther, 1981; Putney et al., 1984; Negro-Vilar & Lapetina, 1985; Kojima et al., 1983; Lin, 1985; Kawai & Clark, 1985; Brunswig et al., 1986), this view of protein kinase C as a mediator of intracellular secretory events is strengthened.

To test the interaction between protein kinase C and cellular endomembranes directly, several requirements must be met. First, one must have in possession a purified form of protein kinase C that is totally free of contaminating cytosolic and

†Supported by a grant from the Research Service of the Veterans Administration.

[1] Abbreviations: CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; NP-40, Nonidet P-40 [(octylphenoxy)poly(ethoxyethanol)]; Zwittergent 3-10, zwitterionic (Zwittergent) detergent 3-10; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; protein kinase C, calcium-activated and phospholipid-dependent protein kinase; protein kinase A, adenosine cyclic 3′,5′-monophosphate dependent protein kinase; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone [1-chloro-3-(tosylamido)-7-amino-L-2-heptanone]; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone [L-1-(tosylamido)-2-phenylethyl chloromethyl ketone]; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/87/0426-7047$01.50/0

membrane proteins. Second, one must have obtained this purified enzyme from a tissue in which it is also possible to isolate membranes from individual secretory compartments (e.g., endoplasmic reticulum, Golgi membranes, plasma membranes, coated and uncoated vesicles, etc.) for use in in vitro membrane reconstitution experiments. Rat liver, due to its size, its content of protein kinase C, and its previous use as a model tissue for subcellular membrane fractionation (Fleischer & Kervina, 1974), seemed an ideal tissue source for this purpose.

Accordingly, we report here on the purification and characterization of a calcium- and phospholipid-dependent protein kinase (protein kinase C) from rat liver. The purification process is described in detail since several unexpected properties of protein kinase C were observed. Most important among these is the fact the rat liver protein kinase C exists as three isoenzymes with similar enzymatic properties; one isoenzyme, which we refer to as protein kinase C-II, exists in quantities greater than that of the other two. Of interest, also, are the facts that the molecular weight of these isoenzymes is about 2-fold greater than that previously published for protein kinase C (Kikkawa et al., 1982; Wise et al., 1982; Le Peuch et al., 1983; Schatzman et al., 1983; Uchida & Filburn, 1984; Nishikawa et al., 1985), that the absolute content of the enzyme(s) in liver is 2–5-fold higher than previously observed (Kikkawa, 1982; Kiss & Mhina, 1982; Jergil & Sommarin, 1983), and that a greater proportion of the enzyme appears to be membrane-associated than shown before (Kikkawa et al., 1982).

Experimental Procedures

Materials. [γ-^{32}P]ATP (~3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). The following chemicals were supplied by Sigma Chemical Co. (St. Louis, MO): histone type IIIs, histone Vs, casein, various protease inhibitors, nonionic detergents, bovine brain phosphatidylserine, diolein, phosphoserine, phosphothreonine, phosphotyrosine, ATP, and other nucleotides. Hydroxylapatite (Bio-Gel HTP) and reagents for electrophoresis were obtained from Bio-Rad (Richmond, CA). Fractogel TSK HW-55(F), octyl β-glucoside, and CHAPSO[1] were products of Pierce Chemicals (Rockford, IL). DEAE-cellulose (DE 52) and phosphocellulose papers (P81) were purchased from Whatman (Maidstone, Kent, England). Ultrogel AcA34 was obtained from LKB (Gaithersburg, MD). Ultrapure, density-grade sucrose and ultrapure, enzyme-grade ammonium sulfate were purchased from Beckman Instruments Inc. (Palo Alto, CA) and Chemicon International Inc. (Los Angeles, CA), respectively. PIPES, MOPS, and Zwittergent Kit were supplied by Calbiochem (San Diego, CA). Molecular weight standards for gel filtration were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). All other reagents used were of analytical grade.

Assay of Calcium-Activated and Phospholipid-Dependent Protein Kinase (Protein Kinase C). The activity of protein kinase C was determined by measurement of the enzymatic transfer of ^{32}P from [γ-^{32}P]ATP to histone (H_1) substrate. The standard assay (unless otherwise stated) consisted of incubation of the following components in a final volume of 100 μL: 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 6.8, 10 mM magnesium acetate, 800 μg/mL histone H_1 (type IIIs Sigma), 25 mM 2-mercaptoethanol, 0.1 mM [γ-^{32}P] ATP (200–300 cpm/pmol), 20 mM NaF, 0.75 mM $CaCl_2$, 250 μg/mL phosphatidylserine, 10 μg/mL diolein, and a suitable amount of enzyme. Phosphatidylserine and diolein were dissolved in chloroform and, after evaporation of the solvent (under N_2), were resuspended in 50 mM PIPES, pH 6.8, and sonicated before addition to the assay. Basal activity was measured in the presence of 0.5 mM EGTA instead of $CaCl_2$, diolein, and phosphatidylserine. The reaction was inhibited by [γ-^{32}P] ATP addition. Incubation was carried out at 30 °C for the desired time (2–10 min), and the reaction was terminated by spotting 50 μL of mixture onto 2 × 2 cm phosphocellulose strips (Whatman P81) and immersing the strips in 75 mM phosphoric acid (10 mL per sample) as described by Roskoski (1983). The strips were washed 4 times with 75 mM phosphoric acid, with at least one overnight wash. After the phosphocellulose strips were dried, radioactivity was counted in a Beckman Model LS-3801 scintillation spectrometer in Beta Max (6 mL). Protein kinase C activity was determined by subtracting the amount of ^{32}P incorporation into histone noted in the presence of 0.5 mM EGTA from the amount of ^{32}P incorporation noted in the presence of $CaCl_2$, phosphatidylserine, and diolein. Protein kinase C activity is expressed as pmol of ^{32}P incorporated into histone·min^{-1}·(mg of protein)$^{-1}$.

Assay of cAMP-Dependent Protein Kinase (Protein Kinase A). Enzyme activity was assayed by measuring ^{32}P incorporation from [γ-^{32}P]ATP into lysine-rich histone (f1) by a slight modification of the procedure of Corbin and Reimann (1974). The assay system contained in a final volume of 100 μL: 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, 10 mM magnesium acetate, 1 mg/mL histone f1 (type Vs Sigma), 40 mM NaF, 0.25 mM [γ-^{32}P]ATP (100–200 cpm/pmol), 1 mM theophylline, a suitable aliquot of enzyme, and 10 μM cAMP (if desired). The reaction was stopped after 10 min of incubation (unless otherwise stated) at 30 °C as described above. Subsequently, strips were washed and processed for radioactivity determinations as described above under Assay of Calcium-Activated and Phospholipid-Dependent Protein Kinase (Protein Kinase C). Samples incubated without enzyme served as blanks. Basal protein kinase activity was determined in the absence of cAMP. cAMP-dependent protein kinase was determined by subtracting the ^{32}P incorporation into histone noted in the absence of added phospholipids and cAMP from the amount radioactivity incorporation noted in the presence of cAMP. Enzyme activity is expressed as pmol of ^{32}P transferred from [γ-^{32}P]ATP to histone·min^{-1}·(mg of protein)$^{-1}$.

Miscellaneous Enzyme Assays. cAMP-independent protein kinase activity was assayed by a modification of the procedures of Kitagawa and Racker (1982) and Nakajo et al. (1984), using dephosphorylated casein (Ashby & Walsh, 1974) as a substrate. Glycogen synthase and phosphorylase activities were assayed by the methods of Thomas et al. (1968) and Tan and Nuttal (1975), respectively. Phosphorylase kinase activity was measured according to Chrisman et al. (1982).

Subcellular Fractionation of Rat Liver Homogenate. Initially, fractionation, of rat liver homogenate was carried out by differential centrifugation according to the method of de Duve et al. (1955). Briefly, liver was sliced and homogenized 3 times in 0.25 M sucrose buffered with 3 mM imidazole, pH 7.4 (3 volumes), with a Potter–Elvejhem type homogenizer rotating at 1300 rpm for 20 s at 4 °C. The nuclear (N) fraction (10000*g* min), the mitochondrial (M) fraction (33000*g* min), and the light mitochondrial–lysosomal (L) fraction (250000*g* min) were pelleted by using a JA-20 fixed angle rotor and Beckman J2-21 refrigerated centrifuge (Beckman Instruments, Palo Alto, CA). Separation between the microsomal pellet (P) and the cytosol (S) was achieved by centrifugation at (4 × 10^6)*g* min using a type 50.2 Ti rotor and

a Beckman L8-M ultracentrifuge. All sedimented fractions were washed an additional 2 times in sucrose–imidazoles solution and finally resuspended to a desired volume in the same buffer. Various membrane fractions were assayed immediately for "marker" enzymes, protein kinase C, and protein kinase A. DNA and the activity of the enzymes 5′-nucleotidase, succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (succinate–INT) reductase, β-*N*-acetylglucosaminidase, galactosyltransferase, glucose-6-phosphatase, and lactate dehydrogenase were used as markers for nuclei, plasma membranes, mitochondria, lysosomes, Golgi, endoplasmic reticulum, and soluble fraction, respectively.

5′-Nucleotidase and glucose-6-phosphatase activities were assayed according to Aronson and Touster (1974). Succinate-INT reductase activity was measured by the procedure of Pennington (1961). Galactosyltransferase activity was measured by a slight modification of the procedure of Bretz et al. (1980) as described previously (Reaven & Azhar, 1981; Azhar et al., 1983). β-*N*-Acetylglucosaminidase was assayed according to Beaufay et al. (1974) and lactate dehydrogenase according to Rip et al. (1981). For the determination of protein kinase C and protein kinase A activities subcellular fractions were treated with the detergent 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) to unmask and maximally solubilize these activities. Suitable aliquots of N, M, L, and P fractions (4 mg of protein/mL) were mixed with an equal volume of 2% (w/v) solution of CHAPSO in buffer solution containing 40 mM Tris-HCl pH 7.4, 40 mM 2-mercaptoethanol, 10 mM EDTA, and 10 mM EGTA. After incubation at 4 °C for 30 min, the entire suspension was diluted 5-fold with buffer (20 mM Tris-HCl, pH 7.4, 20 mM 2-mercaptoethanol), and 20-μL aliquots in duplicate were used for protein kinase C and A assays. In some instances membrane–detergent suspensions were centrifuged at $105000g_{av}$ for 1 h to obtain supernatant fractions containing solubilized membrane components.

Density Gradient Centrifugation. The sedimentation coefficient of protein kinases C was determined by the procedure of Martin and Ames (1961). Linear sucrose gradients (5–20%) were formed in 5 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. Beef liver catalase (11.3 S, M_r 232 000), yeast alcohol dehydrogenase (7.6 S, M_r 141 000), bovine serum albumin (4.3 S, M_r 66 000), and lysozyme (2.1 S, M_r 14 388) were used as markers. Samples (200 μL) containing protein kinases C and standard protein markers were layered over 5 mL of sucrose gradients and centrifuged at 38 000 rpm for 18 h at 4 °C in a Beckman SW55 rotor with an L8-M ultracentrifuge. Fractions (200 μL) were collected, and suitable aliquots were assayed for protein kinase C or marker enzymes. Catalase was assayed by the method of Beers and Sizer (1952). Yeast alcohol dehydrogenase and lysozyme were determined by the procedures of Vallee and Hoch (1955) and Martin and Ames (1961), respectively. Bovine serum albumin was determined by its absorbance at 280 nm with a Beckman Model DU-6 spectrophotometer.

Gel Filtration Chromatography and Determination of Stokes Radius. Purified and concentrated preparations of protein kinase C peaks I–III were applied to an Ultrogel AcA34 column (2.6 × 84 cm) which was equilibrated with buffer A and calibrated with the following standard proteins: thyroglobulin (M_r 669 000, a = 85.0 Å), ferritin (M_r 440 000, a = 61.0 Å), catalase (M_r 232 000, a = 52.2 Å), aldolase (M_r 158 000, a = 48.1 Å), ovalbumin (M_r 43 000, a = 30.5 Å), chymotrypsin (M_r 25 000, a = 20.9 Å), and ribonuclease (M_r 13 700, a = 16.4 Å). The column was eluted with the same buffer at a flow rate of 14 mL/h. The void volume (V_0) and the total column volume (V_t) were estimated by elution of blue dextran 2000 and tritiated glycerol, respectively. The elution volumes of the standard proteins were determined spectrophotometrically by the absorbance at 280 nm. K_{av} values for the standard protein were calculated (Laurent & Killander, 1964) from their elution volumes (V_e) by the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$. The data were plotted as $(-\log K_{av})^{1/2}$ versus the stock radius of standard proteins (Laurent & Killander, 1964; Siegel & Monty, 1966). The stock radius and molecular weight of protein kinases C were determined from the standard curve.

Phosphorylated Amino Acid Analysis. Phosphorylated amino acid analysis of histone was carried out by the procedure of Hunter and Sefton (1980). Histone was phosphorylated with the purified enzyme preparations as described under Assay of Calcium-Activated and Phospholipid-Dependent Protein Kinase (Protein Kinase C). The proteins were precipitated with 20% trichloroacetic acid, and the precipitate was washed with organic solvents (Hunter & Sefton, 1980) and dried under N_2. The pellet in each case was resuspended in 0.5 mL of 6 N HCl and hydrolyzed at 110 °C for 2 h in sealed glass ampules. The hydrolyzed material was lyophilized to remove HCl and redissolved in a small volume of electrophoresis buffer. The samples were then subjected to electrophoresis as described by Hunter and Sefton (1980). Phosphoamino acid standards phosphoserine, phosphothreonine, and phosphotyrosine were mixed and spotted with each sample. Standards were visualized with ninhydrin spray while radioactive amino acids were detected by radioautography on Kodak XAR-5 X-ray film with a Du Pont Cromex intensifying screen.

Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970), using 5–20% linear acrylamide gradient. Following electrophoresis at 25 mA for 4–5 h, gels were fixed overnight in 10% trichloroacetic acid (w/v)–50% methanol (v/v). Subsequently gels were stained with Coomassie brilliant blue G (0.1%) in solution containing 20% methanol and 3.5% (v/v) perchloric acid. Gels were destained in 7% acetic acid. Phosphorylase *b* (M_r 92 500), bovine serum albumin (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), and soybean trypsin inhibitor (M_r 21 000) were used as standard proteins.

Buffers Used during Purification of Protein Kinase C. (*1*) *Buffer A* contained 20 mM Tris-HCl, pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA, 10 mM EGTA, 0.2 mM phenylmethanesulfonyl fluoride, 0.1 mM TLCK, 0.1 mM TPCK, 5 μg/mL each of leupeptin, pepstatin, chymostatin, and antipain, 10 μg/mL aprotinin, and 0.5 mM benzamidine.

(*2*) *Buffer B* contained 5 mM Tris-HCl, pH 7.5, 50 mM 2-mercaptoethanol, 1 mM EDTA, 0.5 mM EGTA, and 0.25 μg/mL each of TPCK, antipain, pepstatin A, chymostatin, and leupeptin.

(*3*) *Buffer C* was the same as buffer B but contained 20 mM sodium phosphate, pH 7.4, instead of 5 mM Tris-HCl, pH 7.5.

Miscellaneous Procedures. Protein content of subcellular fractions was carried out by a modification of the procedure of Lowry et al. (1951) as described by Markwell et al. (1981). Protein content during purification of protein kinases C was monitored by the Bradford protein assay (1976) using bovine serum albumin as a standard. The fluorometric procedure of Böhlen et al. (1972) was used to determine the protein content (in the nanogram range) of highly purified enzyme.

Table I: Subcellular Distribution of Protein Kinase C and Protein Kinase A in Rat Liver[a]

cellular fractions	% of total cellular protein	protein kinase C act.[b] [pmol·min^{-1}·(mg of protein)$^{-1}$]	% of total protein kinase C act.	protein kinase A act.[c] [pmol·min^{-1}·(mg of protein)$^{-1}$]	% of total protein kinase A act.
nuclear (N)	12.5 ± 1.9	127.3 ± 13.3	10.6 ± 1.9	143.3 ± 9.2	2.87
mitochondrial (M)	15.9 ± 0.6	22.1 ± 1.6	2.4 ± 0.1	57.5 ± 1.1	1.5 ± 0.1
lysosomal (L)	5.9 ± 0.5	125.1 ± 18	4.8 ± 0.7	100.8 ± 5.3	1.0 ± 0.3
microsomal (P)	18.1 ± 0.5	526.0 ± 50.4	63.0 ± 1.6	232.6 ± 34.3	6.8 ± 0.4
soluble (S)	43.9 ± 1.6	50.3 ± 1.2	15.9 ± 0.2	1237.1 ± 95.2	88.9 ± 1.3

[a] Rat livers were homogenized in 0.25 M sucrose–3 mM imidazole, pH 7.4, and subjected to differential centrifugation to isolate N, M, L, P, and S fractions as described under Experimental Procedures. Particulate and soluble fractions were pretreated with CHAPSO (1% final concentration) before assaying for various kinases. Protein kinase C was measured in the presence of $CaCl_2$ (0.75 mL), diolein (10 μg/mL), and phosphatidylserine (250 μg/mL). Histone type IIIs (Sigma) was used as phosphate acceptor (substrate). Protein kinase A activity was measured in the presence of 2 μM cAMP with histone type Vs (Sigma) as a substrate. All assays were done in duplicate under the optimal concentration of protein and time. Results are mean ± SE of four separate experiments. [b] Protein kinase C activity was obtained by subtracting activity measured in the presence of 0.5 mM EGTA from activity measured with Ca^{2+}, diolein, and phosphatidylserine. [c] Protein kinase A activity was obtained by subtracting activity measured without cyclic nucleotide from activity measured in the presence of cAMP.

Estimates of free Ca^{2+} (when desired) were calculated according to Feldman et al. (1972) as adapted by Paxton and Harris (1982).

Results

Subcellular Distribution of Protein Kinase C and Protein Kinase A in Rat Liver. In this section a series of experiments are described that demonstrate the presence of calcium-activated and phospholipid-dependent protein kinase (protein kinase C) in rat liver, its subcellular distribution, and the effect of detergents on "latency" of the particulate form of the enzyme. In addition, the subcellular distribution profile of protein kinase C is compared with that of cAMP-dependent protein (protein kinase A) and specific enzyme markers. Initially, attempts were made to establish suitable experimental conditions for the study of membrane-associated protein kinase C by using particulate-free supernatant derived from detergent–EDTA-solubilized microsomes. Optimal enzyme activity showed a wide pH range (between 6.6 and 8.0), and PIPES buffer of pH 6.8 was used in subsequent studies. At pH 6.8, linear rates were established with respect to enzyme concentration and incubation time (data not shown). Furthermore, NaF (40 mM) was included in the enzyme assay mixture as a nonspecific phosphatase inhibitor. It was found in these preliminary experiments that most of the membrane-associated activity was latent, with 3–4-fold stimulation in the microsomal fraction dispersed by Triton X-100. Experiments were performed to determine the optimum concentration and specificity of various detergents. Our results show that increasing concentrations of Triton X-100, NP-40, CHAPSO, octyl β-glucoside, and Zwittergent 3-10 activate protein kinase C activity above nondetergent levels. It appears that all the detergents tested were able to stimulate protein kinase C activity in a dose-dependent manner. A significant increase in activity was observed with as little as 0.05% (w/v) of various detergents. For most of the detergents tested, maximal stimulation of protein kinase C was achieved with 0.5–1% of detergent. Furthermore, CHAPSO was found to be most effective in unmasking enzyme activity when tested at a final concentration of 1%. Higher concentrations of CHAPSO, Triton X-100, and NP-40 appeared to inhibit enzyme activity. Neutral detergent, octyl β-glucoside, and Zwittergent 3-10 (although less effective than CHAPSO) also maximally stimulated enzyme activity in the concentration range of 1–10%. Other detergents such as deoxycholate, Zwittergent 3-12, Zwittergent 3-14, sodium taurocholate, etc., were only minimally effective when tested at a concentration of 0.5% (data not given). The optimum concentration of CHAPSO in the presence of metal chelators resulted in 80–85% solubilization of enzyme activity. As a result of these experiments showing the latency of protein kinase C in our system, all subsequent preparations of subcellular fractions were treated with detergent buffer before measurements of the activity of protein kinase C and protein kinase A were carried out.

Results presented in Table I show the distribution of protein kinase C and protein kinase A activities in various subcellular fractions prepared according to de Duve et al. (1955). Approximately 85% of protein kinase C activity was found to be membrane-associated, and only 15% of the activity was present in the soluble fraction. Microsomal and nuclear fractions accounted for 63% and 10% of total activity. In comparison, approximately 90% of the protein kinase A activity was found in the soluble fraction and 10% in the membrane fraction. For protein kinase C the highest enzyme specific activity was in the microsomal fraction, followed by the nuclear, lysosomal, soluble, and mitochondrial fractions. In contrast, the specific activity of protein kinase A was highest in the soluble fraction. The purity of subcellular fractions was characterized by reference to the DNA/protein ratio for the nuclear fraction and to marker enzymes for mitochondrial (succinate–INT reductase), lysosomal (β-*N*-acetylglucosaminidase), Golgi (galactosyltransferase), endoplasmic reticulum (glucose-6-phosphatase), plasma membrane (5′-nucleotidase), and cytosolic (lactate dehydrogenase) fractions. Results in Figure 1 show the distribution of protein kinases C and A and marker enzymes plotted according to de Duve et al. (1955). Again, the majority of protein kinase C sedimented in the microsomal fraction, which was enriched in endoplasmic reticulum and plasma membrane markers. The remaining 10% activity was found in the nuclear (N) fraction, which also showed enrichment for DNA and 5′-nucleotidase.

Since the microsomal fraction (P) contained the highest amount of protein kinase C, initial attempts were made to isolate and purify the enzyme from this fraction. However, this approach met with little success: purification of the enzyme resulted in poor recovery, and in addition, the enzyme was extremely unstable even at initial stages of purification. To overcome these problems, a buffered extraction medium (referred to as buffer A) was devised and utilized for the purification of protein kinase C. Approximately 60–70% of the total activity could be released into the cytosol (100000*g* supernatant) when livers were directly homogenized in this medium. Furthermore, enzyme activity was quite stable in the crude extract and could be stored at 4 °C for at least 1 week without significant loss of activity. The inclusion of various protease inhibitors along with EGTA/EDTA in buffer A effectively blocked the proteolytic degradation of the enzyme.

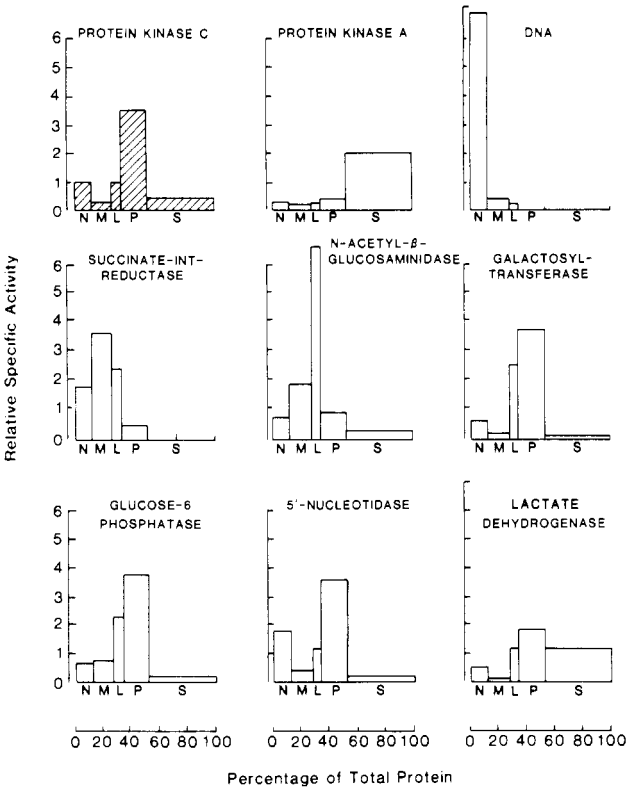

FIGURE 1: Distribution patterns of protein kinase C, protein kinase A, and marker enzymes after fractionation of liver homogenates by different centrifugation. Rat livers were homogenized in 0.25 M sucrose containing 3 mM imidazole (pH 7.4) and subjected to differential centrifugation to isolate nuclear (N), mitochondrial (M), light mitochondrial–lysosomal (L), microsomal (P), and cytosolic (S) fractions as described under Experimental Procedures. Particulate and soluble fractions were pretreated with CHAPSO (1% final concentration) before assay for protein kinase C or protein kinase A activity. Marker enzyme activities were assayed without detergent treatment of subcellular fractions. All assays were done in duplicate under the optimal conditions of concentration of protein and time. In each panel, the abscissa represents the relative protein content in N, M, L, P, and S fractions cumulatively from left to right. The ordinate represents the relative specific activity, i.e., percentage of activity of protein kinases, DNA, or merker enzyme divided by the percent of protein in each fraction. DNA content is used to identify nuclei, whereas the activity of 5′-nucleotidase, succinate–INT reductase, β-*N*-acetylglucosaminidase, galactosyltransferase, glucose-6-phosphatase, and lactate dehydrogenase is used to identify respectively plasma membrane, mitochondrial, lysosomal, Golgi, endoplasmic reticulum, and cytosolic cell compartments. The results are means of four separate experiments. Protein kinase C activity was obtained by subtracting activity measured in the presence of 0.5 mM EGTA from activity assayed with Ca^{2+}, phosphatidylserine, and diolein. Protein kinase A activity was obtained by subtracting activity measured without cAMP from activity measured in the presence of cyclic nucleotide.

Purification of Protein Kinases C from Rat Liver. (*1*) *Homogenization and Preparation of Crude Extract.* Three to four nonfasted male rats (300–350 g) were sacrificed by decapitation. The livers were rapidly removed and placed in ice-cold buffer A. All subsequent steps were carried out at 0–4 °C. The livers were rinsed, weighed (~30–40 g), minced with scissors, and homogenized with 4 volumes of buffer A by using a loose-fitting motor-driven Teflon pestle in a glass tube. The homogenate was centrifuged at 10000*g* for 10 min, followed by centrifugation at 25000*g* for 10 min. The supernatant was then centrifuged at $95500g_{av}$ for 90 min in a Beckman T35 rotor, and the resulting supernatant was designated as "crude extract".

(*2*) *Ammonium Sulfate Fractionation.* Solid enzyme-grade ammonium sulfate (17.6 g/100 mL, 30% saturation) was slowly added with stirring to the crude extract. After 1 h, the mixture was centrifuged at 12000*g* for 20 min. The sediment was discarded and the supernatant was brought to 70% saturation by the addition of solid ammonium sulfate (27.3 g/100 mL). After 1 h, the precipitated enzyme was sedimented by centrifugation at 12000*g* for 20 min. The sediment was resuspended in ~15 mL of buffer B, and the enzyme solution was dialyzed overnight (20–22 h) against 2 L of buffer B with three to four changes or buffer. A small amount of precipitate formed during dialysis was removed by centrifugation (10000*g* for 10 min). More than 75% of protein kinase C activity was contained in this fraction. Although only a small increase in specific activity was noted by this procedure, the ammonium sulfate precipitation concentrated the enzyme and eliminated some of the protein kinase A activity.

(*3*) *DEAE-cellulose Chromatography.* The enzyme solution from step 2 was applied to a DEAE-cellulose column (1.6 × 18 cm) which had been previously equilibrated with buffer B, and the column was washed with the same buffer until the effluent had an absorbancy of 0.02–0.04 at 280 nm. The enzyme was then eluted from the column with a linear gradient of NaCl (0–0.4 M) in 500 mL of buffer B. Fractions of 7 mL each were collected. Each fraction was assayed for protein kinase C and protein kinase A activity. Protein kinase C activity was measured by the incorporation of [^{32}P]phosphate into histone H_1 (Sigma type IIIs) in the presence of phosphatidylserine, Ca^{2+}, and diolein (Figure 2A). Similarly, protein kinase A activity was assayed by following radioactive phosphate incorporation into histone f_1 (Sigma type Vs) (Figure 2B). Results presented in Figure 2A show that in the absence of phosphatidylserine, Ca^{2+}, and diolein (and in the presence of 0.5 mM EGTA) one minor peak (peak a) and one major peak (peak b) of protein kinase were observed (open circles in Figure 2A). When the column fractions were assayed in the presence of Ca^{2+}, diolein, and phosphatidylserine, two peaks (designated peaks b and c) of protein kinase were obtained with histone H_1 (closed circles in Figure 2A). The protein kinase activity with absolute dependence on Ca^{2+}, phosphatidylserine, and diolein coincided with peak c activity and was designated as protein kinase C. In contrast, activities of peak a or b were not modulated by Ca^{2+} and/or phospholipid.

Each column fraction was also assayed for [^{32}P]phosphate incorporation into histone f_1 (the preferred substrate for protein kinase A) in the presence and absence of 2 μM cAMP (Figure 2B). Two major peaks of activity designated as I and II were consistently eluted (Figure 2B). On the basis of their relative elution position from DEAE-cellulose, which is known to separate type I and type II isoenzymes (Corbin et al., 1975), and on the basis of the finding that fractions I and II bound [^{3}H]cAMP and were inhibited when assayed in the presence of 2 μM cAMP and heat-stable protein kinase inhibitor (data not given), it was concluded that type I protein kinase A eluted with peak I and type II isoenzyme with the peak II fraction.

In summary, DEAE-cellulose column chromatography partially resolved the protein kinase C from type I protein kinase A and was devoid of type II protein kinase A (Figure 2B) or cAMP-independent protein kinase (data not shown). The major fractions containing protein kinase C were pooled and concentrated to 8–10 mL on an Amicon concentrating system (PM-10 membrane). The concentrated fractions were subsequently subjected to Fractogel filtration.

(*4*) *Fractogel Filtration Chromatography.* The concentrated solution was applied to a Fractogel column (1.6 × 84 cm) equilibrated in buffer B. The column was eluted with

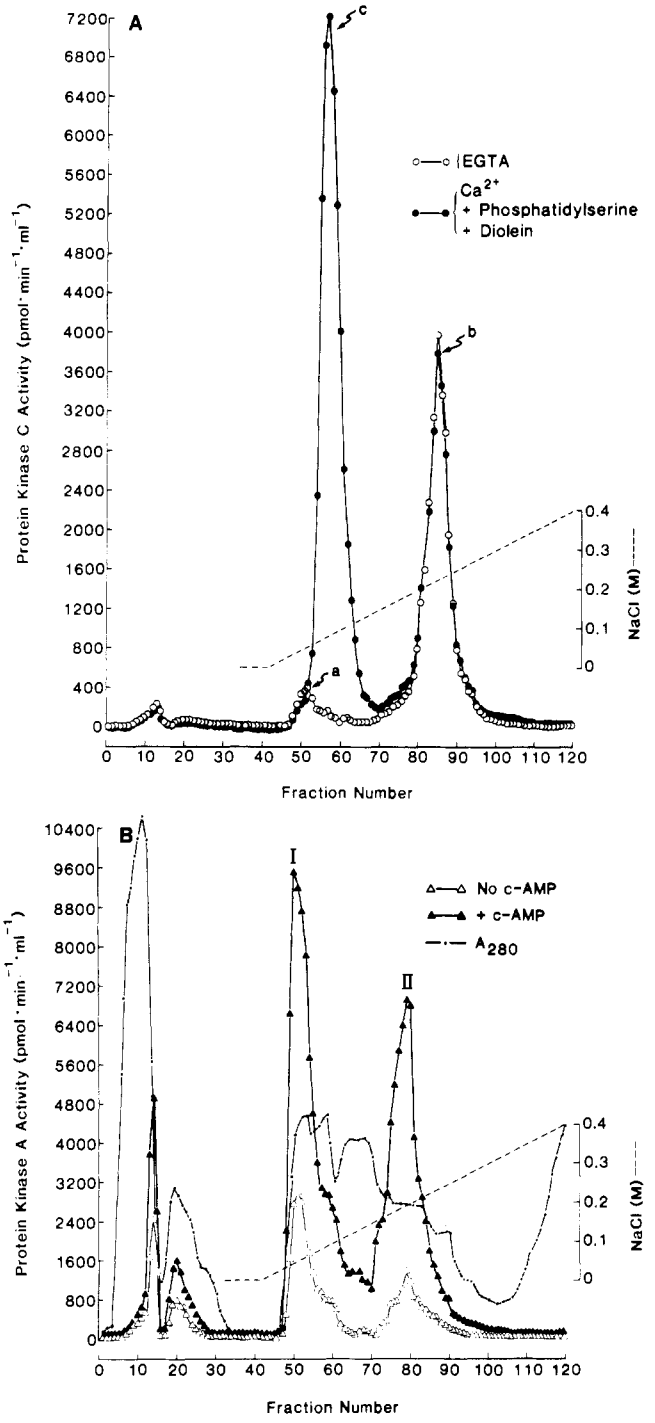

FIGURE 2: DEAE-cellulose column chromatography of protein kinase C, protein kinase A, and cAMP-independent protein kinase activity. Dialyzed ammonium sulfate fraction (~1500 mg of protein, step 2) was applied to the column (1.6 × 18 cm) preequilibrated in buffer B. The column was eluted with a linear gradient of 0–0.4 M NaCl in buffer B. The total gradient volume was 500 mL, and 6.8-mL fractions were collected. Assays for protein kinase C (panel A) and protein kinase A (panel B) were carried out on the eluted fractions as described under Experimental Procedures.

buffer B at a flow rate of 5 mL/h, and 4.5-mL fractions were collected. Protein kinase C activity was identified in fractions 34–42, which was partially resolved from contaminating type I protein kinase A in fractions 28–40.

(5) *Hydroxylapatite Chromatography.* The active fractions were combined and applied to a hydroxylapatite column (1.6 × 12 cm) previously equilibrated with buffer C. The protein was eluted with a linear gradient (20–500 mM potassium phosphate) in 500 mL of buffer C, and 4.8-mL fractions were

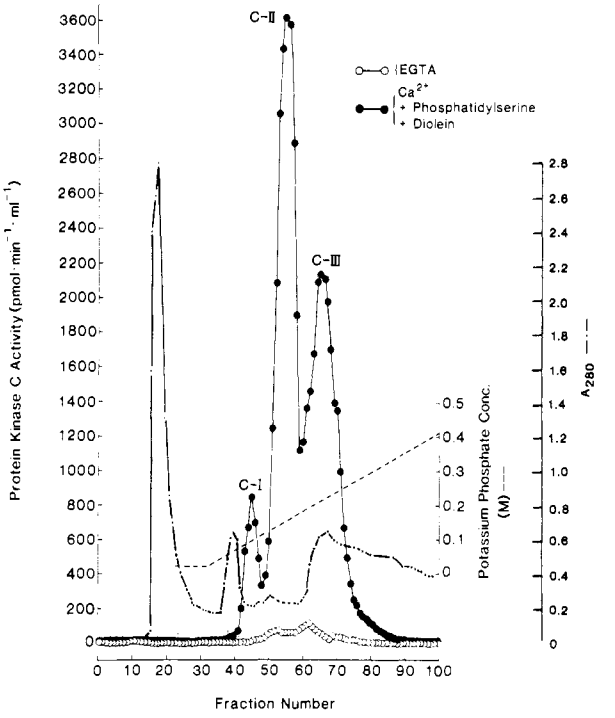

FIGURE 3: Separation of protein kinase C into three forms by hydroxylapatite chromatography. Protein kinase C preparation (~40 mg of protein) obtained by gel filtration (step 4) was applied to a column of hydroxylapatite (1.6 × 12 cm) and equilibrated with buffer C. The column was eluted with a linear gradient of 20–500 mM potassium phosphate in buffer C. The total gradient volume was 440 mL, and 100 fractions (4.8 mL each) were collected and assayed for protein kinase C.

collected. This column resolved protein kinase C activity into three distinct peaks (designated as C-I, C-II, and C-III) as shown in Figure 3. It should be noted that contaminating type I protein kinase A was quantitatively eluted between fractions 35 and 45. Thus, protein kinases C-II and C-III were free from protein kinase A contamination. At this stage all three enzymes were quite stable and could be stored at 4 °C for up to 1 week without significant loss of activity.

(6) *Ultrogel Filtration Chromatography.* Active fractions of protein kinases C-I, C-II, and C-III were concentrated to 5–7 mL by ultrafiltration and applied separately to an Ultrogel AcA34 column (2.6 × 83 cm) equilibrated in buffer B. The column was run for 30 h (flow rate ~14 mL/h), and fractions were assayed for protein kinase C activity. All three protein kinases eluted as single symmetrical peaks. Following rechromatography on an Ultrogel column (2.6 × 83 cm), protein kinase C-II eluted as homogeneous peak and no further purification was attempted for this isoenzyme.

(7) *Polyacrylamide-Immobilized Phosphatidylserine Affinity Chromatography.* Protein kinases C-I and C-III were further purified by affinity chromatography on polyacrylamide-immobilized phosphatidylserine according to Uchida and Filburn (1984) with the exception that the cholesterol/phosphatidylserine ratio was changed to 10:1 instead of 5:1 and the buffer used was 5 mM Tris-HCl, pH 7.5, as suggested by Girard et al. (1985). Each isoenzyme eluted from the affinity column was extremely unstable with a half-life less than 30 h but could be stabilized for several days in the presence of 0.8 M L-arginine (Inagaki et al., 1985). However, all of the experiments reported in this paper were performed within 24 h following the isolation of protein kinases C-I, -II, and -III without the use of arginine.

Table II summarizes the purification schedule of the different forms of protein kinase C from rat liver. The protocol

Table II: Purification of Protein Kinase C from Rat Liver

step	protein (mg)	total act.[a] (pmol·min^{-1})	sp act.[a] [pmol·min^{-1}·(mg of protein)$^{-1}$]	recovery (%)	purification (x-fold)
(1) crude extract	1966.0	586 000	299	100.0	1.0
(2) $(NH_4)_2SO_4$	1428.0	456 138	320	78.0	1.1
(3) DEAE-cellulose (DE-52)	167.0	376 603	2255	64.0	7.5
(4) Fractogel filtration	42.3	338 313	7998	58.0	27.0
(5) hydroxylapatite					
peak I	3.6	34 129	9400	5.8	32
peak II	4.8	168 085	35 018	28.7	117
peak III	2.8	139 552	49 840	23.8	167
(6) Ultrogel filtration					
peak I	0.426	10 236	24 028	1.8	80
peak II[b]	0.256	98 530	384 882	16.8	1287
peak III	0.135	27 339	202 511	4.7	677
(7) affinity chromatography					
peak I	0.029	2329	80 310	0.4	269
peak III	0.025	7381	295 240	1.3	987

[a] One unit of activity is defined as the amount of enzyme that transferred 1 pmol of ^{32}P from [γ-^{32}P]ATP to histone IIIs per minute. [b] This material was chromatographed twice on an Ultrogel column (2.6 × 83 cm).

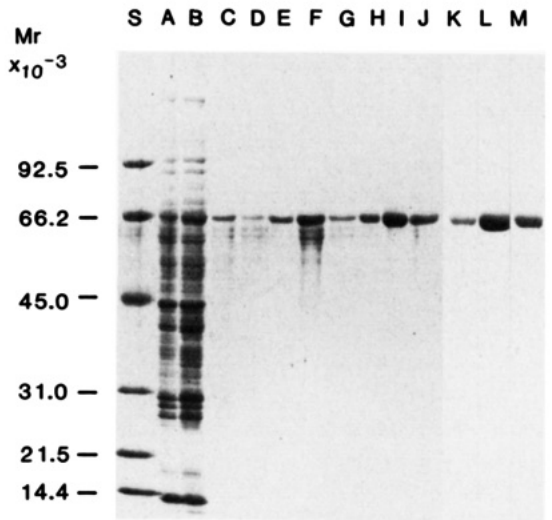

FIGURE 4: SDS–polyacrylamide gel electrophoresis of protein kinase C isoenzymes at various stages of purification. Purification was performed as described under Experimental Procedures. The samples were run on SDS–PAGE (5–20% linear acrylamide gradient), and the gels were stained with Coomassie blue. Lane A, crude starting material (105000g supernatant); lane B, 30–70% ammonium sulfate fraction; lane C, DEAE-cellulose peak fraction; lane D, Fractogel peak fraction; lanes E, F, and G, peak fractions of protein kinases C-I, C-II, and C-III respectively, from the hydroxylapetite column; lanes H, I, and J, peak fractions of protein kinases C-I, C-II, and C-III, respectively, from the Ultrogel column; lane L, protein kinase C-II (peak fractions) preparation rechromatographed on Ultrogel column; lanes K and M, peak fractions of protein kinases C-I and C-III, respectively, from the phosphatidylserine–acrylamide affinity column. Lane marked S represents molecular weight standards, which included phosphorylase b (M_r 92 500), bovine serum albumin (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), and soybean trypsin inhibitor (M_r 21 500).

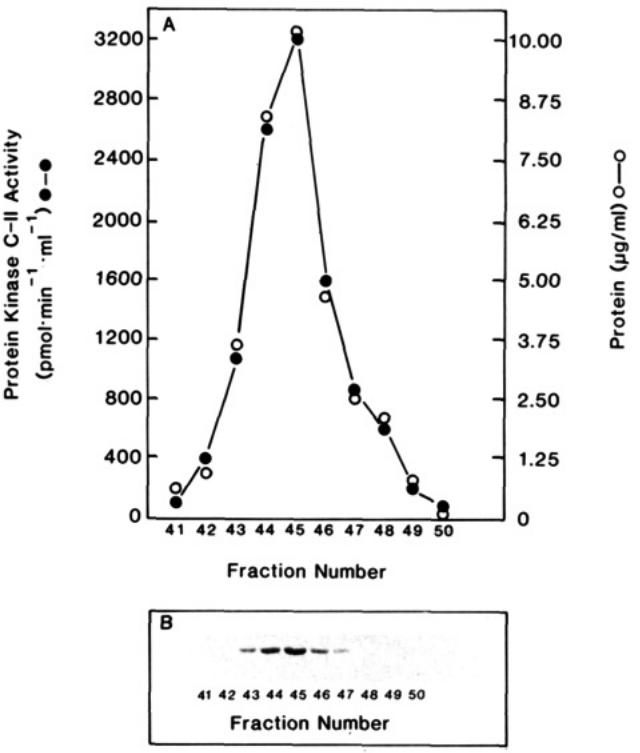

FIGURE 5: Rechromatography of highly purified protein kinase C-II on Ultrogel AcA34 column. Highly purified protein kinase C-II (material from step 6, Table II) was concentrated to ~7 mL by ultrafiltration and reapplied to a column (2.6 × 83 cm) of Ultrogel AcA34 which had been equilibrated and eluted with buffer B. Fractions (~7 mL) were collected, and portions were assayed for Ca^{2+}- and phospholipid-dependent protein kinase activity (●) or protein content (○) (panel A). The samples were analyzed by SDS–polyacrylamide (5–20% linear gradient) gel electrophoresis (panel B). Ca^{2+}- and phospholipid-dependent protein kinase was determined as described under Experimental Procedures. Protein concentration was determined by the fluorometric procedure of Böhlen et al. (1972). The molecular weight of the peptide band shown in panel B is 64K. No other peptide bands were visible on the gels.

results in 269-, 1287-, and 987-fold purification of protein kinases C-I, -II, and -III, respectively, with an approximate 18.5% yield of protein kinase C activity. The polypeptide composition of the fractions obtained during purification was analyzed by SDS–PAGE as shown in Figure 4: i.e., sequential preparative steps showed enrichment and then the identification of homogeneous 64K protein bands for protein kinase C-I (lane K), C-II (lane L), and C-III (lane M).

In order to relate the 64K polypeptide with protein kinase C activity, the highly purified Ultrogel preparations of protein kinase C-II (step 6) were subjected to gel filtration (Ultrogel AcA34), and eluted fractions were assayed for protein kinase C activity and protein content and further characterized by SDS–PAGE. A single peak of enzyme activity was found (fractions 41–50), which coeluted with the protein peak (Figure 5A). On SDS–PAGE the active material showed a single protein band with an average molecular weight of 64K (see legend to Figure 5B). In addition, Figure 5 shows a close

Table III: Substrate Specificity of Purified Protein Kinases C-I, C-II, and C-III

substrate	concn in assay (mg/mL)	rate of Ca^{2+}- and phospholipid-stimulated phosphorylation (% of histone H_1 act.) C-I	C-II	C-III
histone H_1[a]	2.0	100	100	100
protamine	2.0	19	22	39
casein	5.0	10	15	14
troponin	0.8	7	7	11
myosin light chain (rabbit muscle)	0.8	2	3	3
phosvitin	2.5	0	1	5
phosphorylase *b*	2.0	<0.1	<0.2	<0.1
poly(Glu-Na,Tyr) (4:1)	4.0	0	0	0

[a] The rate of Ca^{2+}- and phosphatidylserine-stimulated phosphorylation of histone H_1 was 80 nmol·min^{-1}·(mg of protein)$^{-1}$ for C-I, 421 nmol·min^{-1}·(mg of protein)$^{-1}$ for C-II, and 268 nmol·min^{-1}·(mg of protein)$^{-1}$ for C-III.

correlation between the amount of protein kinase C activity present in each fraction and the intensity of the 64K band.

Physical Properties of Isoenzymic Forms of Protein Kinase C. The molecular weight values of native enzymes (proteins), estimated by gel filtration (Ultrogel), were 225 000, 223 000, and 220 000 for protein kinases C-I, C-II, and C-III, respectively. Likewise, from the data obtained by gel filtration, a Stokes radius of 51.0 Å can be calculated for the three isoenzymes. Also, all three isoenzymes (C-I, C-II, and C-III) sedimented as single peaks as judged by sucrose density gradient ultracentrifugation (data not shown). The sedimentation coefficients ($s_{20,w}$) of the three isoenzymes determined by using catalase, alcohol dehydrogenase, bovine serum albumin, and lysozyme as markers were respectively 9.0 S, 9.2 S, and 9.2 S. On the basis of values for the sedimentation coefficient ($s_{20,w}$) and Stokes radius (a) and the assumed partial specific volume of 0.725 cm^3/g (Siegel & Monty, 1966), the approximate molecular weights of protein kinase C-I, C-II, and C-III were calculated to be about 210 000. These figures are in good agreement with those obtained by gel filtration methods. The fractional ratios (f/f_0) (calculated from molecular weight, sedimentation coefficient, and an assumed specific volume of 0.725 cm^3/g) averaged about 1.3 for the three isoenzymes, suggesting asymmetry of the enzyme molecules.

Electrophoresis of protein kinase C isoenzymes in PAGE containing SDS gave a single band corresponding to M_r 64K for all three isoforms (Figure 4). This suggests that the native isoenzymes (with molecular weight of about 220 000) may be composed of subunits.

Comparison of Enzymatic Properties of Protein Kinases C-I, C-II, and C-III. The reaction requirements, substrate specificity, and catalytic constants of protein kinase C isoenzymes were compared to determine whether their enzymatic properties differ significantly.

(*1*) *Reaction Requirements.* All three isoenzymic forms had absolute requirements for Ca^{2+} and phosphatidylserine (data not shown). Addition of Ca^{2+} and phosphatidylserine at saturating concentrations synergistically stimulated enzyme activities (stimulation ranged from 150- to 300-fold).

(*2*) *Substrate Specificity.* The ability of purified liver protein kinases C-I, C-II, and C-III to phosphorylate a number of protein substrate is shown in Table III. In general, the substrate specificity of the three forms did not differ significantly. The most active substrate other than histone H_1 was protamine. Casein and troponin were also phosphorylated to some extent. All three isoenzymes were more or less inactive against rabbit or chicken muscle myosin light chains, phosvitin, phosphorylase *b*, and poly(Glu,Tyr) (4:1) (a suitable substrate for tyrosine protein kinase).

Table IV: Demonstration of Lack of Effect of cAMP, cGMP, or Calmodulin on Histone Phosphorylation by Protein Kinases C-II and C-III[a]

	enzyme act. [×10^3 pmol·min^{-1}·(mg of protein)$^{-1}$] protein kinase C-II	protein kinase C-III
histone H_1	2.0	3.6
histone H_1 + Ca^{2+} + phosphatidylserine	463.00	332.00
histone H_1 + Ca^{2+} + calmodulin	1.9	3.8
histone H_1 + cAMP	2.1	3.4
histone H_1 + cGMP	1.7	3.5
histone f_1	1.6	6.2
histone f_1 + Ca^{2+} + phosphatidylserine	90.0	73.0
histone f_1 + Ca^{2+} + calmodulin	1.6	6.3
histone f_1 + cAMP	1.5	6.4
histone f_1 + cGMP	1.7	6.1

[a] Assays were conducted under the standard conditions except that the indicated reagents were added. In all cases, assays were conducted in duplicate, in linear range (i.e., 10 min at 30 °C with approximately 100 ng of enzyme protein). The concentrations of histone, Ca^{2+}, phosphatidylserine, calmodulin, cAMP, and cGMP used were 800 μg/mL, 0.5 mM, 250 μg/mL, 0.8 μM, 10 μM, and 25 μM, respectively.

Complete acid hydrolysis of histone H_1 after phosphorylation permitted quantitative analysis of the amino acids that were labeled with [γ-^{32}P]ATP. Eighty-five to ninety percent of the label was detected in phosphoserine, and the remaining label was in phosphothreonine. No significant label was detected in phosphotyrosine (data not given).

No stimulation of histone phosphorylation (H_1 or f_1) by protein kinase C isoenzymes was observed with cAMP, cGMP, or calmodulin (Table IV). Thus, purified protein kinase C preparations (C-I, C-II, and C-III) were devoid of protein kinase specifically stimulated by these agents. Similarly, phosphorylase kinase, glycogen synthase, and glycogen phosphorylase activities were not detected in purified isoenzymic preparations (data not shown).

(*3*) *Catalytic Constants.* For each isoenzyme, apparent K_m values for histone, ATP, and Mg^{2+} as well as apparent K_a values for Ca^{2+} and phosphatidylserine were determined. The apparent K_m values for histone were calculated to be 0.16 mg/mL, 0.30 mg/mL, and 0.05 mg/mL for C-I, C-II, and C-III, respectively. In contrast, the apparent K_m values for ATP (range 10.1–13.3 μM) and Mg^{2+} (range 1.3–1.5 mM) were similar. The apparent K_a for phosphatidylserine was similar for C-I (35 μg/mL) and C-II (40 μg/mL) and was approximately double that of C-III (20 μg/mL). Comparable K_a values were found for Ca^{2+} of C-II (28 μM) and C-III (35 μM). It should also be noted that addition of diolein decreased the K_a for Ca^{2+} and phosphatidylserine without affecting maximal activities of the three isoenzymes (data not shown).

Discussion

This study describes the characteristics, distribution, and purification steps of protein kinase C in rat liver and, for the first time, provides evidence for the existence of multiple forms of the liver enzyme. Since rather specialized techniques were paramount in permitting the final purification of the enzyme to be carried out, these issues will be dealt with in some detail.

The first point has to do with understanding the latency of the enzyme. When livers are homogenized in isotonic sucrose buffered with imidazole, the majority of protein kinase C

activity is recovered in the particulate fraction. However, most of this activity is latent and can be visualized only after solubilization with detergent (possibly protein kinase C is sealed in membrane vesicles impermeable to substrates such as histone). We have found that CHAPSO, which possesses the combined properties of polar (sulfobetaine-containing) and hydrophobic (bile salt, anionic) detergents in a single molecule, is most effective in solubilizing and maximally stimulating this enzyme activity. When CHAPSO is used, we have found that total enzyme activity is 2 (Kiss & Mhina, 1982; Jergil & Sommarin, 1983) to 5 (Kikkawa et al., 1982) times the value reported by others. Furthermore, when CHAPSO is used, we have found that approximately 85% of total protein kinase C activity is located in the particulate fraction of the liver as compared to the 20% of total activity previously shown to be in this fraction by Kikkawa et al. (1982). We feel that the composition of the homogenization buffer is a major factor in unmasking protein kinase C activity and probably explains why our values differ from others.

Within the particulate fraction, the specific activity of protein kinase C is substantially higher in the microsomal fraction than in the others. Since this fraction is enriched in plasma membrane (5′-nucleotidase), Golgi membranes (galactosyltransferase), and endoplasmic reticulum (glucose-6-phosphatase) markers, it would appear that the enzyme is specifically associated with one or more of these cellular membranes but not the membranes of nuclei, mitochondria, and lysosomes. Since the nuclear fraction also shows a substantial amount of 5′-nucleotidase activity (and possibly is also contaminated with plasma membranes), it is not surprising to find rather high levels (10.6%) of total protein kinase C activity associated with this fraction as well.

The second technical issue of importance in the study has to do with the extreme susceptibility of liver protein kinase C to protease action: solution of this problem led to purification of the enzyme from rat liver and identification of the multiple forms of the enzyme. The novel feature of the present procedure was the use of proteolytic inhibitors and metal chelators (EGTA, EDTA) throughout the purification. After purification by ammonium sulfate fractionation and DEAE-cellulose chromatography, Fractogel filtration allowed us to eliminate significant amounts of the contaminating type I cAMP-dependent protein kinase. However, the single most important step in the purification process was the use of hydroxylapatite, which effectively resolved the protein kinase C into isoenzymic forms. The increase in enzyme activity following the Fractogel filtration and hydroxylapatite steps suggests the loss of some inhibitor substance and reactivation of the enzyme during this phase of the procedure.

The unexpected findings of this study bring up a number of questions. An important point is whether the multiple forms of protein kinase C found in liver represent a true phenomenon. Although isoenzyme forms of cAMP-dependent protein kinases (Corbin et al., 1975; Flockhart & Corbin, 1982) have previously been described, none until recently have been identified for protein kinase C, and it is always a concern that modifications of enzymes (protein aggregation or degradation) may occur during isolation procedures. Indeed, Girard et al. (1986) working with rat brain and heart protein kinase C identified both an 80K native enzyme and a 67K catalytically active species. The latter was presumed a fragment of the native enzyme since its activity was independent of phospholipids and Ca^{2+}, and it was not absorbed by phosphatidylserine-coupled affinity gels even in the presence of Ca^{2+}. However, Bazzi and Nelsestuen (1986) have published a preliminary report showing that, in bovine brain, both 80K and 67K kinases C exist and that both molecular species show absolute requirements for calcium and phospholipids. In addition, while the current report on liver protein kinase C was in revision, Huang et al. (1986) reported that rat brain protein kinase C actually exists as three isoenzyme forms, all with similar characteristics and similar molecular weights of ~80K. Given these recent findings about isoenzymes of brain protein kinase C and the reports that several laboratories have cloned and identified multiple protein kinase C cDNAs of brain (Knopf et al., 1986; Mokowske et al., 1986; Nishizuka, 1986; Ohno et al., 1987), it is conceivable that the liver enzyme also exists as multiple isoenzymes. In the case of liver, the molecular weight (SDS–PAGE) of the protein kinase C isoenzymes is in the 64K range, but the proteins nevertheless appear to be native, not degradative forms of the enzyme. This thinking follows from the fact that the purification procedure was conducted in the presence of protease inhibitors to avoid degradation, that all three of the demonstrated isoenzymic forms have comparable physicochemical properties including their molecular weights and sedimentation coefficients, and that the enzymic activities of all three enzymic forms are free of other proteins. That they are totally dependent upon Ca^{2+} and phosphatidylserine is in keeping with the known properties of protein kinase C from other tissues (Kikkawa et al., 1982; LePeuch et al., 1983; Schatzman et al., 1983; Kraft & Anderson, 1983b; Brocklehurst et al., 1985; Noguchi et al., 1985).

Why such isoenzymes coexist in liver is a totally different question. As discussed by Bell and colleagues (Knopf et al., 1986), families of protein kinase C related polypeptides may have many different structural and biochemically relevant functions in tissues. One can easily imagine that in liver, a tissue which simultaneously processes a multitude of cellular proteins, a fine control for protein phosphorylation would provide a needed kind of flexibility.

In summary, we have described three purified isoenzymic forms of protein kinase C in rat liver. All three isoenzymes have similar molecular weights and other physical–chemical properties. The enzymes differ from other identified and purified protein kinases C in their tissue content, tissue distribution, and lower molecular weights and in the fact that each isoenzyme is made up of subunits. To what extent these differences represent methodological variations in isolating the enzymes or represent tissue-specific (or species-specific) changes remains to be seen.

Registry No. 5′-ATP, 56-65-5; Ca, 7440-70-2; Mg, 7439-95-4; protein kinase, 9026-43-1.

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Interaction of the Unique Competitive Inhibitor Imidazole and Related Compounds with the Active Site Metal of Carbonic Anhydrase: Linkage between pH Effects on the Inhibitor Binding Affinity and pH Effects on the Visible Spectra of Inhibitor Complexes with the Cobalt-Substituted Enzyme†

Raja G. Khalifah,* Janice I. Rogers, and Jogeshwar Mukherjee‡

Department of Biochemistry, Kansas University School of Medicine, and Veterans Administration Medical Center, Kansas City, Missouri 64128

Received January 15, 1987; Revised Manuscript Received May 6, 1987

ABSTRACT: Previous studies on the interaction of carbonic anhydrase (CA) with the unique CO_2 competitive inhibitor imidazole and related compounds were all interpreted as showing that an ionizable water ligand on the metal of this zinc metalloenzyme is *not* displaced by inhibitor binding. Internal inconsistencies in the pH dependence of binding and the pH dependence of the visible spectra of complexes with cobalt-substituted enzyme prompted us to reinvestigate this binding. Visible spectroscopy was used to measure the binding of imidazole and 1,2,4-triazole to Co(II)-substituted human CA I and active site carboxymethylated human CA I (CmCA I) and the binding of 1,2,4-triazole to bovine Co^{II}CA II. The limiting visible spectra for these enzyme–inhibitor adducts were also computed and examined for pH dependence. It was shown that the pK_a of visible spectral changes can be independently predicted from studies on the pH dependence of binding. After consideration of possible contributions from effects of His-200 ionization in CA I and CmCA I, and His-64 in CA II, the pH effects on binding affinity and spectra were found to be of the correct magnitude to establish linkage between binding and an ionization. It was also shown, however, that pH effects on binding and spectra cannot distinguish whether neutral imidazole binds to both ionization forms of the enzyme (Zn-OH_2 and Zn-OH) or whether neutral imidazole and its anion both bind to only the acid form of the enzyme, presumably after displacing the water. These findings have implications to the crystallographic interpretations on the imidazole–enzyme complex and to the catalytic mechanism of CO_2 hydration.

It was discovered some 15 years ago that imidazole is a carbonic anhydrase (EC 4.2.1.1) isozyme I inhibitor that is uniquely competitive with the physiological substrate carbon dioxide (Khalifah, 1971). To date, only one other inhibitor has been demonstrated to be similarly competitive with carbon dioxide, this being phenol in its inhibition of the other major isozyme II (Simonsson et al., 1982). The understanding of the mode of binding of imidazole to CA I[1] is thus of special mechanistic significance (Kannan et al., 1977; Silverman & Vincent, 1983). This is made even more so by the difficulty of studying an isolated enzyme–carbon dioxide complex (Riepe & Wang, 1968; Khalifah, 1971; Williams & Henkens, 1985; Stein et al., 1977) and by the absence of true homologues of this small substrate.

Besides being competitive with CO_2 and being an uncommon neutral inhibitor, imidazole differs from most other inhibitors of carbonic anhydrase in three important respects. First, we note that the overwhelming majority of anionic inhibitors [cf. reviews by Maren (1967, 1976)], including the ionizable sulfonamides (Kanamori & Roberts, 1983; Blackburn et al., 1985), are known to bind by displacing the ionizable water ligand in the coordination sphere of the active site zinc (Lindskog, 1982, 1983; Lindskog et al., 1971; Bertini & Luchinat, 1983). However, when Kannan and co-workers carried out crystallographic studies of the imidazole–CA I complex, they found that imidazole does not displace the catalytically essential water ligand of the metal (Kannan et al., 1977; Kannan, 1980). Instead, it appears to bind as a

† Financially supported by the Medical Research Service of the Veterans Administration.

* Address correspondence to this author at Research Service (151), Veterans Administration Medical Center, Kansas City, MO 64128.
‡ Present address: Department of Radiology, University of Chicago, Chicago, IL 60637.

[1] Abbreviations: CA I and II, carbonic anhydrase isozymes I and II (formerly referred to as B and C type); CmCA I, CA I carboxymethylated at its active site His-200; Bis-Tris, [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane; MES, 4-morpholineethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-7057$01.50/0