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Phorbol diester receptor copurifies with protein kinase C

(carcinogenesis/tumor promotion)

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ABSTRACT **The phorbol diester receptor present in the particulate fraction of rat brain was solubilized by divalent ion chelation in the absence of detergents. The soluble receptor was partially purified by $(NH_4)_2SO_4$ precipitation, DEAE-cellulose, and gel filtration chromatography. The purified receptor required exogenous phospholipid for activity and displayed a K_d of 7 nM for [^{3}H]phorbol 12,13-dibutyrate. Biologically active phorbol analogs inhibited binding, whereas inactive analogs did not. The Ca^{2+}-dependent, phospholipid-sensitive protein kinase C copurified with the phorbol receptor. Purified protein kinase C was activated directly by phorbol 12-myristate 13-acetate in the presence of phospholipid.**

The tumor-promoting phorbol diesters are agents that are noncarcinogenic but will induce tumor formation when administered after subthreshold doses of a carcinogen (1). In an attempt to understand the mechanism of tumor promotion, the phorbol diesters have been studied in many systems and have been shown to induce a myriad of *in vitro* functional and biochemical changes. These changes, which may be related to cocarcinogen activity, include morphological transformation (2), enhanced hexose transport (3), enhanced prostaglandin synthesis (4), altered phospholipid and protein synthesis (5, 6), loss of fibronectin (7), increased ornithine decarboxylase activity and polyamine production (8, 9), and altered rates of DNA synthesis (10–13). The phorbols also induce secretion (14–15), superoxide production (16), and alteration of surface receptors (17–19) and influence differentiation of cells *in vitro;* most notably, all human myeloid leukemia cells can be induced to differentiate into macrophage-like cells (20–22). For induction of many of these responses, similar structure–function relationships were shown for a series of phorbol diester analogs, suggesting that each of these diverse responses was mediated via a common pathway. Further support for a common pathway has come from studies demonstrating high affinity, saturable, stereospecific receptors that mediate the activity of the phorbol diesters in many different cell types (23–28). Although the binding properties of these receptors have been characterized extensively, their biochemical structure or possible mechanism of transduction is unknown.

Recently, Castagna *et al.* were able to show direct activation of partially purified protein kinase C by active phorbol diester analogs (29). Based on these observations, they put forth the exciting hypothesis that protein kinase C may be a membrane target of the phorbol diesters. In this manuscript we present data that extends this hypothesis. The phorbol diester receptor of rat brain membranes, solubilized by divalent ion chelation in the absence of detergents, copurifies with protein kinase C. In the latter steps of purification, both activities require added phospholipid.

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MATERIALS

Sprague–Dawley female weanling rats were a gift from P. M. Conn; Sephacryl S-200 was from Pharmacia (Uppsala, Sweden); DE 52 was from Whatman; AcA 44 Ultrogel was supplied by LKB; [γ-^{32}P]ATP was from ICN; HA filters were from Millipore; Aquasol scintillation fluid was from New England Nuclear; and histone III-S, bovine serum albumin fraction V, diolein, and phosphatidylserine were from Sigma. Diolein and phosphatidylserine were diluted in chloroform to 50 mg/ml and 10 mg/ml, respectively. The chloroform was evaporated from an aliquot and each compound was resuspended with sonication to 500 μg/ml in deionized water. [^{3}H]Phorbol 12,13-dibutyrate (PDB) (17.5 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from New England Nuclear. The stock solution was diluted to 1 μM in 20 nM Tris·HCl, pH 7.5/0.01% Pentex bovine serum albumin from Miles. Unlabeled PDB, phorbol 12,13-didecanoate, 4α-phorbol didecanoate, and 4α-phorbol were from P-L Biochemicals, and phorbol 12-myristate and phorbol 13-acetate were from L. C. Services (Woburn, MA) (all were gifts from J. B. Weinberg).

METHODS

Partial Purification of Phorbol Diester Receptor and Protein Kinase C. Fresh rat brains were homogenized for 2 min in a commercial blender in 3 vol of 20 mM Tris·HCl, pH 7.5/50 mM 2-mercaptoethanol/1 mM $CaCl_2$/2 mM phenylmethylsulfonyl fluoride/0.01% leupeptin. Debris was removed by centrifugation at $1,000 \times g$ for 20 min and the resulting supernatant was centrifuged at $100,000 \times g$ for 1 hr. The pellet was suspended and sonicated briefly in extraction buffer containing 20 mM Tris·HCl (pH 7.5), 5 mM EGTA, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 0.01% leupeptin, gently stirred for 1 hr, and centrifuged at $100,000 \times g$ for 1 hr. $(NH_4)_2SO_4$ was added to the supernatant to 21% (wt/vol) and the resulting suspension was centrifuged at $37,000 \times g$ for 15 min. $(NH_4)_2SO_4$ was again added to the supernatant to achieve a final concentration of 45% (wt/vol); the mixture was centrifuged and the resulting pellet was suspended in 20 mM Tris·HCl, pH 7.5/50 mM 2-mercaptoethanol/2 mM EGTA/2 mM EDTA.

After dialysis against 20 mM Tris·HCl, pH 7.5/50 mM 2-mercaptoethanol, 2 ml of the 21–45% $(NH_4)_2SO_4$ fraction was passed through a 1.0 × 90 cm Sephacryl S-200 column equilibrated with the same buffer. Blue dextran and bovine serum albumin were run separately as markers. Another 2-ml aliquot of the 21–45% $(NH_4)_2SO_4$ fraction was loaded on a 1.0 × 5.0 cm DE 52 column equilibrated with the Tris/mercaptoethanol buffer. The column was washed with 10 ml of equilibration buffer and then a 60-ml linear gradient of 0–0.6 M NaCl in equilibration buffer was applied. All procedures were performed at 4°C.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; PDB, phorbol 12,13-dibutyrate.

Table 1. Distribution of phorbol receptor

Homogenization conditions	Distribution in homogenate	
	Cytosol	Particulate
$CaCl_2$	2.6 (34%)	5.1 (66%)
EGTA/EDTA	6.8 (84%)	1.3 (16%)

Rat brains (10 g wet weight) were homogenized in 30 ml of either 20 mM Tris·HCl, pH 7.5/5 mM EGTA/2 mM EDTA/0.01% leupeptin/1 mM phenylmethylsulfonyl fluoride or the same buffer with 1 mM $CaCl_2$ replacing the EGTA/EDTA. Debris was removed at 2,500 × *g* for 10 min, the supernatant was centrifuged at 100,000 × *g* for 1 hr, and the pellet was collected and suspended in a volume of 20 mM Tris·HCl (pH 7.5) equal to the cytosol volume. Fifty-microliter aliquots were assayed in duplicate for receptor by using the gel filtration assay as described. Specific binding is reported as fmol of [^{3}H]PDB bound per 50-μl aliquot. The percentage of total receptor activity in each fraction is shown in parentheses. The total volumes of all fractions were equal (≈30 ml).

Soluble Phorbol Diester Receptor Assay. For the soluble receptor assay, 50 μl of receptor preparation was added to a 150-μl reaction mixture containing 10 pmol of [^{3}H]PDB (50 nM) in 20 mM Tris·HCl, pH 7.5/1.6 mM $CaCl_2$/phosphatidylserine at 20 μg/ml. This mixture was incubated at 22°C for 15 min and receptor-bound and free [^{3}H]PDB were separated by rapid gel filtration at 4°C through a 1.8-ml AcA 44 column equilibrated with 20 mM Tris·HCl at pH 7.5. The 200-μl incubation mixture was applied to the column and then was washed sequentially with 1.0 ml and 2.0 ml of 20 mM Tris·HCl at pH 7.5. The first 1.2 ml was collected in about 2 min and contained the receptor-bound [^{3}H]PDB. Nonsaturable binding was assessed in the presence of a 100-fold excess (5 μM) of nonradioactive PDB. Total binding and nonsaturable binding were done in duplicate and the results are reported as saturable binding (total minus nonsaturable binding). Radioactivity was measured in 15 ml of Aquasol in a Beckman LS6800 scintillation counter with efficiencies between 38% and 42%.

Protein Kinase C Assay. Protein kinase C activity was quantitated by measuring incorporation of ^{32}P from [γ-^{32}P]ATP into histone type III-S, as modified from Castagna *et al.* and Wise *et al.* (29, 30). The reaction was carried out for 10 min at 30°C; protein was precipitated by 25% trichloroacetic acid after the addition of albumin at 500 μg per assay and the precipitate was collected and washed on Millipore HA filters (0.45-μm pore). The filters were dried, suspended in 10 ml of Aquasol, and ^{32}P was counted in a Beckman LS6800 scintillation counter.

Table 2. Fractionation of protein kinase C and phorbol receptor

Step	Total kinase activity		Protein kinase C	Phorbol receptor
	With EGTA	With Ca^{2+} and lipids		
EGTA/EDTA extract	22,950	24,540	1,600	3.0
$(NH_4)_2SO_4$ pellet				
0–21%	20,960	18,450	−2,500	0.7
21–45%	9,700	26,200	16,500	2.2
45% supernatant	1,540	1,940	400	<0.1

Protein kinase activity is reported as ^{32}P cpm incorporated into the acceptor protein per 50-μl aliquot assayed in the presence of EGTA or Ca^{2+} and phosphatidylserine as described. Phorbol receptor was assayed as described and is reported as fmol of [^{3}H]PDB specifically bound per 50 μl. The volumes of the four fractions were kept constant at ≈30 ml so the results are directly comparable.

RESULTS

Extraction of Phorbol Diester Receptors. Based on the assumption that protein kinase C may bind to membranes in a Ca^{2+}-dependent manner, we prepared crude membrane fractions from rat brain in the presence of Ca^{2+} or divalent ion chelators. These preparations then were assayed for soluble and membrane-bound phorbol diester receptors. As seen in Table 1, with both methods of preparation, receptors were distributed between the cytosolic and particulate fractions. However, with homogenization in the presence of chelators, only 16% of the total receptors were membrane-associated, whereas 66% were membrane-associated when brain homogenization was done in a Ca^{2+}-containing buffer. These latter membrane-associated receptors (Ca^{2+}-prepared membranes) then were extracted with the EGTA/EDTA buffer. Phorbol binding in the 100,000 × *g* supernatant was 3.0 fmol of [^{3}H]PDB per 50 μl versus 1.3 fmol per 50 μl of a suspension of the pellet, demonstrating that ≈60% of the receptors in the Ca^{2+}-prepared membranes had been solubilized by the EGTA/EDTA treatment. Preparation of membranes in the presence of Ca^{2+}, with subsequent receptor extraction with chelators, was chosen as the preferred method for obtaining soluble phorbol diester receptors.

Similar studies of the distribution of protein kinase C were not possible. Although protein kinase activity was extracted by these procedures, stimulation of activity by Ca^{2+} and lipids was dependent on further purification of the kinase (Table 2). Significant kinase activity was present in the extract, but only a 10% increase was evident in the presence of added lipids and Ca^{2+}.

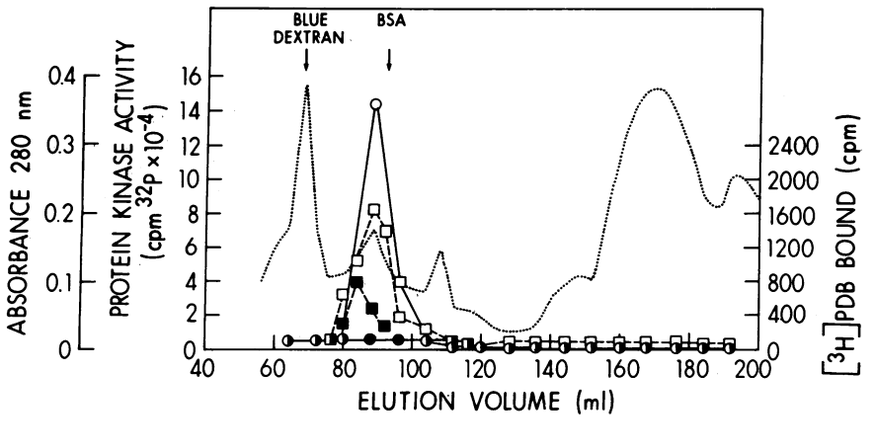

FIG. 1. Sephacryl S-200 chromatography. The column was equilibrated and run as described in *Methods*. Protein kinase activity was determined in the presence of 1 mM EGTA (●——●) or 1 mM Ca^{2+} and phosphatidylserine at 20 μg/ml (○——○). [^{3}H]PDB binding activity also was assessed in the presence of 1 mM EGTA (■– – –■) or 1 mM Ca^{2+} and phosphatidylserine at 20 μg/ml (□– – –□). The vertical arrows denote the elution positions of blue dextran and bovine serum albumin (BSA). Absorbance at 280 nm (·····).

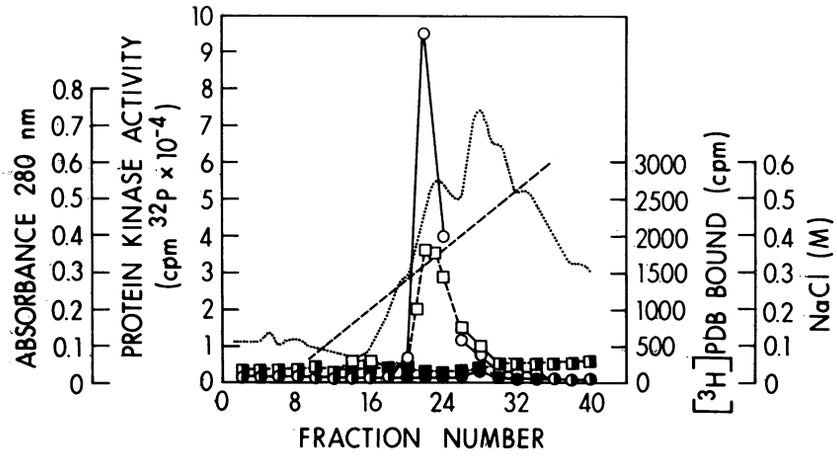

FIG. 2. DEAE-Cellulose chromatography. The DE 52 column was equilibrated and eluted as described in *Methods*. Protein kinase activity was determined in the presence of 1 mM EGTA (●——●) or 1 mM Ca^{2+} and phosphatidylserine at 20 μg/ml (○——○). [^{3}H]PDB binding activity also was assessed in the presence of 1 mM EGTA (■---■) or 1 mM Ca^{2+} and phosphatidylserine at 20 μg/ml (□---□). Absorbance at 280 nm (·····). NaCl gradient (---).

Most of this Ca^{2+}- and lipid-independent kinase was precipitated by 21% $(NH_4)_2SO_4$, whereas the Ca^{2+}- and lipid-stimulated kinase was found in the 21–45% $(NH_4)_2SO_4$ pellet. An absolute increase in total kinase and protein kinase C was apparent during this fractionation procedure, suggesting that endogenous inhibitors were being removed. This is in agreement with the observations of others (30). Phorbol binding activity also was fractionated by these methods; 75% of the receptors in the extract were found in the 21–45% pellet.

Partial Purification of Protein Kinase C and Phorbol Diester Receptors. Aliquots of the 21–45% $(NH_4)_2SO_4$ fraction were further purified by DEAE-cellulose and gel filtration chromatography. The Sephacryl S-200 elution profile is shown in Fig. 1. The phorbol receptor and kinase C coeluted in a single, symmetrical peak, slightly ahead of bovine serum albumin. At this stage of purification, kinase activity was absolutely dependent on added lipid and Ca^{2+}. Phorbol binding also was stimulated 3- to 4-fold by the addition of lipid and Ca^{2+}.

The DEAE elution profile is shown in Fig. 2. Again, the phorbol receptor and protein kinase C coeluted as a single peak and both activities required exogenous lipids and Ca^{2+}. We have run variations of this column four times with similar results. In one experiment in which the column was overloaded, the ratios of activities in the breakthrough and retained peaks were identical.

Characteristics of Partially Purified Phorbol Diester Receptors. An assay for the soluble phorbol receptor was developed, based on rapid separation of receptor-bound from free [^{3}H]PDB by using a series of 1.8-ml AcA 44 columns (Fig. 3A). The first 1.2 ml of elution volume was collected and counted for receptor-bound [^{3}H]PDB, whereas the next 2 ml contained the free [^{3}H]PDB. Nonsaturable binding in the presence of 5 μM nonradioactive PDB was surprisingly low. The phospholipid-containing incubation buffer did not cause elution of

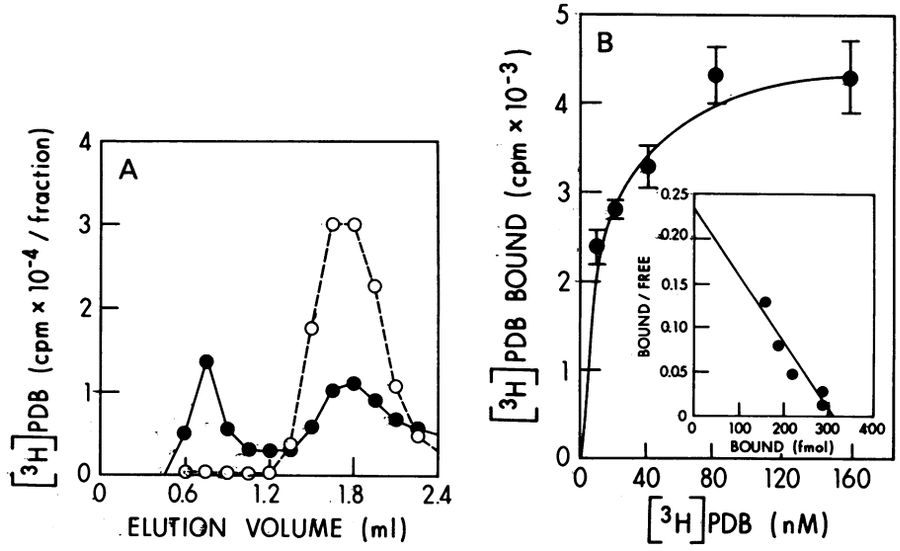

FIG. 3. (*A*) Separation of receptor-bound and free [^{3}H]PDB by gel filtration. The pooled fractions from DEAE were incubated with 50 nM [^{3}H]PDB in the absence (●——●) or presence (○---○) of 5 μM nonradioactive PDB and were fractionated on a 1.8-ml AcA 44 column as described. Each 150 μl was assayed for total [^{3}H]PDB. (*B*) Binding of [^{3}H]PDB as a function of increasing concentration. The assay conditions were as described with the indicated concentrations of [^{3}H]PDB. Data points were taken in duplicate and the data represent the mean ± SEM for two separate experiments. (*Inset*) Scatchard analysis of these data.

Table 3. Specificity of [^{3}H]PDB binding

Compound	Concentration	% inhibition
PMA	1 nM	29
	10 nM	96
	100 nM	100
Phorbol 12,13-didecanoate	100 nM	70
4α-Phorbol 12,13-didecanoate	100 nM	0
4α-Phorbol	100 nM	0
Phorbol 12-myristate	100 nM	50
Phorbol 13-acetate	100 nM	0
Diolein	1 μg/ml	11
	10 μg/ml	38

To the standard soluble receptor incubation mixture described in *Methods* was added the indicated concentration of phorbol analog or diolein.

[^{3}H]PDB in the void volume in the absence of added receptor. By using this technique, a saturation curve for binding of [^{3}H]PDB to the pooled fractions of DEAE was performed (Fig. 3*B*). Saturation was evident above 100 nM, with ≈50% of the sites occupied at 10 nM [^{3}H]PDB. Scatchard analysis of these data suggests a curvilinear relationship. However, for this initial study we chose to fit these data by least squares analysis and thereby derived an approximate K_d of 7 nM.

Specificity of the soluble receptor is defined in Table 3. Phorbol 12-myristate 13-acetate (PMA) is the most potent biologically and completely inhibited binding of [^{3}H]PDB at 10 nM. Phorbol 12,13-didecanoate also is quite potent biologically and completely inhibited binding at 100 nM. The 4α analog of phorbol 12,13-didecanoate is biologically inert and did not inhibit at 100 nM. Other biologically inactive analogs, including 4α-phorbol and phorbol 13-acetate, also did not inhibit. At 100 nM, phorbol 12-myristate caused ≈50% inhibition. The biological activity of this analog has not been determined. Diolein, an activator of protein kinase C, produced slight inhibition of binding (38% at 10 μg/ml). In preliminary experiments, the receptor was stable between pH 6 and 7.5 and displayed maximal activity over the same range. Receptor at the $(NH_4)_2SO_4$ step or beyond was not stable to freezing at −20°C or −70°C.

Phorbol Diester Activation of Protein Kinase C. We were able to confirm the finding of Castagna *et al.* that PMA could activate directly protein kinase C. In the presence of diolein or PMA alone, increasing Ca^{2+} concentration did not activate significantly the kinase (Fig. 4). However, in the presence of phosphatidylserine, increasing Ca^{2+} concentration produced a dose-dependent increase in enzyme activity. Diolein enhanced slightly this effect. However, the combination of PMA and phosphatidylserine was clearly the best activator of the kinase activity. Even in the presence of 1 mM EGTA, 16 nM PMA produced ≈75% of the maximal kinase activity.

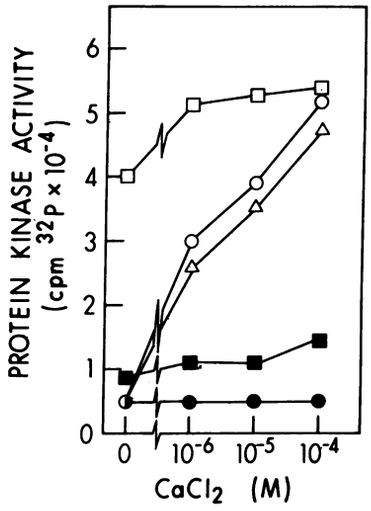

FIG. 4. Activation of protein kinase C by PMA and lipids as a function of $CaCl_2$ concentration. The pooled DEAE fractions were dialyzed overnight against Tris·HCl, pH 7.5/1 mM 2-mercaptoethanol. Protein kinase activity was assayed in the presence of 1 mM EGTA or added $CaCl_2$ at 1 μM to 0.1 mM. Incubation mixtures contained the following: diolein alone, 0.8 μg/ml (●——●); PMA alone, 16 nM (■——■); phosphatidylserine alone, 20 μg/ml (△——△); phosphatidylserine, 20 μg/ml, and diolein, 0.8 μg/ml (○——○); phosphatidylserine, 20 μg/ml, and PMA, 16 nM (□——□).

DISCUSSION

The phorbol diester receptor from rat brain was solubilized by divalent ion chelation in the absence of detergents. A rapid receptor assay was developed and used to demonstrate that the soluble receptor displayed the appropriate affinity (K_d = 7 nM for [^{3}H]PDB) and specificity for phorbol analogs. The soluble receptor was quite stable and could be partially purified by salt precipitation and ion exchange and gel filtration chromatography. The ease of extraction from the membranes suggests that the receptor is a peripheral, rather than integral, membrane protein. Whether it is related to the soluble phorbol-binding protein found in serum is unknown (31). We have used similar techniques to solubilize and characterize the phorbol diester receptor from human promyelocytic leukemia cells (HL-60) (unpublished data).

The studies presented here were based on the hypothesis that protein kinase C was, in fact, the phorbol receptor. The tissue distribution of the kinase and receptor is identical (32–35). More important, Castagna *et al.* demonstrated that active phorbol derivatives would activate directly the purified kinase (29). We have confirmed these observations and also have shown that the kinase and receptor copurify through several fractionation steps. Additionally, both kinase and receptor activities develop a dependency on added phospholipid as the purification proceeds; both activities are absolutely dependent on added phospholipid and Ca^{2+} after DEAE chromatography. Taken together, these data represent strong circumstantial evidence in support of the identity of protein kinase C and the phorbol receptor. Clearly, a final assessment awaits the purification of a homogeneous protein that contains both activities. In this regard, the EGTA/EDTA extract of Ca^{2+}-prepared membranes will have advantages as a starting material over the usual cytosolic preparation.

If protein kinase C is proven conclusively to be the phorbol receptor, rapid advances in our understanding of the mechanism of tumor promotion will follow. Phorbol-induced phosphorylation of a 40,000-dalton protein in platelets has already been documented (29). This protein appears to be involved in platelet secretion and aggregation. It is likely that the varied consequences of phorbol binding will depend in part on the acceptor proteins recognized by protein kinase C in different tissues. Therefore, normal growth control, viral oncogenesis, and tumor promotion may involve similar mechanisms—the activation of membrane-associated protein kinases.

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