Purification of PKC-I, an endogenous protein kinase C inhibitor, and types II and III protein kinase C isoenzymes from human neutrophils

Kenneth J. BALAZOVICH,* Edward L. McEWEN, Mary L. LUTZKE, Laurence A. BOXER and Timothy WHITE

Department of Pediatrics, Section of Hematology/Oncology, M7510 Medical Science Research Building I, University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A.

Human neutrophil protein kinase C (PKC) activity is inhibited by an endogenous protein found primarily in the pellet fraction from homogenized specific granules, which was both heat- and proteinase-sensitive [Balazovich, Smolen & Boxer (1986) *J. Immunol.* **137**, 1665–1673]. We now report that two PKC isoenzymes and the endogenous PKC inhibitor, which we named PKC-I, were purified from human neutrophils. A neutrophil soluble fraction that was subjected to DEAE-Sephacel chromatography yielded highly enriched PKC because, by definition, enzymic activity was strictly dependent on Ca^{2+} and phosphatidylserine. Hydroxyapatite chromatography resolved two peaks of PKC activity. Type II and Type III PKC isoenzymes were each identified on Western blots by using isoenzyme-specific monoclonal antibodies. Unlike rat brain, from which PKC isoenzymes were also purified, Type I PKC was not detected in human neutrophils. Western blots indicated that both Type II and Type III PKC isoenzymes had molecular masses near 80 kDa. In agreement with other reports, PKC was autophosphorylated *in vitro*. PKC-I, an endogenous neutrophil inhibitor of PKC, was purified to apparent homogeneity by DEAE-Sephacel and S-400 Sephacel chromatography. PKC-I had a molecular mass of 41 kDa. PKC-I inhibited purified PKC activity stimulated by 1,2-diacylglycerols in a concentration-dependent manner, and inhibited PKC-dependent phosphorylation of proteins present in neutrophil cytosol.

INTRODUCTION

The Ca^{2+} - and phospholipid-dependent family of enzymes known as protein kinase C (PKC) has been described in several types of blood cells, including lymphocytes [1], platelets [2] and neutrophils [3–5]. PKC becomes mostly insoluble (i.e. translocates) in response to increased intracellular Ca^{2+} [6,7]. PKC is subsequently activated by 1,2-diacylglycerols, which are products of phospholipase activity that remain associated with the membrane. PKC is also activated by certain phorbol esters by a reportedly Ca^{2+} -independent mechanism [8,9]. PKC activity has been quantified *in vitro* in crude fractions of human neutrophils by using an assay that measures histone phosphorylation. Qualitatively, a large number of different endogenous PKC substrates have been described by observing their phosphorylation *in situ* [5,10–12], some of which may be involved in cellular functional responses. PKC activity assayed by both methods has been correlated with degranulation and superoxide-anion production in neutrophils [13–15], although recent investigations [16–18] suggest that there may not be an absolute requirement for its activity to initiate these functions.

Seven isoenzymes of PKC have been reported. Types I, II and III PKC can be resolved by hydroxyapatite chromatography and are encoded by the γ , β and α genes respectively [19,20]. Two forms of Type II PKC result from alternative splicing of a single RNA transcript [21,22]. Three other members of the PKC family, designated δ , ϵ and ζ , have been identified by molecular cloning and sequence analysis [23]. These recently identified isoenzymes show biochemical similarities to Type I, Type II, and Type III PKC isoenzymes, but some of their properties and their cDNA sequences suggest that they represent a distinct subfamily of

PKC isoenzymes. Over 75 % similarity exists between Type I, II and III PKC isoenzymes, but less than 60 % similarity is found between Type III and the δ , ϵ or ζ species [23]. PKC has been purified from several sources, including brain [24,25], heart [26] and both bovine [27] and human [28] neutrophils.

We [29] and others [30–32] have previously described endogenous inhibitors of PKC which may modulate PKC activity and thereby certain cellular functional events *in vivo*. The neutrophil PKC inhibitor, PKC-I, is heat- and proteinase-sensitive, is not itself a proteinase, and appears to be present primarily in the membrane fraction of specific granules and in the plasma-membrane fraction [29]. In support of its cellular localization, both neutrophil cytoplasts and specific-granule-deficient neutrophils appear to lack PKC-I activity [29]. Functionally, PKC-I may modulate PKC activity *in vivo*, because resting specific-granule-deficient neutrophils display translocated PKC and increased endogenous protein phosphorylation compared with normal neutrophils [29]. With regard to the other endogenous PKC inhibitors which have been reported, no characteristic appears common to the group; for example, reported molecular masses range from 0.6 to 67 kDa, only about half are heat-sensitive, and some interact with the regulatory fragment of PKC, whereas others interact with the catalytic fragment. Clearly, much more work will be required to characterize and classify these potential regulators of cellular PKC.

We considered it of paramount importance to purify PKC isoenzymes from human neutrophils because they are one of the few cells thus far reported to contain an endogenous inhibitor of this enzyme. The present paper concerns the purification of two PKC isoenzymes and PKC-I from human neutrophils in order that we might more carefully study their properties.

Abbreviations used: OAG, 1-oleoyl-2-acetyl-glycerol; PKC, protein kinase C; PKC-I, endogenous protein kinase C inhibitor.

* To whom reprint requests should be addressed.

MATERIALS AND METHODS

Materials

Aprotinin, pepstatin, leupeptin and trypsin inhibitor were obtained from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Phosphatidylserine and 1,2-diacylglycerols were obtained from Avanti Polar Lipids (Pelham, AL, U.S.A.). Sephadex resins and DEAE-Sephacel were obtained from Pharmacia (Piscataway, NJ, U.S.A.). Hydroxyapatite, the silver-staining kit, the horse-radish peroxidase immunoblot kit and the reagents used in electrophoresis were obtained from Bio-Rad (Rockville Center, NY, U.S.A.). Radiolabelled reagents were obtained from New England Nuclear (Boston, MA, U.S.A.). Immobilon membrane was obtained from Millipore Corp. (Bedford, MA, U.S.A.). Monoclonal antibodies specific for PKC isoenzymes (clones MC-1a, MC-2a and MC-3a) were obtained from Seikagaku America (St. Petersburg, FL, U.S.A.) or from Amersham (Arlington, IL, U.S.A.) (clone MC-5). All other reagents were obtained from Sigma.

Cells

Neutrophils were harvested from granulocyte-rich plasma (kindly provided by the American Red Cross, Lansing, MI, U.S.A.). Purified neutrophils were obtained by Ficoll centrifugation, dextran sedimentation and hypo-osmotic lysis of residual erythrocytes [5,29,33]. Cells were > 95% neutrophils, and were > 97% viable according to the Trypan Blue exclusion assay.

Cell disruption and solubilization of PKC

Purified neutrophils were suspended at 10^8 /ml in homogenization buffer (0.25 M-sucrose, 20 mM-Tris/HCl, pH 7.5, 5 mM-dithiothreitol, 2 mM-EDTA, 2 mM-EGTA, 0.1 mM-phenylmethanesulphonyl fluoride and 1.0 μ g each of aprotinin, leupeptin, trypsin inhibitor and pepstatin/ml). All homogenization and chromatography procedures were carried out at 4°C. Neutrophils were disrupted in a melting-ice bath with a Potter-Elvehjem homogenizer driven by a Wheaton motor. Homogenates were centrifuged at 500 g for 10 min to remove unbroken cells and nuclei; the pellet fraction from this step was defined as the post-nuclear pellet fraction. The supernatant fraction was centrifuged at 220 000 g for 30 min. The resulting supernatant fraction, defined as the soluble fraction, contained > 81% of the total cellular PKC activity and was used as a source of crude PKC. The pellet obtained from high-speed centrifugation of the post-nuclear supernatant fraction was resuspended in homogenization buffer by sonication and was defined as the insoluble fraction. Cytosol fraction was obtained from human neutrophils by nitrogen cavitation and Percoll-gradient centrifugation exactly as described previously [34]. Rat brain soluble fraction was obtained in an identical manner, except that the tissue was finely minced in 10 ml of buffer/g of tissue before homogenization.

Solubilization of PKC-I

We previously reported that PKC-I was found primarily in the specific-granule-enriched fraction from human neutrophils [29]. For the present studies, a granule-enriched fraction was prepared by disruption of neutrophils in homogenization buffer as described above. Homogenates were centrifuged first at 500 g for 10 min, and the resulting supernatant was centrifuged at 220 000 g for 30 min. The pellet fraction, containing both primary and secondary granules, was used as a source of PKC-I. Alternatively, a crude granule fraction was prepared from neutrophils by nitrogen cavitation [29]; the resulting preparation was centrifuged at 500 g for 10 min to remove unbroken cells and nuclei, and finally at 10 000 g for 60 min. The resulting pellet fraction

contained > 90% of the cellular vitamin B12-binding protein and lysozyme, and > 95% of the cellular PKC-I activity, and was used as a source of PKC-I [29]. Granule fractions were disrupted in homogenization buffer (5×10^8 cell equivalents/ml), containing an additional 1 μ g each of aprotinin, leupeptin, trypsin inhibitor and pepstatin/ml, with a Potter-Elvehjem homogenizer driven by a Wheaton motor. Homogenates were centrifuged at 100 000 g for 60 min; the resulting granule soluble fraction was used as an enriched source of PKC-I. Granule breakage was monitored by assaying for the presence of vitamin B12-binding protein, lysozyme and β -glucuronidase in the supernatant fraction [5,29]; typically < 5% of the total granule enzyme activity was found in this fraction, indicating that minimal granule breakage was occurring.

Purification of PKC

The clarified soluble fraction (5–40 ml) from either polymorphonuclear leucocytes or rat brain was applied to a 2.5 cm $\times$ 10 cm column containing DEAE-Sephacel, which was equilibrated with buffer A (25 mM-Tris base, pH 7.5, 5 mM-dithiothreitol, 2 mM-EDTA, 2 mM-EGTA and 0.1 mM-phenylmethanesulphonyl fluoride). The column was washed with buffer A until the A_{280} reading returned to the established baseline. Material which was bound to the column was subsequently eluted with a 0–0.5 M linear gradient of KCl in buffer A, in 6.0 ml fractions. Gel-filtration chromatography was performed by using buffer A and Sephacryl S-400 resin (1.5 cm $\times$ 82 cm column); 0.5 ml fractions were collected. The molecular mass of PKC activity was estimated with respect to standard proteins which were chromatographed in parallel. Alternatively, molecular masses were determined by subjecting protein kinase-containing samples to SDS/PAGE (as described below) and obtaining the R_F values with reference to standard proteins. Hydroxyapatite chromatography was performed by modifications of published methods [24,25]. DEAE-Sephacel-purified PKC (1–2 ml) was applied to a 0.5 cm $\times$ 7.0 cm column containing hydroxyapatite resin which was equilibrated with 10 mM-potassium phosphate buffer (pH 7.4)/10% glycerol/10 mM-dithiothreitol/1 mM-EGTA/0.1 mM-phenylmethanesulphonyl fluoride. Material bound to the column was eluted with a linear gradient of 10–200 mM-potassium phosphate. All fractions were assayed for PKC activity. Those fractions containing PKC isoenzymes were pooled, concentrated with an Amicon filtration apparatus and YM10 membranes (Amicon Corp.), and stored at -70°C in 10% glycerol.

Purification of PKC-I

The supernatant fraction obtained by homogenization of granules was subjected to DEAE-Sephacel chromatography exactly as described above for PKC, except that a 0–0.75 M gradient of KCl was used to elute bound proteins. Fractions were tested for PKC-I activity as described below. Those fractions containing PKC-I were pooled, concentrated and stored at -70°C in 10% glycerol. PKC-I was further purified by gel-filtration chromatography, first on Sephadex G-200 (2.5 cm $\times$ 60 cm column) with buffer A, and finally on Sephacryl S-400 (1.5 cm $\times$ 82 cm column) in buffer A. In each case, 2.0 ml fractions were collected and assayed for PKC-I activity. The final preparation of PKC-I was concentrated and stored at -70°C in 20% glycerol.

Determination of PKC and PKC-I activities

PKC activity was assayed *in vitro* as previously described [5,29] with lysine-rich histone protein (Type IIIS; Sigma Chemical Co.) as a substrate, 2 μ g of 1-oleoyl-2-acetyl-glycerol/ml as an activator, and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (30 $\mu\text{Ci}/\text{mmol}$) as the phosphate donor.

PKC activity was defined as pmol of ^{32}P incorporated into substrate/minute; one unit of PKC activity is defined as that volume of PKC required to catalyse the transfer of 1 pmol of phosphate/min to Type IIIS histone protein. Specific activity for protein kinases was calculated after protein concentrations were determined by the Bio-Rad protein assay. Ca^{2+} /diacylglycerol/phospholipid-independent protein kinase activity was determined by omitting these components from the reaction mixture and subtracting the resulting activity from the total protein kinase activity to yield PKC activity (where appropriate). PKC-I activity was defined as the inhibition *in vitro* of histone phosphorylation catalysed by purified human neutrophil PKC (approx. 100 units/assay). PKC-I assays (250 μl total volume) contained 200 μl of the PKC assay mixture, 25 μl of purified neutrophil PKC and 25 μl of purified or partially purified PKC-I. Assays were conducted under conditions optimized for PKC activity as described above. In some cases PKC and PKC-I were mixed together and incubated for 5 min before their addition to the assay mixture. For phosphorylation *in vitro* of neutrophil cytosolic proteins, an equal volume of neutrophil cytosolic fractions was substituted for histone.

SDS/PAGE

Samples of concentrated PKC or PKC-I were precipitated with 12.5% trichloroacetic acid. Precipitates were washed with acetone, dried, dissolved in electrophoresis sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 62.5 mM-Tris/HCl, plus Bromophenol Blue) and heated to 100 °C for 5 min. Samples were subjected to SDS/PAGE in 10%-polyacrylamide gels by using a discontinuous buffer system [35] and an Idea Scientific (Corvallis, OR, U.S.A.) mini-gel apparatus.

Western blotting and immunochemistry

Proteins obtained by various chromatography techniques were separated by SDS/PAGE (10% gels) and transferred to Immobilon membranes (Millipore Corp.) in 25 mM-Tris base (pH 8.3)/192 mM-glycine/20% methanol by using a Bio-Rad apparatus operating at 0.5 A for 50 min. Non-specific binding of the primary antibody reagent was blocked with a 3% gelatin solution. Blots were incubated with monoclonal antibodies that specifically recognized only the Type I, Type II or Type III isoenzymes (γ , β and α respectively), or with an antibody that recognized both Type II and Type III PKC (clone MC-5). The bound antibodies were detected with a Bio-Rad Immuno-Blot horseradish peroxidase kit and photographed with Kodak Technical Pan film.

Autophosphorylation

Autophosphorylation of PKC was initiated by addition of 50 μl of PKC to 200 μl of a reaction mixture containing 25 mM-Tris/HCl, pH 7.4, 50 mM-ATP (including 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$), 10 mM- Mg^{2+} , 0.6 mM- Ca^{2+} , 20 μg of phosphatidylserine/ml and 2 μg of 1-oleoyl-2-acetylgllycerol/ml. The reaction mixture was incubated at 30 °C for 10 min and stopped with 1 ml of 25% trichloroacetic acid. The precipitated proteins were separated by SDS/PAGE and detected by autoradiography.

RESULTS

Purification of PKC

PKC, which has been purified from brain, heart and bovine neutrophils, among other cells, was purified from human neutrophils because they are one of the few cells thus far reported to contain an endogenous inhibitor of this enzyme, PKC-I [29]. Neutrophil homogenates were centrifuged to remove insoluble cellular materials. When assayed *in vitro*, the resulting soluble

fraction contained > 81% of the total cellular PKC activity. The insoluble fraction had 12% of the total activity, and the balance of the PKC activity (7%) was found in the post-nuclear pellet fraction. These results suggest that most of the PKC activity in unstimulated neutrophils was not tightly associated with cellular membranes and other insoluble material. The soluble fraction was chosen as a source of PKC because it contained most of the cellular enzymic activity measured by our assay *in vitro*. However, in the absence of corroborating data by using other methods of analysis, the actual intracellular distribution of PKC, as opposed to the distribution of enzymic activity, is not known.

DEAE-Sephacel anion-exchange chromatography of the neutrophil soluble fraction yielded a single peak of Ca^{2+} /phospholipid-dependent protein kinase activity at about 0.10 M-KCl; by definition, this activity was attributed to PKC. For comparative purposes, anion-exchange chromatography of rat brain soluble fraction gave similar results. Anion-exchange chromatography achieved about a 59-fold purification of PKC enzymic activity compared with the starting material.

DEAE-Sephacel-purified neutrophil PKC subjected to hydroxyapatite chromatography yielded two peaks of PKC activity. These peaks were eluted at 0.09 M- and 0.13 M-potassium phosphate respectively, and were designated Type II and Type III on the basis of their elution profile compared with published information on PKC isoenzyme purification from other tissues. Under similar conditions, rat brain Types II and III PKC isoenzymes were eluted at 0.07 M- and 0.095 M-potassium phosphate. The specific activity of Type II PKC purified by hydroxyapatite chromatography was 28.7 nmol/min per mg; reliable specific-activity data for Type III PKC were not obtained, owing to the low concentration present in neutrophils. Because our apparatus was limited to analytical-scale purification, about 3 μg of PKC was obtained from a portion of soluble fraction representing 3×10^8 cells.

An immunochemical approach was used to detect PKC after equal amounts of rat brain or human neutrophil PKC were subjected to SDS/PAGE and transferred to Immobilon membranes. Western blots performed with isoenzyme-specific monoclonal antibodies on DEAE-Sephacel-purified rat brain and human neutrophil PKC activity identified both Type II and Type III PKC isoenzymes (Fig. 1). A second antibody reagent (clone MC-5; Amersham), which recognizes both Type II and Type III, but not Type I PKC, confirmed these results (not shown). Although Type I PKC was detected in rat brain fractions (Fig. 1), no positive reaction was observed with anti-Type I PKC antibodies on blots of fractions from human neutrophils. Western blots of material from two peaks of PKC activity obtained by hydroxyapatite chromatography of neutrophil soluble fraction confirmed that the first peak contained Type II PKC (results not shown). Owing to the limited quantity of Type III PKC present in neutrophils, we were not successful in our attempts to identify this isoenzyme on Western blots by using fractions from hydroxyapatite chromatography.

PKC purified by DEAE-Sephacel chromatography was subjected to gel-exclusion chromatography either on Sephadex G-200 or on S-400 Sephacel, where it was resolved into a single peak (result not shown) corresponding to a molecular mass of 78 kDa (with lactoperoxidase as a standard). The predominant band on SDS/PAGE of PKC from hydroxyapatite chromatography had a molecular mass near 80 kDa. In addition, a single protein at 80 kDa was phosphorylated when the enzyme preparation was incubated with Mg^{2+} plus radiolabelled ATP; autophosphorylation of PKC has been reported [36].

Purification of PKC-I

The granule soluble fraction from neutrophil granule homo-

Fig. 1. Western blots of partially purified PKC obtained from rat brain and human neutrophils

Equal amounts of DEAE-Sephacel-purified PKC were separated on a 10% polyacrylamide gel and transferred to an Immobilon membrane. The blot was separated into three panels, and each panel was treated with a monoclonal-antibody reagent specific for Type III (a), Type II (b) or Type I (c) PKC isoenzymes. Primary antibodies were detected with a horseradish-peroxidase-conjugated secondary-antibody reagent. Abbreviations: R, rat brain; HU, human neutrophil. Positions of molecular-mass markers are shown on the left: phosphorylase *b*, 97 kDa; BSA, 68 kDa; actin, 43 kDa.

genates was subjected to DEAE-Sephacel chromatography. Proteins bound to the column were eluted with a linear gradient of 0–0.75 M-KCl, and the resulting fractions were tested for PKC-I activity. The results of this experiment are summarized in Fig. 2(a). Two peaks of PKC-I activity were observed in fractions from anion-exchange chromatography. The first peak of PKC-inhibitory activity was detected in the run-through fractions, and the second was eluted in a broad peak at about 0.4 M-KCl. The first peak of activity may be a proteolytic fragment of PKC-I, a second endogenous PKC inhibitor, or a modified PKC-I which did not bind to the column. This peak of activity had a much lower specific activity than the second peak (e.g. more than 100 times the amount of protein was required to inhibit PKC compared with the inhibitor in the second peak), and contained no visible band on silver-stained SDS/PAGE gels in the region of 40–50 kDa (results not shown). This peak of inhibitory activity requires further investigation. The second peak of activity inhibited PKC in neutrophil cytosol and soluble fractions [29] as well as partially purified PKC. The second peak of inhibitory activity was defined as PKC-I, and was pooled and concentrated. Anion-exchange chromatography resulted in a 12-fold purification of PKC-I activity. DEAE-Sephacel-purified PKC-I was subjected to gel-filtration chromatography on Sephadex G-200, followed by Sephacryl S-400. The results of this chromatography are reflected in Fig. 2(b). A single peak of PKC-I activity was eluted from each gel-filtration column. The molecular mass of PKC-I from each column was estimated to be 41 kDa on the basis of the retention volumes of standard proteins chromatographed in parallel. Silver-stained SDS/PAGE gels of purified PKC-I from Sephacryl S-400 chromatography contained a single band of protein having an estimated molecular mass of 40 kDa (Fig. 2b, insert). The final preparation of PKC-I represented a purification of > 350-fold and a yield of 28%. Approx. 2 µg of PKC-I was obtained from 6×10^9 neutrophils.

PKC-I purified by gel-filtration chromatography inhibited both DEAE-Sephacel-purified PKC and PKC activity in the neutrophil soluble fraction *in vitro*. For example, purified PKC-I inhibited PKC activity in our histone-phosphorylation assay in

Fig. 2. Purification of PKC-I from human neutrophil soluble fraction

(a) Neutrophil granule soluble fraction was applied to a column (2.5 cm × 7 cm) packed with DEAE-Sephacel and equilibrated with buffer A. After extensive washing, bound proteins were eluted from the column with a linear gradient of 0–0.75 M-KCl (—) in buffer A (beginning with fraction 20). Resulting 5 ml fractions were tested for inhibition of purified neutrophil PKC activity (●—●) as described in the Materials and methods section. (b) A fraction containing pooled concentrated PKC-I activity from DEAE-Sephacel chromatography was subjected first to chromatography on Sephadex G-200 (not shown) and then on S-400 Sephacel in a 1.0 cm × 80 cm column equilibrated with buffer A. The resulting 2.5 ml fractions were tested for inhibition of purified neutrophil PKC activity as described in the Materials and methods section. Results are expressed as the percentage inhibition of PKC observed in the indicated fraction. Numbers above the arrows indicate the molecular masses (kDa) and retention positions of standard proteins. Insert: silver-stained SDS/PAGE (10% gel) of pooled fractions 55–58 from S-400 Sephacel chromatography. Molecular-mass markers: 97 kDa, phosphorylase *b*; 66 kDa, BSA; 43 kDa, actin; 31 kDa, carbonic anhydrase.

Fig. 3. Inhibition of purified neutrophil PKC activity by purified PKC-I

A sample (80 ng) of DEAE-Sephacel-purified neutrophil PKC was incubated with purified neutrophil PKC-I at the indicated final concentration for 5 min at 30 °C. The mixture was assayed for PKC activity as described in the Materials and methods section. The results are means (±s.d.) from three different experiments which contained triplicate determinations, and are expressed as the percentage inhibition of control (i.e. in the absence of PKC-I) PKC activity; control PKC activity was 129 ± 30 pmol/min.

Fig. 4. Inhibition of PKC-dependent phosphorylation of neutrophil cytosolic proteins by purified PKC-I

A sample ($40 \mu\text{l}$; 4×10^7 cell equivalents) of neutrophil cytosol was combined with $2 \mu\text{g}$ of OAG/ml, $600 \mu\text{M}$ - Ca^{2+} , $10 \mu\text{g}$ of phosphatidylserine/ml and $50 \mu\text{M}$ -ATP (containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$) plus buffer (lane A), 80 ng of DEAE-Sephacel-purified neutrophil PKC plus 30 ng of purified PKC-I (lane B), PKC-I alone (lane C), or PKC alone (lane D) for 5 min at 30°C . Protein present in assay mixtures was precipitated with 10% trichloroacetic acid and separated by SDS/PAGE. Phosphoproteins were detected by autoradiography.

a concentration-dependent manner, giving 100% inhibition at less than 40 ng of PKC-I/ 80 ng of PKC fraction (Fig. 3). Neutrophil PKC phosphorylated many different endogenous substrates present in the neutrophil cytosol (Fig. 4, lane D), confirming and extending our previous results from experiments on endogenous PKC-dependent phosphorylation [5,29]. PKC-I at 30 ng/ml inhibited the PKC-dependent phosphorylation of most endogenous proteins in neutrophil cytosol (Fig. 4, lane B). Protein phosphorylation in the cytosolic fraction which was not decreased by the presence of PKC-I may be the result of protein kinase activity other than PKC. These results indicate that PKC-I modulates PKC-dependent phosphorylation in neutrophils. We showed that purified exogenous PKC induced contraction of permeabilized gastrointestinal-tract smooth-muscle cells [37]. Preliminary results (not shown) from our laboratory indicate that PKC-I inhibits PKC-dependent contraction, strongly suggesting that PKC-I regulates PKC activity *in vivo*.

DISCUSSION

It is becoming increasingly clear that PKC is a multifunctional enzyme family, members of which have been found in every cell type examined, including such genetically removed organisms as wheat [38] and *Drosophila melanogaster* [39]. Although seven isoenzymes have been reported, only four have been purified and characterized. With respect to the present report, PKC activity has been described in neutrophils from several mammalian species [3–5,29], and has been purified to near homogeneity from bovine neutrophils [27]. Recently, a novel PKC isoenzyme, named nPKC, has been isolated from human neutrophils [40]. nPKC was not recognized by antibodies against any of the known PKC isoenzymes, had different cofactor requirements from those of known protein kinase isoenzymes, and phosphorylated unique endogenous substrates. Thus the full range of the PKC enzyme family may not yet be known. Because

we are interested in the regulation of PKC during neutrophil activation, and in light of results discussed above suggesting that all PKC isoenzymes have not yet been discovered, we considered it important to identify and characterize PKC isoenzymes from human neutrophils in order to study more carefully their regulation by PKC-I.

The soluble fraction from neutrophil homogenates was used as a source of PKC, because that fraction contained $> 80\%$ of the total PKC activity. However, because the precise subcellular localization of PKC isoenzymes in human neutrophils is not known, future work will need to be focused on the identification of PKC isoenzymes retained in the insoluble fraction of homogenates. A Ca^{2+} - and phospholipid-independent but catalytically active proteolytic fragment of PKC (PK-M) has been reported [41,42]. However, the presence of the serine proteinase inhibitor leupeptin in our homogenization buffer, which reportedly inhibits the calpain-dependent cleavage of PKC to PK-M [28], negated the possibility of PKC contamination by this cofactor-independent kinase. This hypothesis is substantiated by immunological results showing that PK-M is not present in unstimulated neutrophils [43]. A second peak of protein kinase activity was obtained from anion-exchange chromatography of either neutrophil or rat brain soluble fraction. This activity was Ca^{2+} - and phospholipid-independent, did not contain PKC, according to biochemical and immunochemical criteria, nor did it contain PK-M, the catalytic fragment of PKC; this enzyme is a 44 kDa protamine kinase and is the subject of a separate study [43a].

The complete cellular homogenate had little PKC activity, an expected finding owing to the presence of PKC-I [29]. That notwithstanding, PKC activity measurements in other subcellular fractions are likely to be quantitatively low owing to the presence (and presumed inhibitory activity) of PKC-I, especially in particulate fractions which contain high concentrations of that protein [29].

Western blots using isoenzyme-specific monoclonal antibodies of preparations containing PKC activity obtained during our purification scheme suggest that human neutrophils contain proteins which are immunologically related to Types II and III PKC (Fig. 1). We have confirmed these results, using a second antibody reagent (clone MC-5, Amersham Corp.) which recognizes a common epitope present on both Types II and III PKC isoenzymes but does not recognize Type I (results not shown). We did not detect Type I PKC in any human neutrophil fraction, although we demonstrated the presence of this isoenzyme in rat brain fractions (Fig. 1). Since rat brain has been shown to have similar amounts of both Type II and Type III PKC isoenzymes [44], and since our Western blot of rat brain and human neutrophil Type II and Type III PKC isoenzymes produced bands of similar density, we infer that the antibody affinities for the different isoenzymes are also similar. On that basis we estimate by scanning densitometry that $> 85\%$ of neutrophil PKC is Type II. Consistent with our own data, Pontremoli *et al.* [28] reported that Type II PKC was present in human neutrophils at a greater concentration than Type III PKC (60% and 35% of the total respectively). In a third study [40], Type III PKC was identified as a faint 80 kDa band on immunoblots, whereas Type II PKC was much more easily identified and purified. Therefore all available reports agree that there is more Type II PKC present in human neutrophils than the Type III isoenzyme. The total amount of PKC obtained was limited compared with the amounts obtained from heart [26] and brain [24,25]. However, since neutrophils are one of the few cells reported to contain an endogenous inhibitor, purification of PKC from this source was important for future studies of the modulation of phosphotransferase activity by PKC-I.

PKC inhibitors have been described in several tissues, the most

well studied of which has been brain. One of the first endogenous PKC inhibitors reported was partially purified from rat brain [45]. This protein had an estimated molecular mass of 20 kDa, was both proteinase- and heat-sensitive, was isolated from a soluble fraction, and was a competitive inhibitor with respect to protein substrates. A PKC inhibitor was isolated from bovine brain as a 17 kDa Ca^{2+} -binding protein [30], but has since been studied with respect to amino acid sequence and biochemistry [46]. This protein is sensitive to proteinases and is obtained from a soluble fraction, but is heat-stable and is a zinc-binding protein which interacts with a unique site (i.e. not a cofactor or catalytic site) on PKC. From its molecular mass and solubility alone, this protein is probably not PKC-I. In addition, an antibody prepared against the 17 kDa bovine brain protein (generously given by Dr. M. P. Walsh) did not cross-react on Western blots or in immunoprecipitation experiments with purified preparations of PKC-I (K. J. Balazovich & J. R. McDonald, unpublished work). Calmodulin isolated from bovine brain also inhibits PKC [47], in addition to a protein of 600–700 kDa which is proteinase-resistant and Ca^{2+} -independent, and interacts with PKC through the ATP-binding site [47]. Most interestingly, a group of three 29–33 kDa proteins isolated from sheep brain, which were heat-sensitive at 100 °C but not at 70 °C, were not sensitive to changes in cofactor, ATP, or substrate concentration, but showed amino acid sequence similarity to neuronal protein 14-3-3 and annexins [48]. From its molecular mass and solubility characteristics, PKC-I is probably not identical with inhibitors thus far reported in brain tissue.

Three endogenous PKC inhibitors, in addition to PKC-I, have been described in neutrophils. First, a 33 kDa protein was isolated from guinea-pig peritoneal neutrophils [49]. This protein associates reversibly with phosphatidylserine-containing liposomes in a Ca^{2+} -dependent manner, resembling an annexin, and translocates to the plasma membrane in stimulated cells. It is not known if PKC-I binds to phosphatidylserine-containing vesicles, but preliminary results (not shown) from our laboratory suggest that PKC-I binding to PKC prevents its association with phosphatidylserine. Second, a soluble PKC inhibitor from rabbit peritoneal neutrophils has a reported molecular mass of 67 kDa [31]. Finally, the low-molecular-mass antibiotic peptides known as defensins HNP-1, HNP-2 and HNP-3 (< 3.5 kDa) all inhibited PKC non-competitively with respect to cofactors, ATP and substrate [21]. Defensins did not inhibit the binding of phorbol 12,13-dibutyrate to PKC, but did inhibit PKC activity stimulated by phorbol 12-myristate 13-acetate [21]. Finally, PKC-I was found to be inactive as an inhibitor of cyclic-AMP-dependent protein kinase and Mn^{2+} -dependent kinase [29], but is not necessarily specific for PKC, as inhibition of a purified endogenous neutrophil 44 kDa protamine kinase by PKC-I was observed (E. L. McEwen & K. J. Balazovich, unpublished work).

PKC-I appears to be a novel protein inhibitor of PKC, on the basis of its solubility and molecular mass. Information on the amino acid sequence of PKC-I and its specific mechanism of inhibition will be required to define further its similarity to other PKC inhibitors and its potential role in regulation of PKC activity in neutrophil function.

We thank the American Red Cross Blood Services Unit (Lansing, MI, U.S.A.), especially Pam Sommers, for their assistance in the preparation of blood products. This work was supported by grants from the National Institutes of Health (K.J.B., AI25641; L.A.B., AI20065).

REFERENCES

- Kraft, A. S., Anderson, W. B., Cooper, H. L. & Sando, J. J. (1982) *J. Biol. Chem.* **257**, 13193–13196
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
- Sha'afi, R. I., White, J. R., Molski, T. F. P., Shefcyk, J., Volpi, M., Naccache, P. H. & Feinstein, M. B. (1983) *Biochem. Biophys. Res. Commun.* **114**, 638–645
- Wolfson, M., McPhail, L. C., Nasrallah, V. W. & Snyderman, R. (1985) *J. Immunol.* **135**, 2057–2062
- Balazovich, K. J., Smolen, J. E. & Boxer, L. A. (1986) *Blood* **68**, 810–817
- May, W. S., Sahyoun, N., Wolf, M. & Cuatrecasas, P. (1985) *Nature (London)* **317**, 546–549
- Wolf, W. S., LeVine, H., May, W. S. & Cuatrecasas, P. (1985) *Nature (London)* **317**, 549–551
- Niedel, J. E., Kuhn, J. L. & Vandenbark, G. R. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 36–40
- Nishizuka, Y. (1983) *Trends Biochem. Sci.* **8**, 13–16
- Andrews, P. C. & Babior, B. M. (1983) *Blood* **61**, 333–340
- Kramer, C. M. & Sando, J. J. (1986) *Cancer Res.* **46**, 3040–3045
- Tauber, A. I. (1987) *Blood* **69**, 711–720
- Goldstein, I. M., Hoffstein, S. T. & Weismann, G. (1975) *J. Cell Biol.* **66**, 647–652
- McPhail, L. C. & Snyderman, R. (1983) *J. Clin. Invest.* **72**, 192–200
- Tauber, A. I., Cox, J. A., Curnutte, J. T., Carrol, P. M., Nakauma, H., Warren, B., Gilbert, H. & Blumberg, P. M. (1989) *Biochem. Biophys. Res. Commun.* **158**, 884–890
- Wright, C. D. & Hoffman, M. D. (1986) *Biochem. Biophys. Res. Commun.* **135**, 749–755
- Berkow, R. L., Dodson, R. W. & Kraft, A. S. (1987) *J. Leukocyte Biol.* **41**, 441–446
- Balazovich, K. J., Almeida, H. I. & Boxer, L. A. (1991) *J. Lab. Clin. Med.* **118**, 576–584
- Kikkawa, U., Ono, Y., Ogita, K., Fujii, T., Asaoka, Y., Sekiguchi, K., Kosaka, Y., Igarashi, K. & Nishizuka, Y. (1987) *FEBS Lett.* **217**, 227–231
- Huang, F. L., Yoshida, Y., Nakabayashi, H., Knopf, J. L., Young, S. W. & Huang, K.-P. (1987) *Biochem. Biophys. Res. Commun.* **149**, 946–952
- Ono, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarashi, K., Kikkawa, U., Ogita, K. & Nishizuka, Y. (1986) *FEBS Lett.* **206**, 347–352
- Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K. & Nishizuka, Y. (1987) *Science* **236**, 1116–1120
- Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. & Nishizuka, Y. (1988) *J. Biol. Chem.* **263**, 6927–6932
- Shearman, M. S., Ogita, K., Kikkawa, U. & Nishizuka, Y. (1989) *Methods Enzymol.* **168**, 347–351
- Sawamura, S., Ase, K., Berry, N., Kikkawa, U., McCaffrey, P., Minowada, J. & Nishizuka, Y. (1989) *FEBS Lett.* **247**, 353–357
- Wise, B. C., Raynor, R. L. & Kuo, J. F. (1982) *J. Biol. Chem.* **257**, 8481–8488
- Christensen, N. O. & Juhl, H. (1986) *Biochim. Biophys. Acta* **885**, 170–175
- Pontremoli, S., Melloni, E., Sparatore, B., Michetti, M., Salamino, F. & Horecker, B. L. (1990) *J. Biol. Chem.* **265**, 706–712
- Balazovich, K. J., Smolen, J. E. & Boxer, L. A. (1986) *J. Immunol.* **137**, 1665–1673
- McDonald, J. R. & Walsh, M. P. (1985) *Biochem. J.* **232**, 559–567
- Huang, C.-K. & Oshana, S. C. (1986) *J. Leukocyte Biol.* **39**, 671–678
- Charp, P. A., Rice, W. G., Raynor, R. L., Reimund, E., Kinkade, J. M., Ganz, T., Selsted, M. E., Lehrer, R. L. & Kuo, J. F. (1988) *Biochem. Pharmacol.* **37**, 951–956
- Boym, A. (1986) *Scand. J. Lab. Clin. Invest.* **21**, Suppl. **97**, 77–84
- Borregaard, N., Heiple, J. M., Simons, E. R. & Clark, R. A. (1983) *J. Cell Biol.* **97**, 52–61
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Flint, A. J., Paladini, R. D. & Koshland, D. E. (1990) *Science* **249**, 408–411
- Bitar, K. N., Hillemeier, C., Biancani, P. & Balazovich, K. J. (1991) *Am. J. Physiol.* **260**, G537–G542
- Olah, Z. & Kiss, Z. (1986) *FEBS Lett.* **195**, 33–37
- Rosenthal, A., Rhee, L., Yadegari, R., Paro, R., Ullrich, A. & Goeddel, D. V. (1987) *EMBO J.* **6**, 433–441
- Majumdar, S., Rossi, M. W., Fujiki, T., Phillips, W. A., Disa, S., Queen, C. F., Johnson, R. B., Jr., Rosen, O., Corkey, B. E. & Korchak, H. M. (1990) *J. Biol. Chem.* **266**, 9285–9294
- Kishimoto, A., Kajikawa, N., Shista, M. & Nishizuka, Y. (1982) *J. Biol. Chem.* **258**, 1156–1164

42. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B. & Horecker, B. L. (1986) *J. Biol. Chem.* **261**, 4101–4105
43. Pontremoli, S., Michetti, M., Melloni, E., Sparatore, B., Salamino, F. & Horecker, B. L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3705–3707
- 43a. McEwen, E. L. & Balazovich, K. J. (1992) *Biochem. J.*, in the press
44. Jaken, S. & Kiley, S. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4418–4422
45. Schwantke, N. & Le Peuch, C. J. (1984) *FEBS Lett.* **177**, 36–40
46. Pearson, J. D., DeWald, D. B., Mathews, W. R., Moziew, N. M., Zurcher-Neely, H. A., Heinrikson, R. L., Morris, M. A., McCubbin, W. D., McDonald, J. R., Fraser, E. D., Vogel, H. J., Kay, C. M. & Walsh, M. P. (1990) *J. Biol. Chem.* **265**, 4583–4591
47. Pribilla, I., Kruger, H., Buchner, K., Otto, H., Schiebler, W., Tripler, D. & Hucho, F. (1988) *Eur. J. Biochem.* **177**, 657–664
48. Toker, A., Ellis, C. A., Sellers, L. A. & Aitken, A. (1990) *Eur. J. Biochem.* **191**, 421–429
49. Sato, E. F., Morimoto, Y. M., Matsuno, T., Miyahara, M. & Utsumi, K. (1987) *FEBS Lett.* **214**, 181–186

Received 12 September 1991/19 December 1991; accepted 2 January 1992