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CONVERSION OF PROTEIN KINASE C FROM A Ca^{2+}-DEPENDENT TO AN INDEPENDENT FORM OF PHORBOL ESTER-BINDING PROTEIN BY DIGESTION WITH TRYPSIN

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Summary: Tryptic fragments of protein kinase C containing the kinase(45 KDa) and phorbol ester-binding activity(38 KDa) were separated by Mono Q column chromatography. The purified phorbol ester-binding fragment exhibits a higher affinity for phosphatidylserine than the native enzyme but comparable K_d for [^{3}H]phorbol 12,13-dibutyrate as the native enzyme. This proteolytic fragment binds phorbol ester equally efficient either in the presence or absence of Ca^{2+} and the addition of the kinase fragment did not restore the Ca^{2+}-requirement for the binding. These results indicate that protein kinase C is composed of two functionally distinct units which can be expressed independently after limited proteolysis with trypsin.

Protein kinase C has been implicated in the transmembrane signalling to regulate many cellular functions (1, 2). This kinase was first described by Nishizuka and co-workers as a proteolytically activated enzyme (3, 4). The native enzyme was subsequently identified as a Ca^{2+}-activated and phospholipid-dependent protein kinase (5, 6) which can be further activated by diacylglycerol (7) generated from the hydrolysis of inositol phospholipids by phospholipase C. Protein kinase C has also been identified as a receptor for the tumor-promoting phorbol esters (8, 9). These compounds activate the kinase in an analogous fashion as diacylglycerol in reducing the K_a for Ca^{2+}. Both tumor-promoting phorbol esters and synthetic diacylglycerols have been used to stimulate cellular responses by a process thought to involve translocation of protein kinase C from cytosol to membrane (10, 11). This activation process frequently results in a subsequent degradation of this kinase by endogenous proteases (12, 13). In vitro, protein kinase C can be degraded by Ca^{2+}-dependent proteases or trypsin to generate a Ca^{2+}/phospholipid-independent kinase designated M kinase (14). The phorbol ester receptor function of the protease-degraded kinase has not been investigated.

Abbreviations: PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate, 13-acetate; PS, phosphatidylserine; DG, 1,2-diolein; DTT,dithiothreitol; EGTA, ethyleneglycol bis(β-aminoethylether) N,N,N',N'-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; and PEBF, phorbol ester-binding fragment.

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In this study we have made an attempt to answer the question concerning the consequence of protein kinase C degradation on the binding of phorbol ester. Our results demonstrate that the native protein kinase C can be degraded by trypsin to generate two functionally independent units, namely, a protein kinase and a phorbol ester-binding protein. These two units can be separated by ion exchange column chromatography on Mono Q. The purified phorbol ester-binding protein binds [^{3}H]phorbol 12,13-dibutyrate(PDBU) independently of Ca^{2+} and has a higher affinity for phosphatidylserine(PS) than the native enzyme.

MATERIALS AND METHODS: The following materials were obtained from the indicated sources: histone IIIS, EGTA, 4 α-phorbol, PDBu, and phorbol 12-myristate, 13-acetate(PMA) from Sigma; [γ-^{32}P]ATP and [^{3}H]PDBu from New England Nuclear; TPCK-treated trypsin from Worthington; PS and 1,2-diolein(DG) from Avanti Polar Lipids; and DEAE-cellulose(DE52) and GF/C glass microfibre filter from Whatman.

Rat brain protein kinase C was purified and assayed by modified procedures (15) of Kikkawa et al. (16). Measurement of ^{32}P-incorporation into histone IIIS was performed as previously described (17). Binding of [^{3}H]PDBu was measured in 0.2 ml reaction mixture containing 30 mM Tris-Cl buffer (pH 7.5), 6 mM magnesium acetate, 0.5 mg/ml of bovine serum albumin, 20 μg/ml of PS, 0.25 mM EGTA, 0.5 mM $CaCl_2$, 50 nM of [^{3}H]PDBu, and 0.3-0.4 μg of protein kinase C in 10 μl of Buffer A (20 mM Tris-Cl buffer, pH 7.5, containing 1 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, 10% glycerol, 10% ethyleneglycol, and 0.2% NP-40). The reaction mixture was incubated at room temperature for 30 min and followed by incubation at 4° for 30 min after adding 0.5 ml of 30% DEAE-cellulose in 20 mM Tris-Cl buffer, pH 7.5. Bound [^{3}H]PDBu was separated from free ligand by filtering through Whatman GF/C glass fibre filter and washed 5 times with 1 ml of 20 mM Tris-Cl buffer, pH 7.5. Measurement of [^{3}H]PDBu binding was carried out in duplicate and the non-specific binding was determined under the same condition with the addition of 100 μM of non-radioactive PDBu. The ligand binding parameters (18) and the concentration of free Ca^{2+} (19) were estimated by using established computer programs. Tryptic digestion was carried out at 30° for 8 min in 0.3 ml of reaction mixture containing 20 mM Tris-Cl buffer (pH 7.5), 1 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, 10% glycerol, 0.6 mg/ml of protein kinase C, and 7.5 μg/ml of trypsin. Tryptic digestion was terminated by the addition of 120 μg of soybean trypsin inhibitor. The proteolytic fragments were purified with a Mono Q column using a linear salt gradient (0-0.7 M KCl) in Buffer A at a flow rate of 0.5 ml/min. Protein concentrations were determined by the method of Bradford (20) using bovine plasma albumin as standard. SDS-PAGE was carried out according to the method of Laemmli (21).

RESULTS

Tryptic digestion of protein kinase C results in a conversion of the Ca^{2+}/phospholipid-dependent to an independent form of the kinase and an increase in the Ca^{2+}-independent [^{3}H]PDBu binding (Fig. 1). Upon prolonged incubation with trypsin, the total phorbol ester binding in the presence of Ca^{2+} was reduced by 20%. The binding activity in the absence of Ca^{2+}, however, became comparable to that in the presence of Ca^{2+}, indicating the formation of a Ca^{2+}-independent phorbol ester-binding protein. In contrast to a reduction in the [^{3}H]PDBu binding by proteolysis, the total kinase activity was increased by 40%. It seems likely that the kinase fragment becomes a better catalyst

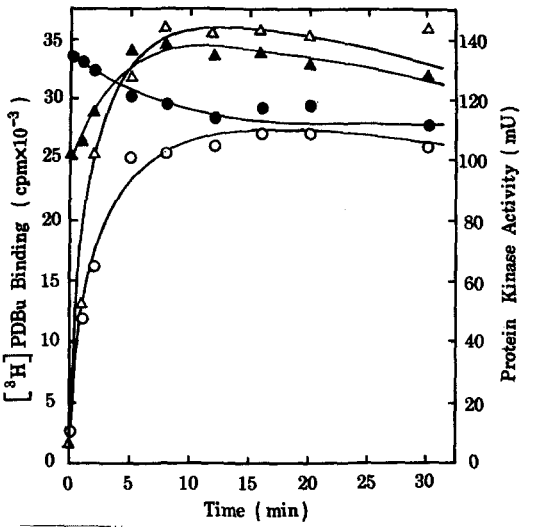

Fig. 1. Changes in the kinase activity and binding of [^{3}H]PDBu resulting from limited proteolysis of protein kinase C by trypsin. Protein kinase C (60 ug/ml) was incubated with trypsin (0.75 µg/ml) at 30°. At time intervals, samples were taken and the reaction was terminated by the addition of 10 µg of soybean trypsin inhibitor to 25 µl of the reaction mixture. These samples were used for the measurements of protein kinase activities in the presence of 0.4mM Ca^{2+}, 20 µg/ml PS, and 4 µg/ml DG (-▲-) or in the presence of 1 mM EGTA without Ca^{2+} and lipid (-△-), and the binding of [^{3}H]PDBu in the presence of PS with Ca^{2+} (-●-) or with 5 mM EGTA (-○-).

than the native enzyme which is only active when in association with the water-insoluble lipid vesicles.

In order to determine the proteolytic fragments carrying the kinase and phorbol ester-binding activities, we submitted the proteolytic digest to chromatography on Mono Q column (see Methods). A phorbol ester-binding and a Ca^{2+}/phospholipid-independent protein kinase activity peaks were detected at 0.2 M and 0.4 M KCl, respectively. The phorbol ester-binding fragment(PEBF), contained no protein kinase activity and the kinase fraction was free of phorbol ester-binding activity. SDS-PAGE (Fig. 2) shows that the native enzyme (82 KDa) (Fig. 2, lane 1) was degraded by trypsin into two major fragments of 45 KDa and 38 KDa (Fig. 2, lane 2). The purified phorbol ester-binding protein is the 38-KDa fragment (Fig. 2, lane 3) and the kinase is the 45-KDa fragment (Fig. 2, lane 4).

Both the native protein kinase C and its proteolytic fragment require PS for [^{3}H]PDBu binding (Fig. 3). The concentration of PS required for half-maximal binding by the isolated 38-KDa PEBF is 0.4 µg/ml, which is approximately 3-fold lower than that for the native enzyme (1.25 µg/ml). The PS requirement for the degraded enzyme containing both the 45- and 38-KDa fragments is similar to that for the 38-KDa fragment alone. These results indicate that the kinase fragment(45 KDa) does not influence the affinity of PEBF for PS. Similarly, addition of increasing amount of PEBF to the kinase fragment did not restore

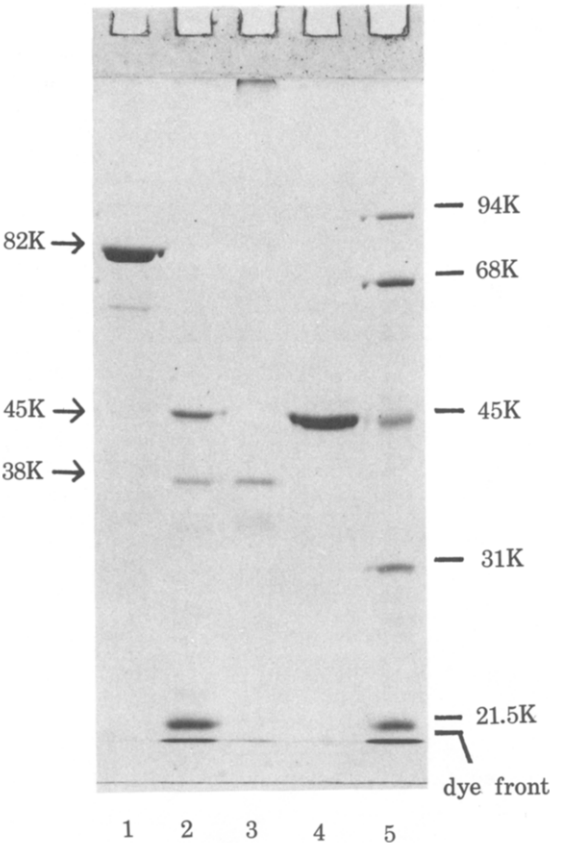

Fig. 2. SDS-PAGE of the purified proteolytic fragments containing the kinase and phorbol ester-binding activity. Lane 1, native protein kinase C; lane 2, trypsin-degraded protein kinase C containing both 45- and 38-KDa fragments and soybean trypsin inhibitor (21.5 KDa); lane 3, PEBF purified from Mono Q column; lane 4, kinase fragment purified from Mono Q column; and lane 5, standard marker proteins.

the Ca^{2+}/PS-requirement for the kinase (data not shown). Binding of [^{3}H]PDBu by both the native enzyme and PEBF follows a saturation kinetics (Fig. 4A). The binding of [^{3}H]PDBu by these proteins was reduced by tumor-promoting phorbol ester, PMA, but not affected by the inactive analog, 4α-phorbol. Scatchard plot analysis of the binding of [^{3}H]PDBu by protein kinase C and PEBF shows that the slopes of these two curves are similar (Fig. 4B). The K_d of PDBu for the native enzyme and PEBF are 12.6 ± 2.8 and 11.7 ± 2.8 nM, respectively. The native protein kinase C differs most significantly from PEBF in the requirement of Ca^{2+} for [^{3}H]PDBu binding. In the presence of EGTA, the binding of phorbol ester by the native enzyme is very low (Fig. 5); the concentration of Ca^{2+} required for half-maximal phorbol ester binding is approximately 0.6 uM. In contrast, neither PEBF alone nor PEBF and the kinase fragment together display

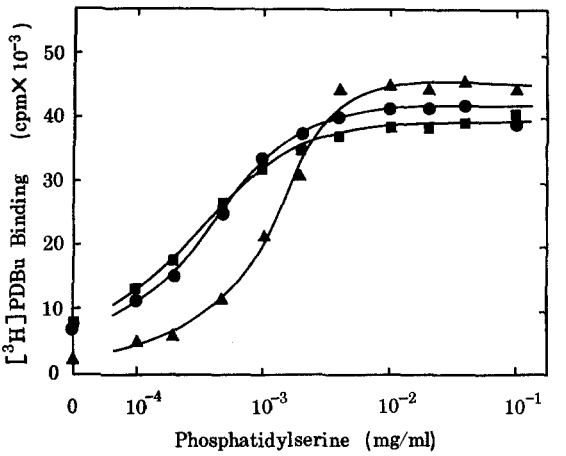

Fig. 3. Requirement of PS for the binding of [^{3}H]PDBu by the native and degraded protein kinase C. Measurement of [^{3}H]PDBu binding was carried out as described in "Method" with variable concentrations of PS from 0.1 to 100 ug/ml. Symbols are: -▲-, native protein kinase C; -●-, purified PEBF; and -■-, tryptic digest of protein kinase C containing both the kinase fragment and PEBF.

a requirement of Ca^{2+} for [^{3}H]PDBu binding. These results indicate that the Ca^{2+}-requirement for phorbol ester binding requires an intact conformation.

DISCUSSION

The results presented in this report demonstrate that tryptic degradation of rat brain protein kinase C generates a Ca^{2+}/phospholipid-independent protein kinase and a Ca^{2+}-independent/phospholipid-dependent phor-

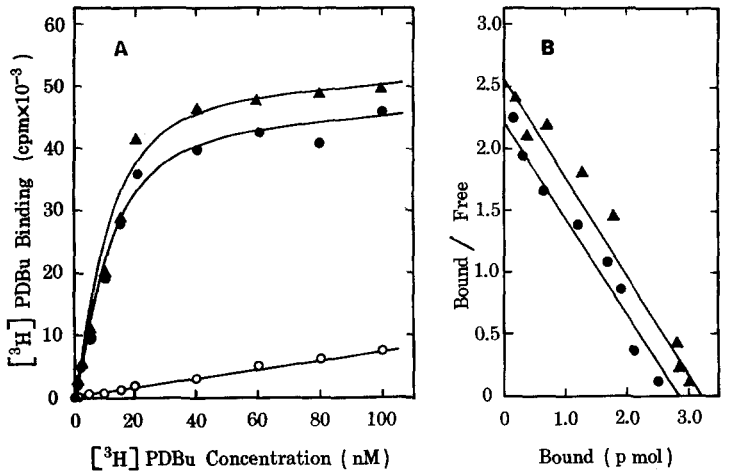

Fig. 4. Scatchard plot analysis of [^{3}H]PDBu binding by the native protein kinase C and PEBF. Panel A shows the [^{3}H]PDBu binding saturation curves for the native enzyme (-▲-) and PEBF (-●-) and the non-specific binding (-o-) with increasing PDBu concentration. Panel B shows the Scatchard plot of [^{3}H]PDBu binding by the native enzyme (-▲-) and PEBF (-●-). The kinetic parameters were obtained by computer analysis (18).

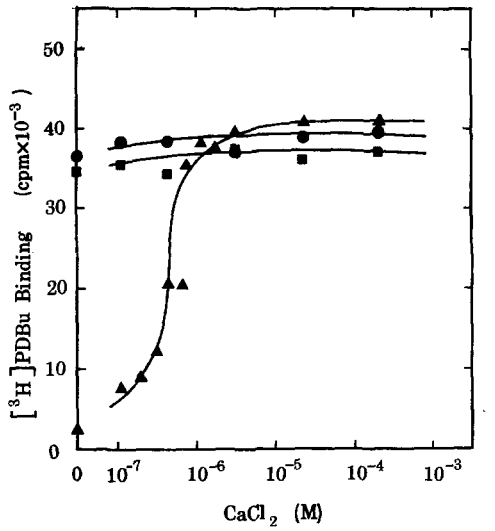

Fig. 5. Requirement of Ca^{2+} for the binding of [3H]PDBu by the native and degraded protein kinase C. Binding of [3H]PDBu by the native enzyme (-▲-), PEBF (-●-), and the tryptic digest of protein kinase C containing both the kinase fragment and PEBF (-■-) was carried out under standard assay condition with increasing concentration of $CaCl_2$. The concentration of free Ca^{2+} was obtained by computer analysis (19).

bol ester-binding protein. The resulting protein kinase, a 45-KDa fragment, has been characterized previously (3, 4). We have identified, for the first time, the residual fragment of 38 KDa as a phorbol ester-binding protein. The binding of [3H]PDBu by this fragment, PEBF, requires less PS than the native enzyme for half-maximal activity. This result can be interpreted that proteolytic degradation of protein kinase C results in an enhanced phorbol ester binding by PEBF at sub-optimal concentration of PS. The increase in the affinity of PEBF for PS may result from cleavage of a hydrophilic domain from the native enzyme. The hydrophilic domain, which contains the kinase, may hinder the binding of native protein kinase C to a hydrophobic environment, such as lipid vesicles. The kinase-containing domain can be considered as a regulatory unit for PEBF in an analogous fashion as PEBF for the kinase.

Tumor-promoting phorbol esters have been shown to induce a number of biological responses (22, 23). It has been proposed that these compounds may induce their effects by intercalating into the cell membrane to activate protein kinase C, replacing the transient natural activator, diacylglycerol (1, 24). Protein kinase C bound to the cell membrane induced by either tumor-promoting phorbol esters or diacylglycerol may serve as a signal transducer by phoshorylating endogenous membrane proteins (1). The association of protein kinase C to the membrane also triggers a rapid degradation of this enzyme (12, 13). Because proteolytic degradation of protein kinase C also activates the phorbol ester binding by increasing the affinity of PEBF for PS and eradicating

the requirement for Ca^{2+}, we speculate that PEBF may also participate in the signal transduction. Investigation of the functional role of the PEBF should lead to a better understanding of the action of tumor-promoting phorbol esters.

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