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Potential Role of Protein Kinase C on the Differentiation of Erythroid Progenitor Cells

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The effect of protein kinase C inhibitors, staurosporine and 1-(5-isoquinolinyl sulfonyl)-2-methyl piperazine(H7) on *in vitro* differentiation of erythroid progenitor cells which were isolated from spleens of mice infected with the anemia-inducing strain of Friend virus were examined. Erythropoietin-mediated differentiation of erythroid progenitor cells, as determined by the incorporation of ^{59}Fe into protoporphyrin, was inhibited by staurosporine and H7 in a concentration-dependent manner. Scatchard analysis of the ^{3}H-phorbol-12,13-dibutyrate binding to erythroid progenitor cells revealed that at the high affinity sites the dissociation constant was 22nM and the maximum number of ^{3}H-phorbol-12,13-dibutyrate binding sites per cell was approximately 3.7×10^{5}. Cytosolic protein kinase C was isolated from erythroid progenitor cells and then purified by sequential column chromatography. Two isoforms of protein kinase C were found. Photoaffinity labeling of the purified protein kinase C samples with ^{3}H-phorbol 12-myristate 13-acetate followed by analysis of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autofluorography showed radiolabeled 82-KDa peptides. Radiolabeling of the 82-KDa peptides with ^{3}H-phorbol 12-myristate 13-acetate was almost completely blocked by excess unlabeled phorbol 12-myristate 13-acetate. Results of phorbol 12-myristate 13-acetate-promoted phosphorylation with the purified protein kinase C samples showed that the phosphorylation of 82-KDa peptides was increased as the concentration of phorbol 12-myristate 13-acetate was increased from 10^{-8} M to 10^{-4} M. In light of the findings that erythroid progenitor cells possessed an abundance of protein kinase C and that staurosporine and H7 inhibited erythroid differentiation, it seemed likely that protein kinase C would play a role in the erythroid progenitor cell development.

Key words : Erythropoietin, Protein Kinase C, ^{3}H-phorbol-12,13-dibutyrate Binding, Photoaffinity labeling, Autophosphorylation, Erythroid Progenitor Cell

INTRODUCTION

Protein kinase C is an ubiquitous enzyme mediating signal transduction pathways for the regulation of remarkably diverse biological functions (Nishizuka, 1986). Diacylglycerol, produced from hydrolysis of membrane phospholipids, increases the affinity of protein kinase C for Ca^{++} thereby activating the enzyme. Tumor promoting phorbol esters, for which protein kinase C acts as the receptor (Ashendel *et al.*, 1985; Kikkawa *et al.*, 1983; Niedel *et al.*, 1983), activate the enzyme by promoting the migration of protein kinase C from cytosol to the membrane (Franklin *et al.*, 1989) and by increasing its affinity for Ca^{++} in a manner analogous to diacylglycerol (Ebel-ing *et al.*, 1985). The use of activators and inhibitors of the enzyme such as phorbol esters, staurosporine and H7 had been useful in gaining insight into biological roles for protein kinase C.

Using a recently developed model for erythropoietin-directed differentiation of erythroid cells (Kim *et al.*, 1989), a possible role for protein kinase C in hematopoiesis was investigated. Here we got the results on the inhibition of erythropoietin-dependent *in vitro* differentiation of erythroid progenitor cells by staurosporine and H7, ^{3}H-phorbol-12, 13-dibutyrate binding to erythroid progenitor cells, partial purification of protein kinase C, and the characterization of the enzyme by photoaffinity labeling and by phorbol

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12-myristate 13-acetate-promoted phosphorylation. The results suggest a potential role for protein kinase C in erythropoietin-directed differentiation of erythroid progenitor cells.

MATERIALS AND METHODS

Materials

Anemia-inducing strain of Friend virus was kindly provided by Dr. Stephen T. Sawyer (Vanderbilt University, Nashville, TN). Erythropoietin was a generous gift from Genetics Institute (Cambridge, MA). Phosphatidylserine, 1,2-dioleoylglycerol, histone type III-S, γ-globulin, bovine serum albimin, phorbol-12,13-dibutyrate, phorbol 12-myristate 13-acetate, leupeptin, soybean trypsin inhibitor, pepstatin, aprotinin, phenylmethylsulfonylfluoride, staurosporine, H7, Drabkin's reagent, Iscove's modified Dulbecco's medium, and molecular weight markers for SDS-PAGE were purchased from Sigma Chemical Company (St. Louis, MO). Reagents for SDS-PAGE were obtained from Bio-Rad Laboratories (Richmond, CA), and human transferrin from CalBiochem (San Diego, CA). Polyethylene glycol was obtained from Fisher Scientific Co. (Pittsburgh, PA). DEAE-Sephacel, Phenyl-Sepharose CL-4B, AH-Sepharose 4B, Sepharose CL-6B and the molecular weight markers for the gel filtration chromatography from Pharmacia Fine Chemicals (Pisca-taway, NJ). ^{3}H-phorbol-12,13-dibutyrate, ^{3}H-phorbol 12-myristate 13-acetate, and $^{59}FeCl^{3}$ from duPont-New England Nuclear (Boston, MA), and [γ-^{32} P]-ATP from Amersham (Arlington Heights, IL).

Preparation of erythroid progenitor cells

Anemia-inducing strain of Friend virus injection into mice tail veins and the separation of mice splenic erythroid progenitor cells by gravitational separation were carried out according to the procedure of Sawyer *et al.* (1987). The erythroid progenitor cells which were separated by velocity separation at unit gravity were used for the *in vitro* differentiation experiments. The erythroid progenitor cells used for the purification of protein kinase C and also for the phorbol-12,13-dibutyrate binding experiments were prepared as previously reported (Im *et al.*, 1990). Briefly, 18 days after injection of anemia-inducing strain of Friend virus-containing plasma into BALB/c mice, the enlarged spleens were excised and minced through a nylon sieve (75 μm mesh). The cells were washed three times with cold saline solution by centrifugation for 10 min at 450×g. For the purification of protein kinase C and the phorbol-12,13-dibutyrate binding experiments, the washed cells were used without further purification.

Effect of staurosporine and H7 on the erythropoietin-mediated in vitrodifferentiation of erythroid progenitor cells

Erythropoietin-mediated *in vitro* differentiation of erythroid progenitor cells was carried out according to the procedure of Koury *et al.* (1984, 1988). Briefly, the gravity separated erythroid progenitor cells (2×10^{6} cells) in 1 ml of Iscove's modified Dulbecco's medium containing erythropoietin (0.2 U/ml), 30% fetal bovine serum, 1% bovine serum albumin, penicillin (100 U/ml), streptomycin (100 μg/ml) and 0.1 mM α-thioglycerol were cultured in 16 mm diameter plastic wells at 37°C in humidified air plus 4% CO_2. The incorporation of ^{59}Fe into protoporphyrin (a measure of heme synthesis) was determined at 12-hour intervals. At 12 hours prior to the determination of ^{59}Fe incorporation into protoporphyrin, 50 μl of human transferrin (2.4 mg/ml Iscove's modified Dulbecco's medium), 10 μCi of $^{59}FeCl_3$, and varying amounts of staurosporine or H-7 in 10ml of dimethylsulfoxide were added to 1 ml aliquots of cell culture. The cells were collected, washed, then lysed with Drabkin's solution. To the lysates, HCl was added to give a final concentration of 0.1 N, and then the ^{59}Fe incorporated into heme was extracted with 2 ml of cyclohexanone. The amount of cyclohexanone-extracted ^{59}Fe was determined in a gamma counter. Since cyclohexanone specifically extracts only the ^{59}Fe which was incorporated into heme, and not the free ^{59}Fe, the cyclohexanone extraction method was an excellent assay technique for the determination of heme synthesis.

^{3}H-Phorbol-12,13-dibutyrate binding to erythroid progenitor cells

For the determination of time-course of ^{3}H-phorbol-12,13-dibutyrate binding to erythroid progenitor cells, 100 μl of ^{3}H-phorbol-12,13-dibutyrate was added to 200 μl aliquots of erythroid progenitor cells (4×10^{6} cells) and the samples were incubated at room temperature for 0-45 min in the absence (for total binding) or presense (for non-specific binding) of excess unlabeled phorbol-12,13-dibutyrate (100 μM). After incubation, the samples were chilled on ice and centrifuged for 10 min at 800×g at 4°C. The cell pellet was lysed with 1 ml of 0.2 M NaOH, neutralized with 0.2 ml of 1 M HCl and the radioactivity of the samples was determined in a scintillation counter. Specific binding was calculated by subtracting the non-specific binding from the total binding. For the Scatchard analysis of ^{3}H-phorbol-12,13-dibutyrate binding to erythroid progenitor cells, 100 μl aliquots of ^{3}H-phorbol-12,13-dibutyrate solution containing different amounts of unlabeled phorbol-12,13-dibutyrate were added to 200 μl aliquots of erythroid progenitor

cells and the samples were incubated for 30 min at room temperature. The incubated samples were centrifuged and both the free phorbol-12,13-dibutyrate concentration and the phorbol-12,13-dibutyrate bound to the cells were determined as described in the legend for Fig. 4. The data are plotted according to Scatchard analysis (Scatchard, 1949).

^{3}H-Phorbol-12,13-dibutyrate binding to solubilized protein kinase C

Aliquots (100 μl) of solubilized protein kinase C samples were added to 50 μl of binding (50 mM Tris-HCl, pH 7.4 containing 3 mM $CaCl_2$, 112 mM magnesium acetate, 40 mg/ml bovine serum albumin, 1.6 mg/ml phosphatidylserine and ^{3}H-phorbol-12,13-dibutyrate). The samples were incubated for 30 min at 37°C in the absence (for total binding) or presence (for non-specific binding) of 600 nM unlabeled phorbol-12,13-dibutyrate. After incubation, the samples were chilled on ice, and then 10 μl of ice-cold γ-globulin solution (15% w/v) and 90 μl of ice-cold polyethylene glycol solution (30% w/v) were added. The samples were kept onice for 15 min followed by filtration through Milipore STSV 096 NS membranes under vacuum. The membranes were washed three times with 150 μl of ice-cold 10.8% polyethylene glycol solution. Radioactivity of the Milipore membranes was determined in a Beckman scintillation counter and specific binding was calculated.

Protein kinase C assay

Activity of the enzyme was assayed as described previously (Franklin *et al.*, 1989). Total phosphorylation activity was determined by the phosphorylation of Type III-S histone in the presence of the protein kinase C activators (2.4 nM Ca^{++}, 0.2 μg diacylglycerol and 2 mg phosphatidylserine) at 30°C. Non-specific activity was determined in the absence of the activators and in the presence of 0.5 mM EGTA. Specific protein kinase C activity was determined by subtracting non-specific activity from total activity.

Isolation and purification of protein kinase C from erythroid progenitor cells

Erythroid progenitor cells prepared from 150 anem-ia-inducing strain of Friend virus injected mice were suspended in a 200 ml solution composed of 20 mM Tris-HCl, pH 7.5 containing 0.5 mM EDTA, 0.5 mM EGTA, 10 mM mercaptoethanol, 10 μM leupeptin, 33 mg-/100 ml soybean trysin inhibitor, 1 mg/100 ml pepstantin, 1 mg/100 ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride. The sample was homogenized with a polytron tissue homogenizer in an ice bath and then centrifuged at 100,000×g for 1 h at 4°C. The supernatant was centrifuged again at 160,000×g for 1 h at 4°C and the final supernatant was used for the purification of the enzyme. Protein kinase C was purified by sequential column chromatography on DEAE-Sephacel, Phenyl-Sepharose CL-4B, threonine-Sepharose and Sepharose CL-6B-columns. Buffer A (20 mM Tris-HCl, pH 7.5 containing 0.5 mM EDTA, 0.5 mM EGTA and 10 mM mercaptoethanol) was used as the elution buffer for the sequential chromatography and all the purification procedures were carried out in a cold room.

DEAE-Sephacel chromatography

The isolated crude cytosolic protein kinase C sample (175 ml) was loaded on a DEAE-Sephacel column (5× 5.5 cm) and eluted successively with Buffer A containing 50 mM, 100 mM and 0.8 M NaCl. The flow rate was 48 ml/h and 8 ml fractions were collected. The assay results of ^{3}H-phorbol-12, 13-dibutyrate binding and the protein kinase C activity with aliquots withdrawn from each fraction showed that the enzyme was eluted in the 100 mM NaCl elution fractions. The fractions containing protein kinase C were pooled.

Phenyl-Sepharose CL-4B chromatography

To the pooled protein kinase C sample from DEAE-Sephacel chromatography, NaCl was added to 1M, and then the sample was applied to a phenyl-Sepharose CL-4B column (1.5×15 cm). The column was eluted by the decreasing linear NaCl concentration gradient elution with 100 ml each of Buffer A and Buffer A containing 0.6 M NaCl. After the chromatography, the ^{3}H-phorbol-12,13-dibutyrate binding and protein kinase C activity of each fraction were determined. protein kinase C was eluted at the tail end of the the decreasing linear NaCl concentration gradient elution. The fractions containing protein kinase C were pooled.

Threonine-Sepharose chromatography

The pooled protein kinase C sample from phenyl-Sepharose CL-4B was applied to a threonine-Sepharose column (1.5×9 cm). Threonine-Sepharose was prepared according to the procedure of Kitano et al. (1986). The column was initially eluted with 90 mM NaCl in Buffer A, followed by a linear NaCl concentration gradient elution with 100 ml each of Buffer A containing 90 mM and 0.6M NaCl. After chromatography, the ^{3}H-phorbol-12,13-dibutyrate binding and protein kinase C activity were assayed with aliquots from each fraction. Protein kinase C was found in two peak fractions. Peak I and Peak II fractions containing protein kinase C were separately pooled.

Sepharose CL-6B gel filtration chromatography

The pooled Peak I and Peak II samples from threon-

ine-Sepharose chromatography were concentrated to about 2 ml in an Amicon concentrator with a PM-10 membrane. The concentrated samples were dialyzed overnight against two changes of 2L of the elution buffer for the Sepharose CL-6B chromatography (Buffer A containing 0.1 M NaCl). The dialyzed samples were individually chromatographed on a Sepharose CL-6B column. The Sepharose CL-6B purified protein kinase C samples were used for the photo affinity labeling and the phorbol 12-myristate 13-acetate-promoted phosphorylation experiments.

Photoaffinity labeling of purified protein kinase C with ^{3}H-phorbol 12-myristate 13-acetate

Aliquots (400 μl) of the Sepharose CL-6B purified protein kinase C samples were incubated with ^{3}H-phorbol 12-myristate 13-acetate in the absence and presence of excess unlabeled phorbol 12-myristate 13-acetate under the same conditions as described for the assay for the ^{3}H-phorbol-12, 13-dibutyrate binding to solubilized protein kinase C. After incubation, the sample tubes were transferred to an ice bath and were irradiated from the top with UV light (American Ultraviolet Co., Model 1000 F) through a 7-54 UV filter (Farrand Optical Co., Valhalla, NY) for 2 min at a distance of 25 cm. The UV irradiated samples were subjected to SDS-PAGE under reducing conditions. After electrophoresis, the gel was soaked in Enlightning solution for 30 min, dried and autofluorography was performed.

Phorbol 12-myristate 13-acetate-promoted phosphorylation of protein kinase C

Porbol 12-myristate 13-acetate-promoted phosphor-ylation of the Sepharose CL-6B purified protein kinase C samples was carried out according to a modified procedure of Mochly-Rosen *et al.* (1987) and Newton *et al.* (1987). Briefly, aliquots (106 μl) of the purified protein kinase C samples were added to 64 μl of the phosphorylation buffer (20 mM Tris-HCl, pH 7.5 containing 10 μg of phosphatidylserine, 240 nmol $CaCl_2$, 11.25 mM $MgCl_2$ and 2.5 nmol [γ-32P]ATP, 2-4×105 cpm/nmol). The samples were incubated for 3 min at 30°C in the absence or presence of various amounts of phorbol 12-myristate 13-acetate. The reaction was stopped by adding 130 μl of the reducing SDS-PAGE sample buffer. The samples were heated for 5 min in boiling water and then analyzed by SDS-PAGE and autoradiography.

RESULTS

Effects of staurosporine and H7 on the erythropoietin-mediated *in vitro* differentiation of erythroid progenitor cells

To determine whether protein kinase C inhibitors influence the erythropoietin-directed differentiation of

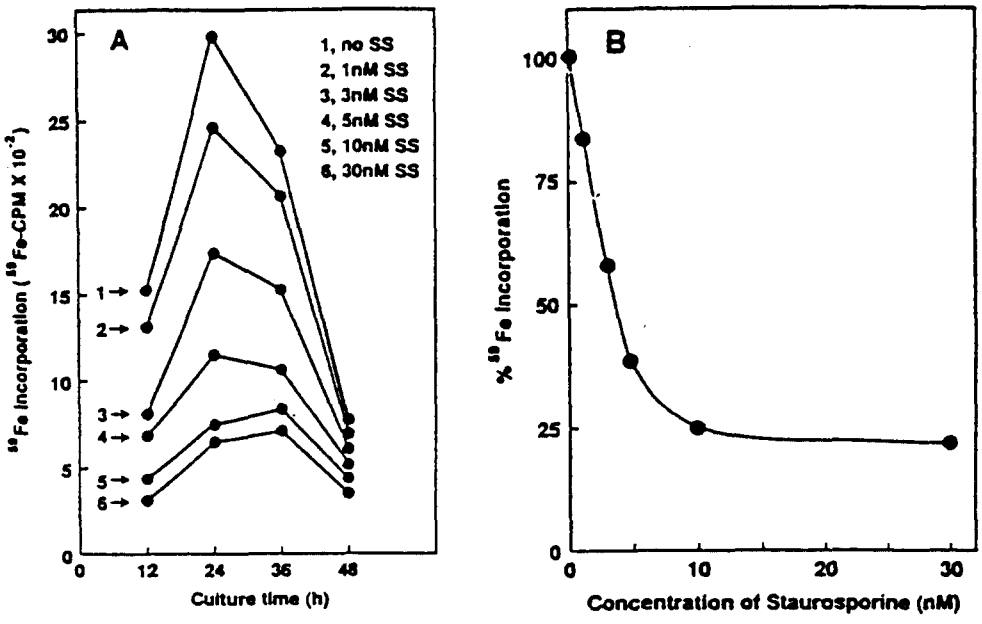

Fig. 1. Effect of staurosporine on the incorporation of ^{59}Fe into heme during culture of erythroid progenitor cells in the presence of erythropoietin. (A) Gravity separated erythroid progenitor cells(2×10^{5} cells/ml) were cultured with erythropoietin (0.2 U/ml). The cells were treated with varying amounts of staurosporine and $^{59}FeCl_3$ 12 hours prior to the determination of ^{59}Fe incorporation into heme. Amounts of ^{59}Fe incorporated into heme were determined at 12 hour intervals as described in "Methods". (B) The ^{59}Fe incorporation data at 24 hour period in A were replotted in terms of % incorporation of ^{59}Fe versus concentration of staurosporine. Data are means of triplicate cultures and standard deviations were all less than 5% of the mean values.

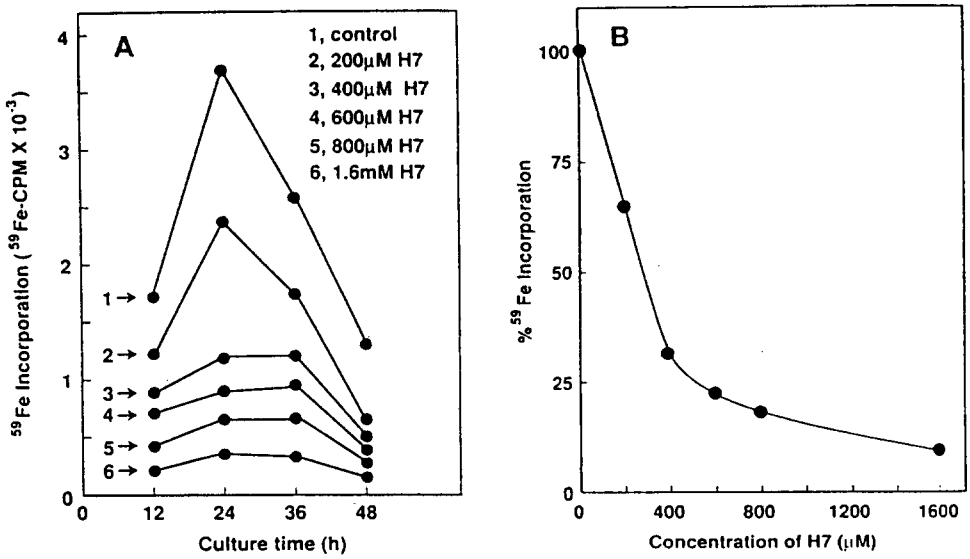

Fig. 2. Effect of H7 on the incorporation of ^{59}Fe into heme during culture of erythroid progenitor cells in the presence of erythropoietin. Experimental procedure was the same as for Fig. 1. (A) Effect of H7 concentration on the ^{59}Fe incorporation into heme. (B) Concentration of H7 versus % ^{59}Fe incorporation at 24 hour culture period. Data are means of quadruplicate cultures and standard deviations were less than 5% of the mean values.

erythroid progenitor cells, effects of staurosporine and H7 on the incorporation of ^{59}Fe into heme during erythroid cell culture were examined. Fig. 1 shows a typical result of three experiments in which the ^{59}Fe incorporation into heme was monitored in the presence of staurosporine. When erythroid progenitor cells were cultured in the medium containing 0.2 U/ml of erythropoietin, initial 59^{Fe} incorporation into heme was low, but increased sharply to a peak during 24 hours of culture. After 24 hours, the ^{59}Fe incorporation was decreased during 36 to 48 hour periods (Fig. 1A). In the absence of erythropoietin in the medium, Virtually no ^{59}Fe incorporation was observed (data not shown). Figure 1A shows that the ^{59}Fe incorporation was inhibited by staurosporine in a concentration dependent manner. The data at 24 hour culture period are replotted in terms of % ^{59}Fe incorporation versus staurosporine concentration as shown in Figure 1B. A maximum inhibition was seen at 10 nM or higher. erythropoietin-mediated ^{59}Fe incorporation into heme was also suppressed by another protein kinase C inhibitor, H7 in a concentration-dependent manner (Fig. 2). However, H7 was far less effective than staurosporine and required more than 4 orders of magnitude higher concentrations than staurosporine to obtain a comparable suppression. The concentrations at which staurosporine and H7 elicited a half maximal ^{59}Fe incorporation were 3.8 nM and 280 μM, respectively (Fig. 1B and Fig. 2B). Dimethylsulfoxide, which was used as a solvent for staurosporine and H7, had no effect on ^{59}Fe incorporation (data not shown).

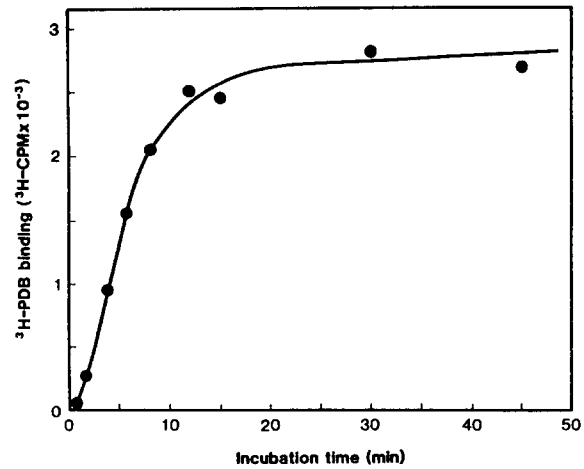

Fig. 3. Time-course of ^{3}H-phorbol-12,13-dibutyrate binding to erythroid progenitor cells.To 200μl aliquots of erythroid progenitor cells (4×10^{6} cells), ^{3}H-phorbol-12,13-dibutyrate (100μl) was added and incubated at room temperature in the absence and presence of excess unlabeled phorbol-12, 13-dibutyrate. The incubated samples were chilled on ice and centrifuged. The precipitated cells were solubilized with NaOH, neutralized with HCl, then radioactivity was determined and specific binding was calculated. For details, see Methods.

Binding of ^{3}H-phorbol-12,13-dibutyrate to erythroid progenitor cells

The time course of ^{3}H-phorbol-12,13-dibutyrate binding to erythroid progenitor cells showed that the specific ^{3}H-phorbol-12,13-dibutyrate binding reached steady state by about 20 min at room temperature

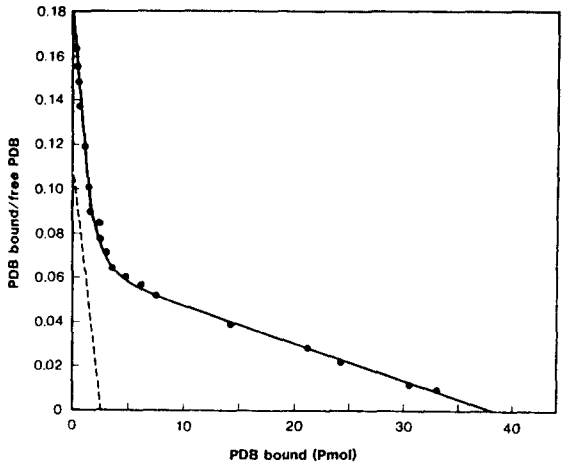

Fig. 4. Scatchard analysis of 3H-phorbol-12,13-dibutyrate binding to erythroid progenitor cells. ^{3}H-Porbol-12,13-dibuyrate(100 μl) was added to 200 μl aliquots of erythroid progenitor cells (5.6×10^{7} cells) and incubated for 30 min at room temperature in the presence of differing amounts of unlabeled phorbol-12,13-dibutyrate. The incubated samples were chilled, centrifuged, then 100 μl of the supernatant was withdrawn and radioactivity was determined. Free phorbol-12,13-dibutyrate concent-rations were calculated from the radioactivity. The remaining supernatant in each centrifuge tube was aspirated off and the inside wall of the tube was wiped with moistened cotton swabs. The precipitated cells were dissolved with NaOH, neutralized with HCl and radioactivity was determined. Corrections for nonspecific binding were made by subtracting the radioactivity obtained from samples containing 100 μM of unlabeled phorbol-12,13-dibutyrate, and the results were analyzed according to the Scatchard plot.

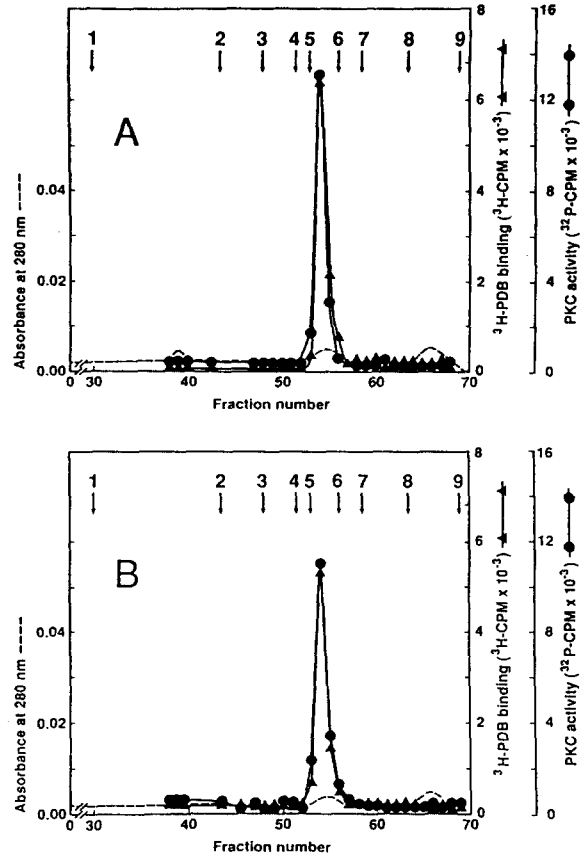

Fig. 5. Sepharose CL-6B gel filtration of Peak I and Peak II protein kinase C samples from threonine-Sepharose chromatography. The protein kinase C containing peak fractions from threonine-Sepharose chromatography were separately pooled and concentrated to about 2 ml in an Amicon concentrator with a PM-10 membrane. The concentrated samples were individually chromatographed on a Sepharose CL-6B column (1.6×95 cm). The flow rate was 6 ml/h and 2 ml fractions were collected. A, Peak I sample. B. Peak II sample. The molecular weight markers used were: 1, blue dextran; 2, thyroglobulin; 3, ferritin; 4, catalase; 5, aldolase; 6, bovine serum albumin; 7, Ovalbumin; 8, carbonic anhydrase; 9, RNase-A.

(Fig. 3). Scatchard analysis of the ^{3}H-phorbol-12,13-dibutyrate binding revealed that at the high affinity sites the dissociation constant was 22 nM and maximum number of ^{3}H-phorbol-12,13-dibutyrate binding sites per cell was appoximately 3.7×105 (Fig. 4).

Isolation and purification of protein kinase C

Cytosolic protein kinase C was isolated from erythroid progenitor cells by homogenization of the cells followed by centrifugation. The supernatant containing protein kinase C was used for the purification of the enzyme by sequential chromatography on DEAE-Sephacel, phenyl-Sepharose CL-4B, threonine-Sepharose and Sepharose CL-6B columns. When the phenyl-Sepharose CL-4B purified protein kinase C sample was chromatographed on a threonine-Sepharose, protein kinase C was separated into two peak fractions (Peak I and Peak II). Peak I and Peak II protein kinase C samples were individually chromatographed on a Sepharose CL-6B column. The results are shown in Fig. 5. Protein kinase C of both Peak I and Peak II samples was eluted at an elution volume corresponding to a Mr of 82,000. The protein kinase C containing fractions of Peak I and Peak II samples were separately pooled and used for the ^{3}H-phorbol 12-myristate 13-acetate photoaffinity labelling and phorbol 12-myristate 13-acetate-promoted phosphorylation experiments.

Photoaffinity labelling of purified protein kinase C samples with ^{3}H-phorbol 12-myristate 13-acetate

Aliquots of the Sepharose CL-6B purified Peak I and Peak II samples were incubated with ^{3}H-phorbol 12-myristate 13-acetate in the absence and in the presence of excess amounts of unlabeled phorbol 12-myristate 13-acetate for 30 min at 37°C. After incubation, the samples were transferred to an ice bath and ir-

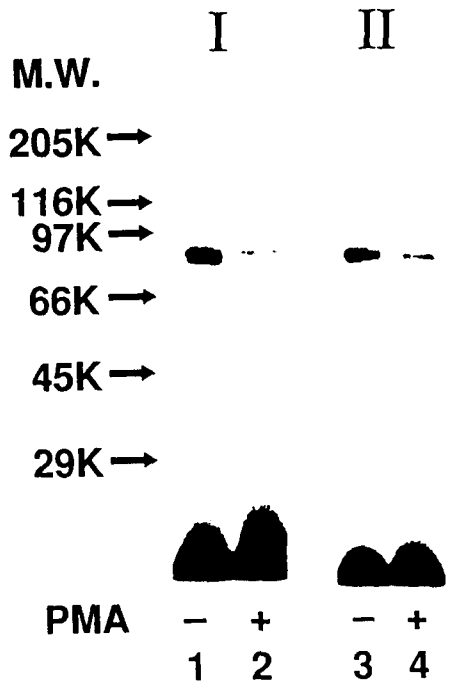

Fig. 6. Photoaffinity labeling of the purified protein kinase C samples with ^{3}H-phorbol 12-myristate 13-acetate. Aliquots of the protein kinase C samples from Fig. 5 were incubated with ^{3}H-phorbol 12-myristate 13-acetate in the absence and presence of an excess amount of unlabeled phorbol 12-myristate 13-acetate. The incubated samples were irradiated with UV light. The UV irradiated samples were analyzed by SDS-PAGE and autofluorography. Lanes 1 and 3,in the absence of phorbol 12-myristate 13-acetate; Lanes 2 and 4, in the presence of excess phorbol 12-myristate 13-acetate.

radiated with UV light for 2 min. The UV irradiated samples were subjected to SDS-PAGE under reducing conditions followed by autofluorography (Fig. 6). In Fig. 6, Panels I and II represent Peak I and Peak II samples, respectively. Fig. 6 shows that in the absence of unlabeled phorbol 12-myristate 13-acetate a radiolabeled peptide with Mr of 82,000 was found in both Peak I and Peak II samples (lanes 1 and 3). However, in the presence of an excess of unlabeled phorbol 12-myristate 13-acetate, radio labelling of 82-KDa peptide of both Peak I and Peak II samples was almost completely blocked (lanes 2 and 4). These results indicated that the photoaffinity labelling of the purified Peak I and Peak II protein kinase C samples with 3H-phorbol 12-myristate 13-acetate was specific.

Phorbol 12-myristate 13-acetate-promoted phosphorylation of purified protein kinase C samples

Aliquots of Sepharose CL-6B purified Peak I and Peak II protein kinase C samples were added to the phosphorylation buffer (20 nM Tris-HCl, pH 7.5 containing phosphatidylserine, $CaCl^{2}$, $MgCl^{2}$ and [γ-^{32}P]-ATP) and incubated for 3 min at 30°C in the absence and presence of various concentrations (10^{-8}-10^{-4}M) of phorbol 12-myristate 13-acetate. The incubated samples were subjected to SDS-PAGE under reducing conditions, followed by autoradiography. The results depicted in Fig. 7 showed that for both Peak I and Peak II samples, the 82-KDa peptides were not phosphorylated in the absence of phorbol 12-myristate 13-acetate. However, as the concentration of phorbol 12-

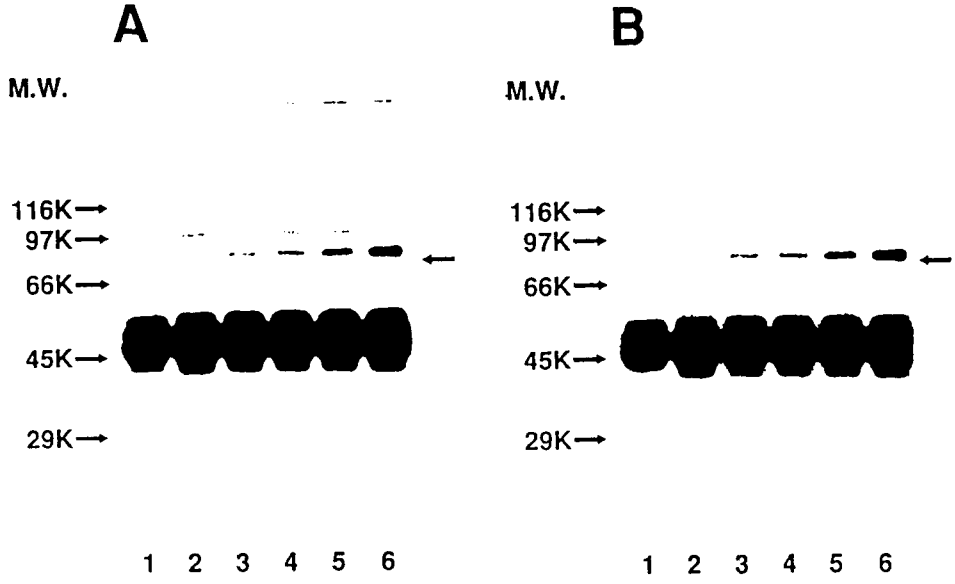

Fig. 7. Phorbol 12-myristate 13-acetate-stimulatedphosphorylation of purified protein kinase C. Aliquots of protein kinase C sample from Fig. 5 were incubated with [γ-^{32}P]-ATP and cofactors of the enzyme in the absence and presence of differing concentrations of phorbol 12-myristate 13-acetate. The incubated samples were analyzed by SDS-PAGE and autoradiography. A, Peak I sample. B, Peak II sample. Concentrations of phorbol 12-myristate 13-acetate: Lane 1, 0M; Lane 2, 10^{-8}M; Lane 3, 10^{-7}M; Lane 4, 10^{-6}M; Lane 5, 10^{-5}M; Lane 6, 10^{-4}M.

myristate 13-acetate was increased from 10^{-8}M to 10^{-4}M, phosphorylation of 82-KDa peptides of Peak I and Peak II samples was increased (lanes 2-6, Fig. 7A and 7B). These results clearly demonstrate that phorbol 12-myristate 13-acetate promotes phosphorylation of both Peak I and Peak II protein kinase C samples of erythroid progenitor cells. The broad radioactive bands corresponding to molecular weight of 48K represente the substrate(s) of the enzyme.

DISCUSSION

Protein kinase C has long been implicated to play a role in the regulation of hematopoiesis (Fibach et al., 1980; Fibach et al., 1979; Nakazawa *et al.*, 1989; Katayama *et al.*, 1989; Sieber *et al.*, 1981). Studies with mouse hematopoietic precursor cells in culture have shown that phorbol 12-myristate 13-acetate stimulates myeloid progenitor cells to form the monocyte/macrophage colonies either with or without colony stimulating factor (Fibach *et al.*, 1980; Fibach *et al.*, 1979; Sieber *et al.*, 1981). By contrast, phorbol 12-myristate 13-acetate action on the differentiation of erythroid progenitor cells was controversial. While Fibach *et al.* (1980) found that phorbol 12-myristate 13-acetate increased the number and size of bursts formed by early erythroid progenitor cells (burst forming unit-erythroid), Sieber *et al.* (1981) reported instead an inhibition of burst formation by bone marrow-derived early erythroid progenitor cells. On the other hand, with respect to the late erythroid progenitor cells (colony forming unit-erythroid) or erythroid cells already committed to terminal differentiation, phorbol 12-myristate 13-acetate was without effect (Katayama *et al.*, 1989; Nakazawa *et al.*, 1989; Sieber *et al.*, 1981). Morever, a suppression of the responsiveness to phorbol 12-myristate 13-acetate was observed after an inducer directed commitment to differentiation of a murine erythroleukemia cell line (Fibach *et al.*, 1979).

The splenic erythroid progenitor cells isolated from mice infected with the anemia causing strain of Friend virus were arrested at the stage of colony forming unit-erythroid which was already committed to terminal differentiation. Under *in vitro* culture conditions, these cells differentiated to hemoglobin-rich reticulocytes within 2-3 days in the presence of erythropoietin and fetal calf serum. We found that staurosporine elicited a dose-dependent inhibition of erythroid progenitor cell differentiation as measured by ^{59}Fe incorporation. Although the identity of the protein kinase which was affected by staurosporine could not be made with certainty, the finding that H7 also suppressed erythroid differentiation suggested protein kinase C as the likely target. Our results on the suppression of erythroid progenitor cell differentiation by protein kinase C inhibitors were at odds with some of the aforementioned findings.

In order to compare protein kinase C content in erythroid progenitor cells with previously reported amounts of the enzyme in various tissues and cell lines (Chida *et al.*, 1988; Horowitz *et al.*, 1981; Shoyab *et al.*, 1981; Sieber *et al.*, 1981), we had characterized phorbol-12,13-dibutyrate binding to erythroid progenitor cells. We found that the maximum number of phorbol-12,13-dibutyrate binding sites per cell and the Kd at the high affinity site were 3.7×10^5 and 22 nM, respectively. It was reported that for rat embryo fibroblasts the maximum number of high affinity phorbol-12,13-dibutyrate binding sites per cell was 1.6×10^5, and the Kd was 8 nM (Horowitz *et al.*, 1981). It was found that the brain and spleen of mice contain high levels of protein kinase C, and the Kd for the phorbol-12,13-dibutyrate binding was 2-4 nM (Shoyab *et al.*, 1981). Recently, It was reported the phorbol-12,13-dibutyrate binding to 41 different cell lines and when the maximum numbers of phorbol-12, 13-dibutyrate binding sites per cell were calculated as a range from 1.8×10^2 to 4.7×10^5 (Chida *et al.*, 1988). It was evident that protein kinase C density in erythroid progenitor cells was one of the highest among the various cell lines. In contrast to the large number of phorbol-12,13-dibutyrate binding sites per erythroid progenitor cell, mature erythrocytes of mice bind a negligible amount of phorbol-12,13-dibutylate (datIlnot shown). Since erythroid progenitor cells possessed a high abundance of protein kinase C as compared to mature mice erythrocytes and protein kinase C inhibitors suppressed the ^{59}Fe incorporation into heme, it was likely that protein kinase C might play an important function for the differentiation of the progenitor cells.

Recently, several procedures for the purification of protein kinase C have been developed (Go *et al.*, 1987; Shearman *et al*, 1989; Walton *et al.*, 1987; Wooten *et al.*, 1987). Several discrete subspecies of protein kinase C (α-, βI-, βII-, γ-, δ-, ε-, and ψ-protein kinase C) have isolated from various tissues (Kikkawa *et al.*, 1989). Following the established procedures, we isolated two isoforms of protein kinase C from erythroid progenitor cells, and the isoforms of protein kinase C were characterized by photoaffinity labeling with ^{3}H-phorbol 12-myristate 13-acetate and by phorbol 12-myristate 13-acetate-promoted autophosphorylation. The described method for the photoaffinity labeling of protein kinase C with ^{3}H-phorbol 12-myristate 13-acetate was simple and would be useful for the identification and characterization of protein kinase C in other cell lines.

Phorbol esters had been known to inhibit the binding

of epidermal growth factor to a variety of cells in culture (Brown *et al.* , 1979; Lee *et al.*, 1978; Shoyab *et al.*, 1980; Shoyab *et al.*, 1979). It had been found that for human OCIMI erythroleukemia cells, phorbol 12-myristate 13-acetate decreased the number of erythropoietin binding sites per cell without altering the affinity of the receptor for erythropoietin (Broudy *et al.*, 1988). It had been shown that phorbol esters catalyze phosphorylation of EGF receptor on threonine (Whitely *et al.* 1986), but block tyrosine-specific phosphorylation of EGF receptor and decreased EGF-stimulated tyrosine kinase activity (Cochet *et al.*, 1984; Friedman *et al.*, 1984). It was found that three isoforms of protein kinase C, which were purified from the rat brain, promoted the phosphorylation of EGF receptor at quite different rates (Ido *et al.*, 1987). We had partially purified erythropoietin receptors from the erythroid progenitor cells and demonstrated the erythropoietin-promoted phosphorylation of erythropoietin receptors (Im *et al.*, 1990). It would be of interest to determine whether protein kinase C inhibitors and phorbol esters had any direct effect on the binding of erythropoietin to purified erythropoietin receptors, and on the erythropoietin-promoted phosphorylation of tyrosine residues of erythropoietin receptors through the protein kinase C-mediated phosphorylation cascade mechanisms.

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