Rat liver nuclei protein kinase C is identified as type II isozyme employing monospecific antibodies obtained against each three types of rat brain protein kinase C isozymes. (Yoshida, Y., Huang, F. L., Nakabayashi, H., and Huang, K. P. (1989) J. Biol. Chem. 264, 1172-1179) is a major contaminant devoid of any protein kinase activity. The ratio obtained between protein kinase C activity over phorbol dibutyrate bound, at various purification steps, indicates that the nuclear enzyme is a phorbol ester receptor. When isolated nuclei were incubated with 12-O-tetradecanoylphorbol-13-acetate, endogenous protein kinase C activity was elevated about 8-10 fold suggesting the existence of phorbol ester signaling pathway at the level of nucleus. The role of nuclear protein kinase C is delineated in the regulation of inducible gene transcription.

One of the major pleiotropic effects of phorbol ester is the activation of protein kinase C (1). Molecular mechanism of its activation remains far from clear. Recently, considerable interests have been focused on the events activated at the site of nucleus during signal transduction (2). Prominent among these is the elevation of a transcription factor AP-1 upon phorbol ester treatment of cells (3-5). The exact mechanism of these events is not known at the present moment. However, a positive regulatory role of protein kinase C at the site of nucleus in cellular transcription (6) seems obvious. It is in this background that we have documented (7) that protein kinase C is located in the rat liver nuclei.

The present paper is a logical extension of our recent work (7) and deals with the properties of phorbol dibutyrate binding, and purification to apparent homogeneity of nuclear protein kinase C. Using monospecific antibodies (8) raised against three types of rat brain cytosolic protein kinase C, it is revealed here that the rat liver nuclei mainly contain protein kinase C isozyme II. The monospecific antibodies obtained against type II isozyme identified 80-kDa immunoreactive protein band at each step of purification. In addition, data presented here indicate that phorbol ester-induced signaling pathway operates at the site of isolated nucleus.

**EXPERIMENTAL PROCEDURES**

**Preparation of Rat Liver Nuclei**—Rat liver nuclei were prepared as described earlier (7). The small pieces of liver were homogenized in 8 volumes of a medium containing 1.5 M sucrose, 1.0 mM MgCl₂, and 10 mM potassium phosphate buffer, pH 6.8. The homogenate was filtered through cheesecloth and centrifuged for 15 min at 1,000 x g. The resulting pellet was suspended in a medium containing 2.4 M sucrose, 1.0 mM MgCl₂, and 10 mM potassium phosphate buffer, pH 6.8, and centrifuged for 1 h at 100,000 x g. The nuclear pellet was suspended in an appropriate amount of medium containing 0.25 M sucrose, 0.5 mM MgCl₂, and 20 mM Tris-HCl, pH 7.5, and centrifuged at 1,000 x g for 10 min. The pellet was further suspended in the extraction medium containing 2 mM EDTA, 0.5% Triton X-100, and 20 mM Tris-HCl, pH 7.5. Following sonication as described earlier (7), the suspension was centrifuged for 30 min at 100,000 x g. The resulting supernatant constituted the starting material for purification of nuclear protein kinase C.

**Purification of Nuclear Protein Kinase C** The DEAE-cellulose, phenyl-Superose (FPLC), and Mono Q chromatographic procedures were carried out as described earlier (7). Fractions rich in protein kinase C activity from Mono Q column were pooled, desalted, and subjected to additional purification step on a 11-DEAE-cellulose column (10 x 2 cm) where the protein kinase C activity was eluted with stepwise pH 6.4, 5.5, and 5.0, employing a medium containing 20 mM bis-Tris, 0.5 mM EGTA, 0.5 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol. Subsequently, the column was further eluted with another medium containing 0.5 mM EGTA, 0.5 mM EDTA, 1.0 mM dithiothreitol, 10% glycerol, and 20 mM Hepes, pH 7.5 (buffer A) supplemented with 150 mM NaCl.
Phorbol Dibutyrate Binding—Phorbol dibutyrate binding was performed as recommended (10) in a volume of 250 µl in 1.5 ml of polypropylene Eppendorf microcentrifuge tubes for 30 min at 37°C. Standard binding assay medium contained 50 mM Tris-HCl, pH 7.4, 0.1 mM CaCl₂, 100 µg/ml phosphatidylserine, 1.0 mg/ml bovine γ-globulin, and 20 nM [³²P]PDBu. Nonspecific binding was determined by including 100 µM phorbol ester (TPA) in the binding assay. The samples were chilled in ice for 5 min at the termination of binding and 187 µl of 35% polyethylene glycol (in 50 mM Tris-HCl, pH 7.4) was added. The samples were further incubated for 15 min in ice to precipitate protein. The precipitates were centrifuged for 15 min at 12,000 rpm at 4°C in a Beckman microfuge 12 centrifuge. From the supernatant, a 100-µl aliquot was removed, and its radioactivity was measured to evaluate the concentration of free [³²P]PDBu. The remaining supernatant was removed by aspiration and the tiny fluid covering the precipitate was blotted out. The tip of the Eppendorf microtube tubes (containing precipitate) were cut off and placed in vials to determine total bound [³²P]PDBu.

Protein Kinase C Assay—Protein kinase C activity was determined as described in Ref. 7. The material rich in protein kinase C activity from the Mono Q column was pooled and was desalted on a Trisacryl GF-45 column preequilibrated with buffer A containing 20 mM Hepes, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol. The desalted material was loaded on a II-DEAE-cellulose column (10 × 2 cm) preequilibrated with buffer A. Elution of the column began with bis-Tris buffer (containing 0.5 mM EGTA, 0.5 mM EDTA, 1.0 mM dithiothreitol, 10% glycerol, and 20 mM bis-Tris) adjusted to pH 6.4, 6.0, and 5.5. 0.5 ml fractions were collected. No enzymatic activity was detected in the fractions eluted either with the buffer adjusted to pH 6.4 or 5.5. Once the elution was switched to buffer at pH 5.0, the protein kinase C was eluted. The column was subsequently eluted with buffer A supplemented with 120 mM NaCl and a profile of this elution is shown above. Almost negligible protein kinase C activity was detected in the fractions eluted with NaCl.

These fractions were pooled and subjected to sodium docetyl sulfate-polyacrylamide gel electrophoresis. The silver nitrate staining of this gel revealed a protein band at 66 kDa (Fig. 2, lane b). Fractions, shaded (during pH elution), were pooled and served as a source of purified protein kinase C. This fraction revealed a single band at 50 kDa by silver nitrate staining of SDS-polyacrylamide gel electrophoresis. The silver nitrate staining of this gel revealed a protein band at 66 kDa (Fig. 2, lane b). Fractions, shaded (during pH elution), were pooled and served as a source of purified protein kinase C. This fraction revealed a single band at 50 kDa by silver nitrate staining of SDS-polyacrylamide gel electrophoresis. The silver nitrate staining of this gel revealed a protein band at 66 kDa (Fig. 2, lane b). Fractions, shaded (during pH elution), were pooled and served as a source of purified protein kinase C. This fraction revealed a single band at 50 kDa by silver nitrate staining of SDS-polyacrylamide gel electrophoresis. The silver nitrate staining of this gel revealed a protein band at 66 kDa (Fig. 2, lane b). Fractions, shaded (during pH elution), were pooled and served as a source of purified protein kinase C. This fraction revealed a single band at 50 kDa by silver nitrate staining of SDS-polyacrylamide gel electrophoresis. The silver nitrate staining of this gel revealed a protein band at 66 kDa (Fig. 2, lane b). Fractions, shaded (during pH elution), were pooled and served as a source of purified protein kinase C. This fraction revealed a single band at 50 kDa by silver nitrate staining of SDS-polyacrylamide gel electrophoresis. The silver nitrate staining of this gel revealed a protein band at 66 kDa (Fig. 2, lane b). Fractions, shaded (during pH elution), were pooled and served as a source of purified protein kinase C. This fraction revealed a single band at 50 kDa by silver nitrate staining of SDS-polyacrylamide gel electrophoresis. The silver nitrate staining of this gel revealed a protein band at 66 kDa (Fig. 2, lane b). Fractions, shaded (during pH elution), were pooled and served as a source of purified protein kinase C. This fraction revealed a single band at 50 kDa by silver nitrate staining of SDS-polyacrylamide gel electrophoresis. The silver nitrate staining of this gel revealed a protein band at 66 kDa (Fig. 2, lane b). Fractions, shaded (during pH elution), were pooled and served as a source of purified protein kinase C. This fraction revealed a single band at 50 kDa by silver nitrate staining of SDS-polyacrylamide gel electrophoresis.
Immunoreactive protein at a lower molecular mass was seen in the enzyme material obtained after phenyl-Superose step (Fig. 3B, lanes C). However, this was a nonspecific interaction with immunoglobulin unrelated with protein kinase C. Such nonspecific interactions are usually seen (14) and their nature remains enigmatic.

**Cofactor Requirements of Nuclear Protein Kinase C**—Detailed characteristics of purified (single band at 80 kDa revealed by silver nitrate staining on SDS-polyacrylamide gel electrophoresis) nuclear protein kinase C are listed in Table II. The enzyme requires calcium plus phosphatidylserine for manifestation of its activity. Relatively higher calcium optimum is sustained in the fully purified preparation. The calcium was replaced by phorbol ester and reasonable kinase C activity was manifested, without any added calcium, in the presence of phosphatidylserine plus TPA or diacylglycerol. However, Ca²⁺ + TPA (or diacylglycerol) at various calcium concentrations tested did not give any appreciable enzymatic activity. It did appear that there was a slight shift for optimum calcium requirement in the enzyme derived from the final purification step (II nd DEAE) as compared with the penultimate step (Mono Q, Ref. 7). Likewise, appreciable activation of the enzyme by phorbol ester or diacylglycerol over the basal activity (i.e. the activity monitored with Ca²⁺ + phosphatidylserine) was observed. An activation to such an extent was not seen with contaminating 66-kDa protein (Ref. 7).

**Phorbol Ester Induced Signal at the Level of Isolated Nuclei**—Isolated rat liver nuclei were incubated with phorbol ester (0.1 or 81 nM) and 6-8-fold rise in the nuclear protein kinase C activity was observed (Table III). Under similar incubations with TPA, Buckley et al. (15) have observed a several hundred-fold rise in protein kinase C activity endogenous to rat liver nuclei. In our hands only 8-10-fold activation by TPA could be observed. We have preincubated the isolated rat liver nuclei with 1 mM calcium as carried out by Buckley et al., but even in that condition we have not been able to see more than 10-fold increase in nuclear protein kinase C activity due to phorbol ester-induced signaling.

**DISCUSSION**

Through the use of a high molarity sucrose (1.3 M) in the homogenization medium and maintaining a higher sucrose molarity (2.3 M) in subsequent suspension and centrifugation procedures, we have isolated nuclei from rat liver devoid of...
The pooled fraction after II-DEAE chromatography (i.e. eluted by stepwise pH changes) was subjected to these measurements. Protein kinase C activity was assayed as described under "Experimental Procedures." These values are based on four independent determinations for which standard errors were not more than 10%. Standard assay medium contained in a total volume of 100 μl: 2 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl, pH 7.5, 1.2 mM EGTA, 81 nM TPA, or 100 μM diacylglycerol (DAG), 16.6 μg of phosphatidylserine (PS), 20 μg histone (type III s SIGMA), 20 μM ATP.

**Table II**

**Cofactor requirements of purified nuclear protein kinase C**

| Additions | [Ca\(^{2+}\)] (1.25 mM) | [Ca\(^{2+}\)] (1.75 mM) | [Ca\(^{2+}\)] (2.5 mM) | [Ca\(^{2+}\)] (3.0 mM) | [Ca\(^{2+}\)] (3.5 mM) |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Ca\(^{2+}\) + PS | 0.1 | 0.1 | 0.1 | 0.15 | 0.15 |
| Ca\(^{2+}\) + PS + TPA | 3.0 | 6.0 | 31.0 | 30.0 | 29.5 |
| Ca\(^{2+}\) + PS + DAG | 4.0 | 6.0 | 46.0 | 44.0 | 44.0 |
| Ca\(^{2+}\) + TPA | 5.0 | 6.0 | 47.5 | 42.8 | 43.0 |
| Ca\(^{2+}\) + DAG | 5.4 | 5.2 | 3.5 | 2.9 | 3.4 |
| PS | 6.8 | 3.5 | 2.5 | 3.6 |
| PS + TPA | 28.5 |
| PS + DAG | 30.4 |

**Table III**

**Activation of nuclear protein kinase C when isolated nuclei were incubated with TPA for 3 min**

Isolated nuclei suspended in a medium containing 0.25 mM sucrose, 20 mM Tris-HCl, pH 7.5, 1 mM MgCl\(_2\) were incubated in 20-ml Erlenmeyer flasks (each set of experiments in quadruplicate) with TPA at 37 °C for 3 min. At the termination of incubation each nuclear fraction was centrifuged at 1000 × g for 15 min. The resulting pellet was suspended in a medium containing 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, and 0.5% Triton X-100. It was sonicated six times for 10 s each with a 1-min interval in between two sonications and centrifuged at 100,000 × g for 30 min. The supernatant served as the source of protein kinase C. The details of protein kinase C assay are described under "Experimental Procedures," except that the assay medium was supplemented with 100 μM diacylglycerol as recommended (11) due to the presence of more than 0.02% Triton X-100.

| Additions | Protein kinase C activity |
|-----------|-----------------|
| Control | 4.0 ± 1.4 |
| 0.1 nM TPA | 25.96 ± 2.2 |
| 60 nM TPA | 33.57 ± 2.4 |

Cytoplasmic contaminants. The protein kinase C activity was found in the nuclei and was partially purified (7).

One of the major thrusts of this paper has been to get rid of the 66-kDa protein band from the protein kinase C. This has been successfully met with employing a second DEAE-cellulose column as a final step. The protein kinase C activity from this column was eluted as a function of pH. The use of classical 120 mM NaCl in Heps buffer, pH 7.5, did elute the 66-kDa protein band from the DEAE column which was not eluted during pH elution (Fig. 1). Considerable speculation has been attached to the 66 (or 67 kDa) protein fragment usually seen in various kinase C preparations. According to a section of opinion 66-kDa protein is a proteolytic product of kinase C having lost its calcium-dependent kinase activity. However, it is clearly demonstrated in this paper that 66-kDa protein is a major contaminant, is devoid of kinase activity (calcium dependent or independent), and is not recognized by protein kinase C antibodies. In fact Parker et al. (16) have documented that the cytosolic protein kinase C has a 67-kDa protein as a major contaminant. It is indeed surprising that Azhar et al. (17) have attributed protein kinase C activity in the liver to a 64-kDa protein band and have failed to see the 80-kDa protein band in their studies on rat liver enzyme.

A perusal of the purification protocol followed in this paper reveals (Table I) that protein kinase C enzymatic activity as well as the phorbol dibutyrate binding parameter ran in parallel at each purification step in terms of percentage of recovery or fold of purification. Furthermore, the ratio between protein kinase C activity over PDBu bound remained identical throughout the purification procedures. This provides strong evidence that the enzyme, under purification, qualified as the receptor of phorbol ester.

Considering cofactor characteristics of the purified nuclear protein kinase C (Table II) one may argue that they are not dissimilar to the partially purified enzyme (7) except that (i) the optimum calcium requirement tends to shift to a lower calcium concentration (2.5 mM instead of 3.5 mM), (ii) the kinase activity elicited (in the absence of added calcium) in the presence of phosphatidylserine + TPA (or diacylglycerol) reached almost identical to the value attained with Ca\(^{2+}\) + phosphatidylserine. This clearly shows that in the case of nuclear protein kinase C, phorbol ester or diacylglycerol could replace calcium. Thus, the elimination of 66-kDa protein band seems to improve the enzymatic characteristics of the nuclear protein kinase C. Nevertheless, one most important cofactor for nuclear enzyme remains the phosphatidylserine. In the absence of phosphatidylserine, enzymatic activity was not detected irrespective of the presence of calcium, or phorbol ester, or their combination.

The second major thrust of this paper is that the rat liver nuclear protein kinase C is the type II isozyme (Fig. 3). The isozyme I antibodies or isozyme III antibodies showed minor immunoreactive protein at some of the steps of purification (Fig. 3, A and C). Distinctly enough isozyme II antibodies revealed a major immunoreactive protein band at 80 kDa (Fig. 3B) at each step of purification starting from nuclear extract up to the final step, i.e. IIId DEAE-cellulose chromatography. It may be recalled that in the rat liver only type II and III protein kinase C isozymes are found. In the cerebellum cells nuclei (18) isozyme type II and type I have been observed, whereas the brain contains all the three types (I, II, and III) of protein kinase C isozymes. These observations do lead to the notion that isozyme II may be responsible for the function of protein kinase C at the site of cell nuclei. It is becoming understandable that each isozyme subserves a different function at various locations within a cell since for the three types the activation kinetics upon stimulation are reported different (19).
Recent studies in rat liver have shown that the protein kinase C endogenous to the nuclei is involved in the signaling pathway initiated by prolactin (20) at the site of the nucleus. Buckley et al. (15) have also shown that there occurs a several hundredfold rise in nuclear protein kinase C activity when isolated rat liver nuclei were incubated with phorbol ester. However, in our hands only 8-fold rise in nuclear protein kinase C activity were seen (Table III) when isolated intact rat liver nuclei were incubated with 0.1 or 81 nM phorbol ester. Irrespective of the discrepancy between our results and that of Buckley et al. (15), it is indeed interesting to be able to initiate a signal by phorbol ester at the site of the nucleus and sustain the nuclear protein kinase C activation. Such an activation provides a tool to address the question of nuclear events associated during signal transduction. The nuclear membrane may not be considered as a wide open frontier passively containing chromatin. It is in fact a hermetic structure with a high selectivity for the molecules passing through it (21). In this context the ATP-stimulated calcium transporting system recently identified in rat liver nuclei is worth citing (22).

The localization in the nucleus of a particular isozyme type of protein kinase C raises questions concerning its role. Based on published observations (6, 23), one obvious role of this enzyme seems to mediate inducible gene expression. The mechanism by which nuclear protein kinase C intervenes the regulation of gene transcription (26) remains to be elucidated.

Acknowledgment—The secretarial assistance of S. Ott is gratefully acknowledged.

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