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Purification and Characterization of an Isoform of Protein Kinase C from Bovine Neutrophils†

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ABSTRACT: Protein kinase C (PKC) from bovine neutrophils was purified 1420-fold. Subcellular fractionation analysis of bovine neutrophil homogenate in the presence of EGTA indicated that more than 95% of the PKC activity was present in the soluble fraction. The purification procedure from cytosol involved sequential chromatographic steps on DE-52 cellulose, Mono Q, and phenyl-Sepharose. Whereas bovine brain PKC could be resolved into four isoenzymatic forms by chromatography on a hydroxylapatite column, bovine neutrophil PKC was eluted in a single peak, suggesting that it corresponded to a single isoform. The apparent molecular weight of bovine neutrophil PKC was 82000, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. By filtration on Sephadex G-150, a molecular weight of 85000 was calculated, indicating that bovine neutrophil PKC in solution is monomeric. Its isoelectric point was 5.9 ± 0.1. Bovine neutrophil PKC was autophosphorylated in the presence of [γ-^{32}P]ATP, provided that the medium was supplemented with Mg^{2+}, Ca^{2+}, phosphatidylserine, and diacylglycerol; phorbol myristate acetate could substitute for diacylglycerol. Autophosphorylated PKC could be cleaved by trypsin to generate two radiolabeled peptides of M_r 48000 and 39000. The labeled amino acids were serine and threonine. During the course of the purification procedure of bovine neutrophil PKC, a protein of M_r 23000, which was abundant in the cytosolic fraction of the homogenate, was found to exhibit a strong propensity to PKC-dependent phosphorylation in the presence of [γ-^{32}P]ATP, Mg^{2+}, Ca^{2+}, phosphatidylserine, and diacylglycerol. This protein was recovered together with PKC in one of the two active peaks eluted from the Mono Q column at the second step of PKC purification. It is suggested that the M_r 23000 protein might be a natural substrate for bovine neutrophil PKC.

Protein kinase C, a Ca^{2+}- and phospholipid-dependent kinase (PKC)[1] of $M_r \simeq 80000$, has been found in a large number of tissues and appears to play a central role in cellular economy [for review, see Nishizuka (1984)]. Recently, the existence of a family of PKC genes has been reported (Knopf et al., 1986; Parker et al., 1986; Coussens et al., 1986; Ono et al., 1986; Ohno et al., 1987). Three cDNA clones were obtained from rat, bovine, human, and rabbit libraries, and two sequences from rat brain were shown to derive from alternative splicing of a single clone (Kubo et al., 1987). Isoforms of PKC have been identified after separation on a hydroxylapatite (HTP) column from rat brain (Huang et al., 1986a; Kikkawa et al., 1987) and rabbit brain (Jaken & Kiley, 1987). PKC isoforms have also been reported in rat liver (Azhar et al., 1987), in murine fibroblasts (McCaffrey et al., 1987), in rat spleen (Brandt et al., 1987), and in bovine adrenocortical tissue (Pelosin et al., 1987).

It is a current view that PKC in neutrophils accomplishes a number of strategic functions, following exposure of these cells to specific stimuli or to phorbol myristate acetate (PMA), in particular the activation of the respiratory burst [for reviews, see Rossi (1986) and Tauber (1987)]. Our group is interested in the activation of the respiratory burst in bovine neutrophils (Ligeti et al., 1988). We therefore decided to explore in more detail the nature, properties, and function of PKC from bovine neutrophils. The present paper describes a simple and rapid method of purification of PKC from bovine neutrophils and some properties of this enzyme. The search for isoforms of neutrophil PKC is also reported, together with the demonstration of a PKC-dependent phosphorylation of a small mo-

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[1] Abbreviations: PKC, protein kinase C; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethane sulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PMA, 4β-phorbol 12-myristate 13-acetate; ATP, adenosine 5′-triphosphate; HTP, hydroxylapatite; M_r, molecular weight; SDS, sodium dodecyl sulfate.

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lecular weight protein from neutrophil cytosol that copurifies with PKC during the initial steps of purification and might be a natural substrate for PKC.

Materials and Methods

Materials. The sources of the chemicals were as follows: [γ-^{32}P]ATP (5000 Ci/mmol) from Amersham (U.K.); histone type IIIS, phosphatidylserine, Mops, EDTA, EGTA, PMSF, leupeptin, soybean trypsin inhibitor, Triton X-100, diacylglycerol, phosphothreonine, phosphoserine, phosphotyrosine, and sucrose from Sigma Chemical Co.; TPCK, TLCK, and SDS from Serva (FRG); ATP from Boehringer (FRG); β-mercaptoethanol from Koch-Light (U.K.); Tween 20, acrylamide, and bis(acrylamide) from BDH (U.K.); DEAE-(DE-52) cellulose from Whatman (U.K.); phenyl-Sepharose CL-4B, Percoll, Mono Q column (HR 5/5), and molecular weight markers from Pharmacia (Sweden); ultrafilters from Amicon; the hydroxylapatite (HTP) column, type HCA (10 × 0.76 cm), from Mitsui Toatsu Chemicals, Inc. (Japan); thin-layer plates (F 1440) from Schleicher & Schuell (FRG); Coomassie blue R 250 from Merck (FRG).

Purification of Bovine Neutrophil PKC. Three types of buffer were used for purification of PKC. Buffer A contained 0.25 M sucrose, 10 mM Mops, pH 7.4, 2 mM EDTA, 10 mM EGTA, 50 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/mL TPCK, 1 μg/mL TLCK, 1 μg/mL soybean trypsin inhibitor, and 10 μg/mL leupeptin. Buffer B contained 10 mM Mops, pH 7.4, 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 10% ethylene glycol, 1 mM PMSF, and 10 μg/mL leupeptin or 1 μg/mL TLCK. Buffer B supplemented with 1.5 M NaCl was referred to as buffer C.

Routine preparations were carried out at 2–4 °C with neutrophils obtained from 10 L of fresh cow's blood (Morel et al., 1985). Medium A used for disruption of neutrophils contained 2 mM EDTA and 10 mM EGTA to prevent translocation of cytosolic PKC to the plasma membrane and to inhibit the hydrolytic activity of calpain, a Ca^{2+}-dependent enzyme abundant in neutrophils, which cleaves PKC to generate a Ca^{2+}- and phospholipid-independent form of kinase C. It also contained protease inhibitors, as cytosol from neutrophils is rich in proteases. Bovine neutrophils suspended in buffer A at a concentration of 10^8 cells/mL were disrupted by four successive sonications of 15 s separated by 30-s intervals in an ice bath, with a Branson sonifier at 60-W output. The supernatant obtained after 1 h of centrifugation at 150000g in a Ti 45 rotor (Beckman) and corresponding to neutrophil cytosol was recovered for further purification. It contained more than 95% of cell PKC. Soluble PKC activity was much lower when cell disruption was performed with a Potter-Elvejhem-type homogenizer.

The purification procedure comprised three chromatographic steps. The first two steps were similar to those described by Yeng et al. (1986) for preparation of PKC from mice brain. In the first step, the cytosolic fraction (40 mL) was applied to a DE-52 cellulose column (10 × 2.7 cm). The column was washed with 200 mL of buffer B at a rate of 24 mL/h and then developed with a linear 0–0.3 M NaCl gradient in 400 mL of buffer B, at the same rate. Active fractions eluted between 0.08 and 0.12 M NaCl were pooled and concentrated to 2 mL by ultrafiltration on Amicon YM-30 filters. The concentrate was diluted to 20 mL with buffer B and applied in a second step to a Mono Q (HR 5/5) column that had been equilibrated with buffer B. The column was eluted with a linear gradient (30 mL) of NaCl (0–0.3 M) made in the same buffer and programmed in an FPLC system. Two peaks of PKC activity, both Ca^{2+} and phospholipid dependent, were eluted at about 0.05 M NaCl (peak 1) and 0.12 M NaCl (peak 2) (Figure 1). Peak 1 contained $^2/_3$ of the total PKC activity and peak 2 the remaining. The third step was chromatographed on phenyl-Sepharose. The material contained in peak 1 and peak 2 of the preceding step was adjusted to 1.5 M NaCl and applied separately to a phenyl-Sepharose column (1.5 × 0.5 cm) equilibrated with buffer C. After being loaded, the column was washed once with 3 mL of buffer C and then eluted with a decreasing linear gradient (6 mL) of NaCl (1.5–0 M). The active fractions were eluted in the absence of salt at the end of the gradient. The material of peak 2 eluted from the Mono Q column could be easily purified to homogeneity by phenyl-Sepharose chromatography as assessed by electrophoretic migration in a SDS–polyacrylamide gel. In contrast, the phenyl-Sepharose step failed to remove efficiently PKC contaminants in peak 1. Whereas the PKC activity recovered at the first steps of the purification procedure was stable at 4 °C, the activity recovered by phenyl-Sepharose chromatography was unstable; it was fully stabilized by addition of 0.05% Tween 20.

Purification of Bovine Brain PKC. Bovine brain removed immediately after death of the animal was immersed in ice-cold phosphate buffer–salt solution. Further operations were carried out at 2–4 °C. The white matter was discarded, and about 100 g of gray matter was dispersed in 2 volumes of buffer A. The brain suspension was homogenized by fractions of 20 mL in a Potter-Elvejhem homogenizer with a tightly fitted Teflon pestle at 1000 rpm. The homogenate was centrifuged at 10000g for 10 min, and the pellet was discarded. The supernatant was centrifuged at 150000g for 1 h. The final supernatant (cytosolic fraction) was filtered on glass wool and used for purification of PKC, following the three same chromatographic steps as for bovine neutrophil PKC. Purified brain PKC was stored at −20 °C in the presence of 0.05% Triton X-100 to protect its activity.

Separation of PKC Isoforms from Bovine Neutrophils and Bovine Brain. Samples obtained from chromatography on Mono Q or phenyl-Sepharose were dialyzed overnight against buffer D which consisted of 0.01 M potassium phosphate, pH 7.4, 10% glycerol, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 1 μg/mL TLCK, 0.5 mM EDTA, 0.5 mM EGTA, and either 0.05% Tween 20 for neutrophil PKC or 0.05% Triton X-100 for brain PKC. The enzyme solution was applied to an HTP column (10 × 0.76 cm) which had been previously equilibrated with a medium containing 0.01 M potassium phosphate, pH 7.4, 10% glycerol, 10 mM β-mercaptoethanol, and 1 μg/mL TLCK or 10 μg/mL leupeptin (buffer E). The column was eluted with a linear 0.010–0.175 M potassium phosphate gradient, pH 7.4, in buffer E, programmed in an HPLC system. Fractions of 0.45 mL were collected in tubes containing either Tween 20 (neutrophil PKC) or Triton X-100 (brain PKC) to obtain a final concentration of detergent of 0.05%. The collected fractions were assayed for conductivity and PKC activity. For convenience, the classification of PKC isoforms adopted in the present work is that proposed by Kikkawa et al. (1987), which corresponds to the order of elution from HTP column chromatography. For example, rat brain PKC consists of three main types of isoforms, numbered I, II with subtypes IIα and IIβ, and III.

Assay of PKC Activity. PKC activity was measured by enzymatic transfer of the terminal radiolabeled phosphate from [γ-^{32}P]ATP to histone type IIIS in the following standard medium: 20 mM Mops, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 50 μg of histone type IIIS, 20 μg/mL phosphatidylserine, and 2 μg/mL diacylglycerol in a final volume of 100 μL (Stasia

Table I: Purification of PKC from Bovine Neutrophil[a]

step	protein (mg)	total act. (units)	sp act. (units/mg)	recovery (%)	purification (x-fold)
cytosolic fraction	1008	393	0.39	100	1
DEAE-cellulose (DE-52)	176	487	2.76	124	7
Mono Q FPLC					
peak 1	2.12	105	49.5	27	127
peak 2	0.38	68	179	17	459
phenyl-Sepharose CL-4B (from peak 2 of Mono Q column)	0.016	9	554	2	1420

[a] One unit of activity is defined as the amount of enzyme that transfers 1 nmol of [^{32}P]phosphate from [γ-^{32}P]ATP to histone type IIIS per minute at 30 °C.

et al., 1987). The reaction was started by addition of [γ-^{32}P]ATP to give a final concentration of 70 μM. Control assays to assess the activity of Ca^{2+}- and phospholipid-independent kinase were systematically included, in which $CaCl_2$, diacylglycerol, and phosphatidylserine were replaced by 2 mM EGTA. Incubation was carried out at 30 °C for 3 and 6 min to ascertain the linearity of the reaction and was terminated by addition of 0.5 mL of ice-cold 10% trichloroacetic acid followed by 0.5 mL of a solution of 2 mg/mL bovine serum albumin. The protein was collected on Millipore HAWP 0.45-μm membrane filters and washed three times with 1 mL of ice-cold 10% trichloroacetic acid. Radioactivity on the filters was measured by liquid scintillation counting (Patterson & Greene, 1965). A blank assay was performed with trichloroacetic acid and bovine serum albumin being added to the incubation medium prior to the enzyme. A unit of PKC activity corresponds to 1 nmol of ^{32}P incorporated into histone per minute at 30 °C.

Autophosphorylation of PKC was performed following the same procedure as that described for measurement of PKC activity except that histone was omitted and the reaction was stopped by precipitation with ice-cold 4% perchloric acid, washed with cold acetone, and processed for SDS gel electrophoresis (see below).

Analysis of Phosphorylated Amino Acids. ^{32}P-Autophosphorylated PKC was precipitated by ice-cold 4% perchloric acid. The precipitate was washed with acetone and solubilized in 0.05 M ammonium acetate and 0.1 M pyridine adjusted to pH 7.8 with acetic acid. This was followed by a tryptic digestion for 24 h at 37 °C with a trypsin to PKC ratio of 1/50. The released peptides were hydrolyzed in 6 N HCl for 1 h at 110 °C. Samples were spotted on thin-layer plates of F 1440 cellulose together with standard phosphorylated amino acids and then subjected to electrophoresis at 800 V for 1 h at 10 °C, in a buffer containing pyridine, acetic acid, and water (1:10:89 v/v), pH 3.6. The plates were stained with ninhydrin to reveal amino acids and subsequently exposed to Fuji X-ray films (Hunter & Sefton, 1980).

Isoelectric Focusing of Bovine Neutrophil PKC. A linear 5–40% sucrose gradient was prepared with 10 mM β-mercaptoethanol, 10 μg/mL leupeptin, and LKB ampholines pH 5–7 in a 110-mL column. The cytosolic fraction containing PKC was applied to the middle of the column. The reservoir at the cathode was filled with 0.01 M NaOH in 50% sucrose (w/v) and that at the anode with 0.01 M sulfuric acid. Isoelectric focusing was carried out at 4 °C for about 40 h at 600 V. Fractions of 2.5 mL were collected and assayed for pH, PKC activity, and protein content.

SDS–Polyacrylamide Gel Electrophoresis. Prior to electrophoresis, protein was precipitated with ice-cold 4% perchloric acid. The precipitate was washed with cold acetone and dissolved in 0.06 M Tris-HCl, pH 6.8, 15% (v/v) glycerol, 5% β-mercaptoethanol, 2.3% SDS, and 0.001% bromophenol blue. After incubation at 60 °C for 30 min, the solubilized samples were subjected to SDS gel electrophoresis as described by Laemmli and Favre (1973), using a 4% stacking gel and a 13% resolving gel. The molecular weight standards used were phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100), and α-lactalbumin (M_r 14 400). Gels were stained with Coomassie blue R 250. For autoradiography, the gels were dried in a vacuum drier and exposed to Fuji X-rays films.

Protein Assay. Protein was routinely determined by the method of Bradford (1976). When the protein concentration was too low, the samples were treated with 10% (w/v) trichloroacetic acid. The precipitate was collected by centrifugation and solubilized in 10% SDS for protein determination by the method of Lowry (1951). In both cases, bovine serum albumin was used as a standard.

Results

Purification of Bovine Neutrophil PKC. A representative purification scheme of bovine neutrophil PKC is documented in Table I. From about 1 g of cytosolic protein, 16 μg of a homogeneous protein with a specific activity of 554 units/mg was recovered. This represents a purification of 1420-fold relative to the initial cytosolic fraction. This value was not corrected for loss of activity which occurs during the enzyme preparation.

The crude cytosolic fraction was first run over a DE-52 column. After this step, the total PKC activity was routinely higher than that of the initial cytosolic fraction, probably due to removal of an endogenous inhibitor (Balazovich et al., 1980). Indeed, PKC inhibitors of protein nature have been purified from brain extracts (Hucho et al., 1987; McDonald et al., 1987). Fractions containing PKC activity were pooled and chromatographed on a Mono Q column with a FPLC apparatus. Two peaks of PKC activity were eluted with 0.05 and 0.12 M NaCl, respectively. As shown by SDS–polyacrylamide gel electrophoresis, PKC in peak 1 was highly contaminated by a number of proteins, whereas the material of peak 2 contained relatively purified PKC (Figure 1). With respect to the fraction recovered from DE-52 cellulose, the factor of PKC enrichment in peak 2 arising from the Mono Q column was 65. After desalting, the material of the first peak rerun over the same Mono Q column gave rise to two peaks of PKC activity eluted with the same concentrations of NaCl as in the first run, namely, 0.05 and 0.12 M NaCl, suggesting that the chromatographic behavior of PKC of the first peak was due to a loose association with other proteins. Only the material of peak 2 which represents about $^1/_3$ of the PKC activity recovered from the Mono Q column was used for a final chromatography on phenyl-Sepharose. In this last step, a further 3-fold purification was achieved. Mono Q chromatography was decisive for PKC enrichment, as it led to a purification factor of 65 for the PKC contained in peak 2.

The overall purification could be achieved in two days.

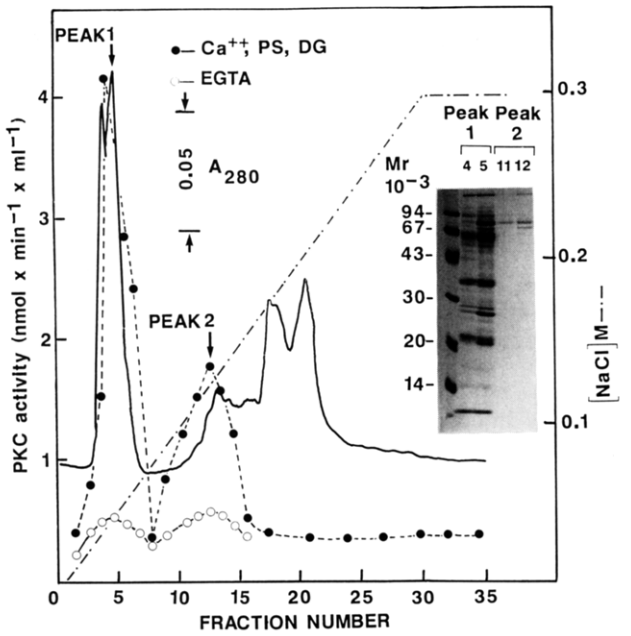

FIGURE 1: Elution profile of bovine neutrophil PKC from the Mono Q column. The PKC-containing fractions eluted from a DE-52 column (cf. Materials and Methods) were pooled and then concentrated and desalted on Amicon YM-30 filter. After dilution, 200 mg of protein in 20 mL was loaded onto the Mono Q (HR 5/5) column. A linear gradient of NaCl was applied, as detailed under Materials and Methods. Fractions of 1 mL were collected. PKC activity was assayed in the presence of Ca^{2+}, phosphatidylserine (PS), and diacylglycerol (DG). Controls were included, where Ca^{2+} and lipids were replaced by EGTA. The absorbance (A) at 280 nm was continuously monitored. (Insert) SDS–polyacrylamide gel electrophoresis of PKC-containing fractions corresponding to peaks 1 and 2 from Mono Q chromatography. After electrophoresis, the gel was stained with Coomassie blue. (Peak 1) Fractions 4 and 5; (peak 2) fractions 11 and 12. Standard M_r markers were included.

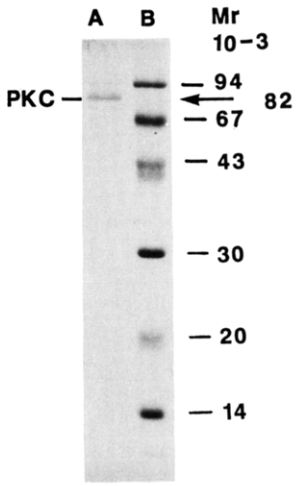

FIGURE 2: SDS–polyacrylamide gel electrophoresis of purified bovine neutrophil PKC stained with Coomassie blue. Conditions are described under Materials and Methods. (Track A) Pure bovine neutrophil PKC after phenyl-Sepharose chromatography; (track B) standard M_r markers.

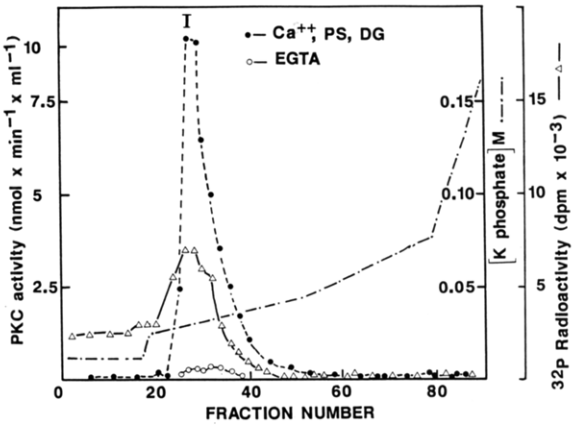

FIGURE 3: Separation of bovine neutrophil PKC isoforms by HTP chromatography. The first PKC-containing peak eluted from the Mono Q column (cf. Figure 1) was applied, after dialysis against buffer D, on an HTP column previously washed with buffer E. Elution was performed with a linear potassium phosphate gradient in buffer E (cf. Materials and Methods). Fractions of 0.45 mL were collected and assayed for PKC activity in the presence of Ca^{2+}, phosphatidylserine (PS), and diacylglycerol (DG). Controls were carried out in the absence of Ca^{2+} and lipids and in the presence of EGTA. Peak I from the HTP column was autophosphorylated by [γ-^{32}P]ATP in the presence of Ca^{2+}, Mg^{2+}, phosphatidylserine, and diacylglycerol and rerun on the same column under the same conditions of elution used for peak I. The elution was monitored by ^{32}P measurement. Note that the peak of radioactivity is virtually at the same position as the peak of PKC activity.

Purified PKC was stored in 0.05% Tween 20.

Storage of Bovine Neutrophil PKC. The 0.05% Tween 20 added to bovine neutrophil PKC allowed full protection of its activity for at least 1 month at −20 °C without loss of its Ca^{2+} and phospholipid dependency. Triton X-100 at the same concentration was much less efficient for protection of activity than Tween 20. For example, after 1 week of storage of bovine neutrophil PKC at −20 °C in 0.05% Triton X-100, the activity had fallen by 40%. Addition of 50% glycerol to Triton X-100 did not improve protection. In the absence of Tween 20, activity was totally lost after storage at −20 °C for 1 night.

Molecular Weight and Isoelectric Point of Bovine Neutrophil PKC. The purity of purified bovine neutrophil PKC was analyzed by SDS–polyacrylamide gel electrophoresis (Figure 2). Coomassie blue staining revealed only one protein with an apparent M_r of 82 000. During filtration on Sephadex G-150, PKC was eluted between enolase (M_r 98 000) and bovine serum albumin (M_r 67 000); its elution volume corresponded to a M_r of 85 000, indicating that PKC in solution is essentially a monomer. With the purification procedure described, there was no evidence for the presence of a degraded form of PKC, analogous to form M of $M_r \simeq 50\,000$ found in preparations of rat brain PKC (Inoue et al., 1977). The isoelectric point of bovine neutrophil PKC, measured by liquid isoelectric focusing, was 5.9 ± 0.1.

Isoenzymatic Patterns of Bovine Neutrophil PKC and Bovine Brain PKC. Separations of isoforms of rat brain PKC have been recently achieved by HTP chromatography (Huang et al., 1986a; Pelosin et al., 1987; Kikkawa et al., 1987). A search was made for the existence of bovine neutrophil PKC isoforms. This study was conducted in parallel with fractionation of isoforms of bovine brain PKC on an HTP column, with a linear gradient of potassium phosphate (0.010–0.175 M), pH 7.4, in buffer E.

The contents of peaks 1 and 2 recovered from the Mono Q column at the second step of the purification of bovine neutrophil PKC were analyzed separately by HTP chromatography. In both cases, PKC activity was eluted in a single peak with about 0.03 M potassium phosphate (Figure 3). As isoform I of rat brain PKC is eluted with this low concentration of potassium phosphate, it was concluded tentatively that the single isoform of bovine neutrophil PKC belongs to type I. To ascertain this conclusion, bovine brain PKC was purified following the same procedure as that described for bovine neutrophil PKC (see Materials and Methods). Chromatography of bovine brain PKC on a Mono Q column yielded two

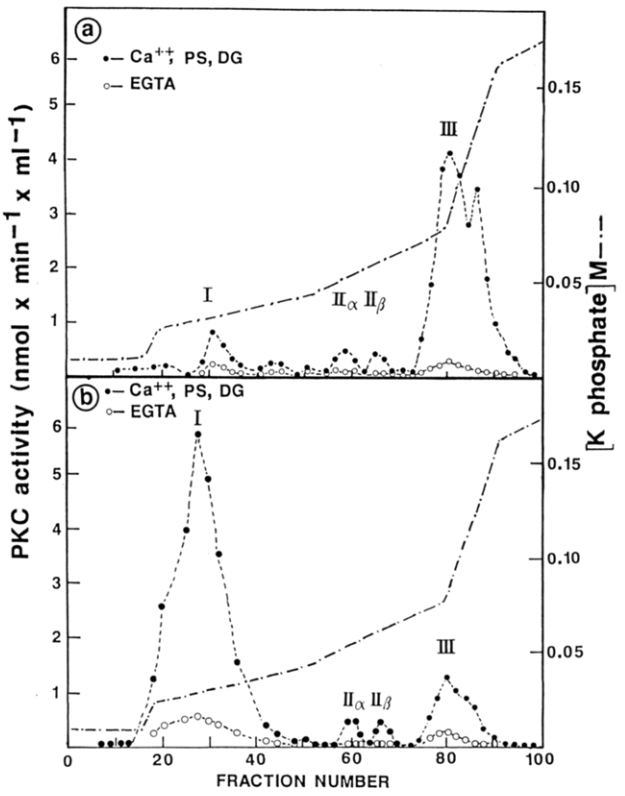

FIGURE 4: Separation of bovine brain PKC isoforms by HTP chromatography. (Panel a) HTP chromatography of the PKC-containing fractions corresponding to peak 1′ eluted from the Mono Q column (HR 5/5). The column was eluted with a linear gradient (0.010–0.175 M) of potassium phosphate in buffer E. (Panel b) HTP chromatography of the PKC-containing fractions corresponding to peak 2′ eluted from the Mono Q column (HR 5/5). PKC activity was assayed in the presence of Ca^{2+}, phosphatidylserine (PS), and diacylglycerol (DG). Controls were carried out in the absence of Ca^{2+} and lipids and in the presence of EGTA. (For details, see text.)

peaks, 1′ and 2′; as in the case of bovine neutrophil PKC, but unlike the latter, the PKC present in each peak was contaminated by other proteins. PKC from peaks 1′ and 2′ could be resolved by HTP chromatography into four peaks of PKC as in the case of rat brain PKC, with the difference that isoform III was predominant in peak 1′ and isoform I in peak 2′ (Figure 4). It is therefore presumed that, as for rat brain (Pelosin et al., 1987), beef brain contains four PKC isoforms of types I, IIα, IIβ, and III (cf. Materials and Methods), which were eluted from the HTP column with 0.03, 0.055, 0.065, and 0.11 M potassium phosphate respectively. These fractionation experiments were repeated at least three times with the same elution profiles.

Autophosphorylation of Bovine Neutrophil PKC. Bovine neutrophil PKC showed a strong propensity to autophosphorylation by [γ-^{32}P]ATP (Figure 5). After SDS–polyacrylamide gel electrophoresis of autophosphorylated PKC, followed by autoradiography, the labeled band on the film was found to coincide with the M_r 82 000 protein band stained in gel by Coomassie blue (Figure 5, track A). Autophosphorylation required Mg^{2+} and either Ca^{2+}, phosphatidylserine and diacylglycerol, or PMA with phosphatidylserine (tracks A and F). No autophosphorylation of PKC could be detected in the absence of Ca^{2+} and lipids (track D) or in the absence of Mg^{2+} (track E).

Partial digestion by trypsin of the autophosphorylated PKC yielded two radiolabeled peptides of M_r 48 000 and 39 000 as revealed by autoradiography (Figure 5, tracks B and C). The M_r 48 000 peptide was readily stained by Coomassie blue. This was not the case for the M_r 39 000 peptide, possibly due to its chemical nature. Labeled peptide fragments of the same size have been recovered from rat brain PKC after autophosphorylation and were ascribed to the N and C termini of PKC (Huang et al., 1986b).

The labeled amino acid residues in autophosphorylated PKC recovered after trypsin digestion and partial acid hydrolysis of the protein were identified as phosphoserine and phosphothreonine (Figure 6).

Ca^{2+}- and Phospholipid-Dependent Phosphorylation of a M_r 23 000 Protein in the Cytosolic Fraction of Bovine Neu-

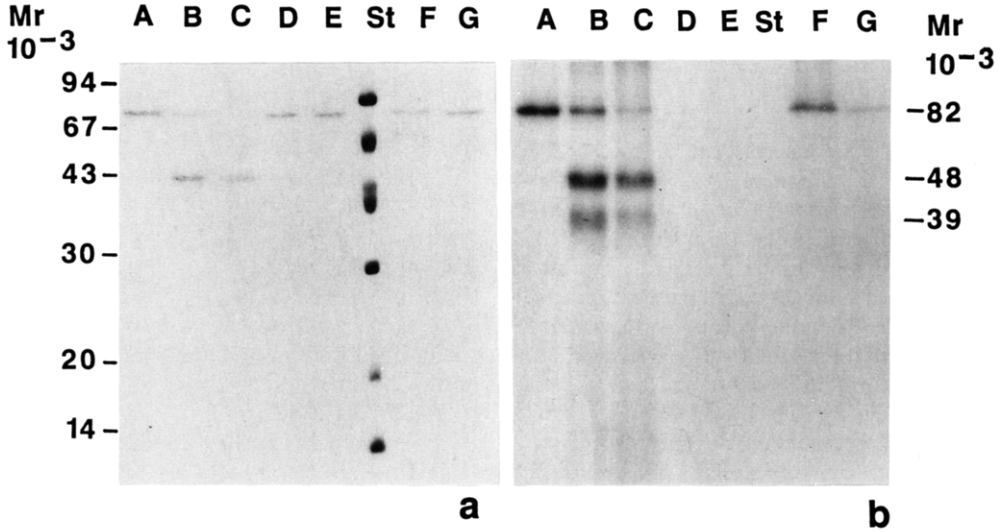

FIGURE 5: Autophosphorylation of bovine neutrophil PKC in the presence of [γ-^{32}P]ATP and cleavage by limited trypsinolysis. PKC eluted from phenyl-Sepharose CL-4B was autophosphorylated with [γ-^{32}P]ATP as described under Materials and Methods. (Panel a) Samples containing PKC activity were incubated in the presence of [γ-^{32}P]ATP under the conditions listed below and then subjected to SDS–polyacrylamide gel electrophoresis and stained with Coomassie blue. Standard M_r markers were included (St). (Panel b) Radiolabeled products were revealed by autoradiography. The different tracks on the gel corresponded to the following treatments of PKC prior to electrophoresis: (A) control PKC autophosphorylated by [γ-^{32}P]ATP in the presence of Ca^{2+}, phosphatidylserine, and diacylglycerol; (B) PKC autophosphorylated as in (A) and then cleaved by trypsin for 10 min at 30 °C at a ratio of enzyme to substrate of 1:50; (C) PKC autophosphorylated as in (A) and cleaved by trypsin for 20 min; (D) PKC autophosphorylated in the absence of Ca^{2+} and lipids; (E) PKC autophosphorylated as in (A) but in the absence of Mg^{2+}; (F) PKC autophosphorylated in the presence of PMA (1 μg/mL) and phosphatidylserine without Ca^{2+}; (G) PKC autophosphorylated in the presence of PMA without Ca^{2+} and phosphatidylserine.

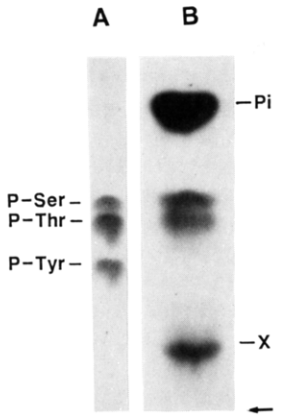

FIGURE 6: Identification of the phosphorylated amino acid residues in the single PKC isoform of bovine neutrophils by thin-layer chromatography analysis, followed by autoradiography. The neutrophil PKC isoform was eluted from an HTP column and autophosphorylated in the presence of Ca^{2+}, phosphatidylserine, diacylglycerol, and [γ-^{32}P]ATP. The autophosphorylated PKC was then subjected to trypsin digestion, followed by partial acid hydrolysis as described under Materials and Methods. The digest was chromatographed on thin-layer cellulose (F 1440). (Track A) Markers corresponding to unlabeled phosphoserine, phosphothreonine, and phosphotyrosine residues were revealed by ninhydrin. (Track B) The chromatoplate was exposed to an X-ray film. The autoradiography shows incorporation of [^{32}P]P_i into phosphoserine and phosphothreonine but not into phosphotyrosine. The labeled spot marked X probably corresponded to labeled peptide(s), arising from not completely hydrolyzed PKC. The arrow indicates the sample origin.

trophils. During the course of a systematic exploration of autophosphorylation of bovine neutrophil PKC at the different steps of purification, it was incidentally found that a soluble protein of M_r 23 000, contained in the high-speed supernatant and probably of cytosolic nature, was strongly radiolabeled by [γ-^{32}P]ATP. As shown in Figure 1, this protein was present in peak 1 recovered from the Mono Q column at step 2 of the purification procedure. Phosphorylation of the M_r 23 000 protein was strictly dependent on the presence of phosphatidylserine and diacylglycerol, Ca^{2+} and Mg^{2+}, or PMA, which strongly suggested that the PKC present in peak 1 had catalyzed phosphorylation of the M_r 23 000 protein. In addition to the M_r 23 000 protein, three other proteins with M_r close to 80 000, 82 000, and 40 000 were predominantly labeled (Figure 7, tracks A and D). When Ca^{2+} and lipids were absent from the medium, labeling decreased significantly in the M_r 82 000 protein (most probably PKC) and nearly totally in the M_r 23 000 protein. In contrast, the labeling of the M_r 80 000 protein was less altered and that of the M_r 40 000 protein not altered at all (tracks B and E); omission of Mg^{2+} resulted in disappearance of labeling (track C); Mg^{2+} was needed for the phosphorylation of the four proteins. This indicates that another kinase other than kinase C was involved in the phosphorylation of the M_r 40 000 and 80 000 proteins and that this kinase like kinase C required Mg^{2+} for activity.

The M_r 23 000 protein was purified by chromatography on Bio-Gel P60 in 6 M urea. As this protein of the neutrophil cytosol might be a natural substrate for bovine neutrophil PKC, it was of interest to compare its rate of phosphorylation to that of histone type IIIS, using as catalyst the unique form of bovine neutrophil PKC. The amounts of the M_r 23 000 protein and histone type IIIS used were fixed at 5 μg, and the activity of bovine neutrophil PKC was adjusted to 0.1 unit. The [γ-^{32}P]ATP-dependent phosphorylation was performed in the presence and absence of lipids and Ca^{2+}. The labeled proteins were separated by SDS–polyacrylamide gel electrophoresis and visualized by autoradiography. Interestingly, the

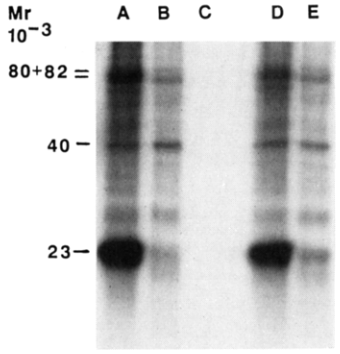

FIGURE 7: Autoradiography of PKC-phosphorylated neutrophil components eluted together with PKC from the Mono Q column. Fractions containing PKC activity, eluted from the Mono Q column (HR 5/5) at 0.05 M NaCl (peak 1) (cf. Figure 1), were pooled and incubated with [γ-^{32}P]ATP under the following different conditions: (A) addition of phosphatidylserine, diacylglycerol, Ca^{2+}, and Mg^{2+} as described under Materials and Methods for the assay of PKC activity; (B) absence of Ca^{2+} and lipids; (C) absence of Mg^{2+}; (D) addition of PMA (1 μg/mL) and phosphatidylserine; (E) addition of PMA (1 μg/mL) without phosphatidylserine. The different samples were precipitated by ice-cold 4% perchloric acid and washed with cold acetone. The precipitates were solubilized by SDS and subjected to SDS–polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue and then exposed to X-ray film.

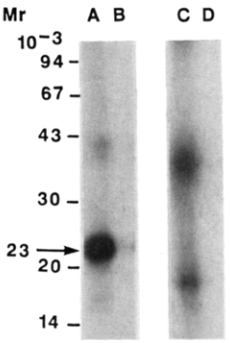

FIGURE 8: Phosphorylation of a neutrophil cytosolic protein of M_r 23 000 and histone type IIIS by the single isoform of bovine neutrophil PKC. The amount of substrates used for phosphorylation by PKC, i.e., the neutrophil protein of M_r 23 000 and histone type IIIS, was 5 μg; 0.1 unit of the single isoform of neutrophil PKC recovered by HTP chromatography (Figure 3) was used for phosphorylation with [γ-^{32}P]ATP, either in the presence of Ca^{2+}, phosphatidylserine, and diacylglycerol or in their absence (cf. Materials and Methods). The samples were then subjected to SDS–polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue and exposed to an X-ray film. The figure shows the autoradiography of the gel and the molecular weight of radioactive bands. The different tracks correspond to the following experimental conditions: (A) incubation of the M_r 23 000 protein with PKC, Ca^{2+}, phosphatidylserine, and diacylglycerol; (B) incubation of the M_r 23 000 protein with PKC and without Ca^{2+} and lipids; (C) incubation of histone type IIIS with PKC, Ca^{2+}, phosphatidylserine, and diacylglycerol; (D) incubation of histone type IIIS with PKC and without Ca^{2+} and lipids.

M_r 23 000 protein was significantly more labeled by bovine neutrophil PKC than histone type IIIS (Figure 8, tracks A and C). No labeling was detected in absence of Ca^{2+} and lipids (tracks B and D). By comparison, the M_r 23 000 protein was used as substrate with the four PKC isoforms from bovine brain under the same experimental conditions as described for bovine neutrophil PKC. In this case, isoforms I and III, but not isoforms IIα and IIβ, of PKC catalyzed phosphorylation of the M_r 23 000. The M_r 23 000 protein from bovine neutrophil was, however, more efficiently labeled by isoform I of bovine neutrophil PKC than by isoforms I and III of bovine brain PKC. A control was performed to check whether the M_r 23 000 protein was phosphorylated by Mg-[γ-^{32}P]ATP in

the absence of PKC and phospholipids; no phosphorylation was observed.

Discussion

Comments on the Purification of Bovine Neutrophil PKC. The method of purification of bovine neutrophil PKC described here is convenient, rapid, and highly reproducible. The first two steps, namely, chromatography on DE-52 cellulose and FPLC chromatography on Mono Q, were similar to those used by Jeng et al. (1986) for the purification of PKC from mouse brain cytosol. In the case of mouse brain PKC, rechromatography of the Mono Q eluate enriched in PKC on a column of Mono Q with an elution buffer supplemented in Mg-ATP resulted in a large increase in purification. We found rechromatography in the presence of Mg-ATP to be inefficient in the case of neutrophil PKC. The recovery of PKC at the last step of the purification, which consisted of phenyl-Sepharose chromatography, was low although the enzyme obtained was electrophoretically homogeneous and its activity was totally Ca^{2+}- and phospholipid-dependent.

Christiansen and Juhl (1986) have reported a partial purification procedure of bovine neutrophil PKC, which resulted in the recovery of two proteins of M_r 88 000 and 72 000. This purification was based on affinity chromatography using polyacrylamide-immobilized phosphatidylserine. In our hands, this type of chromatography did not give reproducible results. In contrast, our purification procedure was highly reproducible and had the considerable advantage of being completed in 2 days, including preparation of neutrophils. The specific activity of our bovine neutrophil PKC, 554 units/mg, compares favorably to specific activities of PKC from other tissues: 600 units/mg for rabbit brain PKC (Jaken & Kiley, 1987); 1500 units/mg for mouse brain PKC (Yeng et al., 1986); 1090–852 units/mg for rat brain PKC (Kikkava et al., 1982; Le Peuch et al., 1983); 2246 units/mg for bovine brain PKC (Walton et al., 1987); 295 units/mg for rat liver PKC (Azhar et al., 1987).

It is known that PKC purified from different sources is very unstable (Yeng et al., 1986). Whereas glycerol and Triton X-100 satisfactorily stabilized bovine brain PKC, they poorly prevented inactivation of bovine neutrophil PKC. In contrast, Tween 20, at a concentration of 0.05%, provided full protection of bovine neutrophil PKC against loss of activity for at least 1 month at −20 °C. The differences in stability of PKC from different tissues and its dependence upon conditions of storage are probably related to the existence of several isoforms of PKC and a different distribution according to the source.

Properties of Bovine Neutrophil PKC Compared to Those of PKC from Other Sources. An isoelectric point of 5.9 ± 0.1 was determined for bovine neutrophil PKC. This is close to the value of 5.6 reported for the isoelectric point of rat brain PKC (Kikkawa et al., 1982).

Cloning analysis has revealed that PKC exists as a family of multiple subspecies, with closely related structures, but individual characteristics. HTP chromatography has recently been used to resolve the different isoforms of PKC. As shown for bovine brain PKC in this paper, four distinct peaks of activity were resolved by HTP chromatography and assigned to four different isoforms by reference to the similar behavior of rat brain PKC isoforms on HTP. In order of elution, the peaks are numbered I, IIα, IIβ, and III [cf. Kikkawa et al. (1987)]. A single peak of PKC activity was recovered from chromatography of bovine neutrophil PKC on HTP. This peak was eluted with 0.03 M potassium phosphate and therefore corresponded to type I of the PKC isoforms from bovine or rat brain. Autophosphorylation of this single form of bovine neutrophil PKC, in the presence of [γ-^{32}P]ATP, Mg^{2+}, Ca^{2+}, phosphatidylserine, and diacylglycerol, results in the labeling of serine and threonine residues. It is noteworthy that types IIα and IIβ of rat brain PKC are phosphorylated on their serine and threonine residues, whereas types I and III are phosphorylated on their serine residues only (Huang et al., 1986b). In brief, although the single isoform of bovine neutrophil PKC has the same chromatographic behavior as type I of rat brain PKC or bovine brain PKC, it differs from them by the additional phosphorylation of threonine residues; in that, it would resemble more type IIα and IIβ of brain PKC. At this point, we would like to draw attention on the fact that, although neutrophil PKC markedly differs from brain PKC by its chromatographic behavior, the criterium used by different authors to resolve brain PKC isoforms, namely, HTP chromatography, may not apply to resolve putative isoforms of bovine neutrophil PKC (Ono et al., 1987). On the other hand, the demonstration of only one peak of bovine neutrophil PKC activity by HTP chromatography might be explained by the high unstability of other isoforms. There is, however, no evidence of differential unstability of PKC isoforms from other tissues, like brain, as shown here and elsewhere (Huang et al., 1986a; Jaken & Kiley, 1987; Pelosin et al., 1987), rat liver (Azhar et al., 1987), and murine fibroblasts (McCaffrey et al., 1987).

The presence of an apparently single form of PKC in bovine neutrophils poses the question as to whether this single form is responsible for one physiological function or, on the contrary, governs a number of cellular activities (Ono & Kikkawa, 1987). Identification of endogenous substrates of bovine neutrophil PKC might be a means to answer this question. In this respect, a protein of M_r 23 000 present in the cytosol of bovine neutrophils has been shown to be very efficiently phosphorylated by neutrophil PKC, but much less by brain PKC. A study of the nature and reactivity of the M_r 23 000 protein is under way.

Acknowledgments

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Registry No. PKC, 9026-43-1.

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Mechanism of Inactivation of Alanine Racemase by β,β,β-Trifluoroalanine†

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ABSTRACT: The alanine racemases are a group of PLP-dependent bacterial enzymes that catalyze the racemization of alanine, providing D-alanine for cell wall synthesis. Inactivation of the alanine racemases from the Gram-negative organism *Salmonella typhimurium* and Gram-positive organism *Bacillus stearothermophilus* with β,β,β-trifluoroalanine has been studied. The inactivation occurs with the same rate constant as that for formation of a broad 460–490-nm chromophore. Loss of two fluoride ions per mole of inactivated enzyme and retention of [1-^{14}C]trifluoroalanine label accompany inhibition, suggesting a monofluoro enzyme adduct. Partial denaturation (1 M guanidine) leads to rapid return of the initial 420-nm chromophore, followed by a slower ($t_{1/2}$ ~30 min–1 h) loss of the fluoride ion and $^{14}CO_2$ release. At this point, reduction by NaB^3H_4 and tryptic digestion yield a single radiolabeled peptide. Purification and sequencing of the peptide reveals that lysine-38 is covalently attached to the PLP cofactor. A mechanism for enzyme inactivation by trifluoroalanine is proposed and contrasted with earlier results on monohaloalanines, in which nucleophilic attack of released aminoacrylate on the PLP aldimine leads to enzyme inactivation. For trifluoroalanine inactivation, nucleophilic attack of lysine-38 on the electrophilic β-difluoro-α,β-unsaturated imine provides an alternative mode of inhibition for these enzymes.

The D-isomer of alanine is an important component of the peptidoglycan, which is the extensively cross-linked, rigid structure essential to the integrity of the bacterial cell wall. The alanine racemases, a group of pyridoxal 5′-phosphate (PLP)[1] containing enzymes, provide bacteria with D-alanine through the racemization of available L-alanine (Adams, 1976; Faraci & Walsh, 1988). Because of the importance of these enzymes for bacterial growth, they are attractive targets for antibacterial drug development.

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TPCK, L-1-chloro-3-(4-tosylamino)-4-phenyl-2-butanone.

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