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MECHANISM OF ACTION OF THE PHORBOL ESTER TUMOR PROMOTERS: SPECIFIC RECEPTORS FOR LIPOPHILIC LIGANDS

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Abstract—Cells and tissue preparations specifically bind the phorbol ester tumor promoters. The agreement in structure–activity relationships between binding and biological response strongly argues that these binding sites function as phorbol ester receptors. Upon subcellular fractionation, the phorbol ester binding activity is particulate. In addition, a phorbol ester apo-receptor can be detected in cytosol which requires phospholipids for reconstitution. This apo-receptor appears to correspond to protein kinase C. Diacylglycerols, the probable natural activators of protein kinase C, competitively inhibit phorbol ester binding, consistent with their being the postulated endogenous phorbol ester analogs. In certain systems, heterogeneity of phorbol ester binding is found. An outstanding issue therefore is whether protein kinase C is *the* phorbol ester receptor or whether it is only the most abundant class of receptor. Although this question remains unresolved, we can demonstrate heterogeneity of phorbol ester binding by reconstitution of apo-receptor into a heterogeneous lipid environment.

The phorbol esters (Fig. 1) are one of the most potent classes of compounds known as tumor promoters [1–3]. Considerable evidence both from human epidemiology and from animal experiments indicates that carcinogenesis is a multi-stage process [4, 5]. In

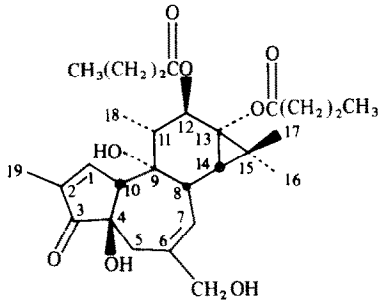

Fig. 1. Structure of phorbol 12,13-dibutyrate.

the mouse skin model system carcinogenesis can be divided into the mechanistically distinct stages of initiation and tumor promotion [6, 7]. The observation is that a single topical application of a high dose of a carcinogen such as 7,12-dimethylbenz[a] anthracene can cause skin tumors in mice. If the dose is decreased sufficiently, no tumors arise. However, subsequent, chronic treatment with a second class of compounds, called tumor promoters, leads to the rapid appearance of tumors. In the absence of the initial exposure to the sub-effective dose of the carcinogen (called the initiator), no tumors arise, indicating that the tumor promoters are not themselves carcinogenic. Tumor promoters further differ from carcinogens in that their action is reversible. If the chronic treatment with the tumor promoter precedes application of the initiator, or if the chronic treatment with the tumor promoter follows that with the initiator, but adequate intervals are permitted to elapse between promoter applications, then no tumors arise. In contrast, as long as a year may elapse between exposure to the initiator and the start of the promotion treatment with little effect on the tumor yield.

Recently, the process of tumor promotion itself has been subdivided into first and second stages, distinguished by different apparent structure–activity relations and profiles of inhibitors for each stage [8–10]. Multiple stages of promotion imply the existence of multiple mechanisms of promotion, specific for the distinct stages, rather than a single mechanism of promotion. A further implication is that the multiple stages of promotion may be mediated by distinct receptors. A possible alternative, however, is that the apparent differences in structure–activity relations may reflect differences in pharmacokinetics rather than in equilibrium potencies.

In addition to their activity as tumor promoters, the phorbol esters have profound effects on a variety of biological systems [see ref. 11–14]. Generalizations about these activities include the following: (1) The phorbol esters often act synergistically with growth factors and other cellular effectors. (2) The phorbol esters frequently affect differentiation and differentiated cell functions. Often differentiation is inhibited, but in some systems, in particular leukemia cells, differentiation may be induced. This latter response is of considerable interest for its possible utility in anti-leukemic therapy. (3) Membrane activities are prominent among those affected. (4)

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In normal fibroblasts the phorbol esters reversibly induce partially mimicry of the transformed phenotype. (5) In transformed fibroblasts the phorbol esters induce further enhancement of transformed properties. Understanding the mechanism of action of the phorbol esters may thus provide insights into a variety of important biological processes outside the specific area of tumor promotion.

Demonstration of specific phorbol ester binding represented a difficult pharmacological problem because of the lipophilicity of the ligand. The most potent phorbol ester, and that most frequently studied in biological systems, is phorbol 12-myristate 13-acetate (PMA). Due to its long fatty acid side chain, PMA readily partitions into membranes to give high, non-saturable binding [15, 16]. The alternative approach that we introduced was to use a different derivative, phorbol 12,13-dibutyrate (PDBu), which we predicted would possess the optimal ratio of binding affinity to non-specific uptake [17]. Our prediction was based on Kubinyi's analysis of the inflammatory potencies of phorbol esters as a function of their octanol–water partition coefficients [18] together with our evidence that inflammatory potency was a relevant measure of biological activity for the derivatives in question, contrary to the general view in the field at the time [19, 20].

Using [^{3}H]PDBu, we and subsequently others have been able to demonstrate specific high affinity binding both to a variety of intact cells and to particulate preparations of cells and tissues [21–23, see 24 for an extensive review]. In most but not all cases, the reported binding data are consistent with a homogeneous class of binding sites (see below for discussion of heterogeneity of binding activity). In intact cells the measured affinities typically range from 7 to 50 nM (Fig. 2A). The number of binding sites is typically 0.5–18 $\times$ 10^5 sites/cell (Fig. 2B). Upon subcellular fractionation the binding sites were found entirely in the particulate fraction [25].

The pharmacological evidence that the binding sites detected with [^{3}H]PDBu mediate biological responses to the phorbol esters is quite strong. First, comparison of binding affinities with half-maximally effective doses for inducing biological response gives quite good agreement. In chick embryo fibroblasts, for example, for a series of 8 derivatives spanning a range of 6 $\times$ 10^4 in biological potency (induction of fibronectin loss) no derivative had a binding affinity differing by more than 3.5-fold from that expected [17].

An objection might be that since these compounds are all structurally related, the correlation simply reflects some physico-chemical property of the compounds which varies co-ordinately with their biological potencies. It is therefore of particular significance that a class of compounds structurally unrelated to the phorbol esters have been identified which both are highly potent tumor promoters and also induce the variety of biological responses previously characterized for the phorbol esters [26]. These compounds, lyngbyatoxin and teleocidin, are cyclic valyltryptophan derivatives rather than diterpenes (Fig. 3). As expected from their parallelism to the phorbol esters in biological effects, lyngbyatoxin and teleocidin block [^{3}H]PDBu binding with affinities

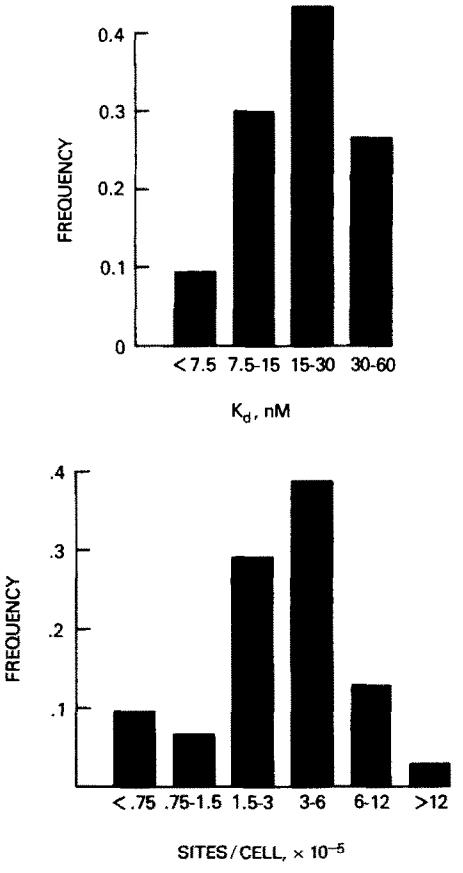


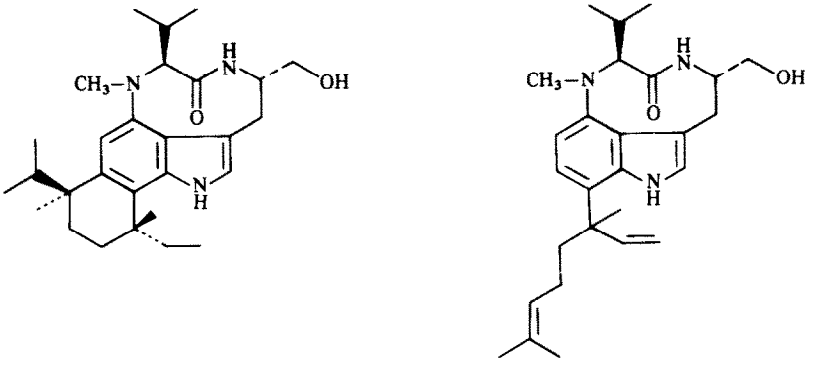
Fig. 2. Summary of values in the literature for [^{3}H]PDBu binding to intact cells. (A) Distribution of binding affinities. (B) Distribution of binding site densities.

corresponding to their biological potencies [27–29].

Although the pharmacological evidence strongly indicates that the binding sites detected with [^{3}H]PDBu mediate phorbol ester responses, quantitative structure–activity analysis is available so far only for a quite limited number of responses. Important unresolved questions therefore are whether *all* phorbol ester responses are mediated by the binding sites and whether additional, quantitatively minor [^{3}H]PDBu binding sites exist with different structure–activity requirements.

A complementary approach to structure–activity analysis for determining the relationship between binding and biological response is genetic, namely the demonstration that a sub-class of variants unresponsive to an agent are lacking receptors for that agent. In the case of the phorbol esters, unresponsive variants have been isolated in a number of cell types. Some of these variants are deficient only in certain responses; others do not respond at all. In no case have variants deficient in binding been obtained [30–34].

Analysis of the relationship between receptor occupancy and response suggests that there are *not* spare receptors for the phorbol esters. In the case of rat pituitary cells, for example, 50% receptor occupancy and 50% biological response (decrease in epidermal growth factor binding) agreed closely [35]



Dihydroteleocidin B

Lyngbyatoxin A

Fig. 3. Structure of the tumor promoting indole alkaloids dihydroteleocidin B and lyngbyatoxin.

In many intact cell systems, the absolute levels of phorbol ester binding reach a maximum and then decline as a function of the duration of incubation of the cells with the phorbol ester [see 24 for citations]. The time course and extent of down modulation depends on the particular system. The rat pituitary cell system has been of particular interest because it shows biological responses to a variety of hormones, including thyrotropin releasing hormone, insulin, and somatostatin. In these cells, thyrotropin releasing hormone causes phorbol ester receptor down modulation to a similar extent and with a similar time course to the homologous down modulation of the receptors in response to phorbol ester itself [36]. For either the homologous or the heterologous down modulation in the rat pituitary cells, the decrease in receptors reflects their conversion to a cryptic state; binding activity is recovered upon lysis of the cells [37].

The phorbol ester receptors have been highly conserved during evolution. Not only are they present in the various vertebrate systems examined but they are also found in sea urchins [23], fruit flies [23], and nematodes [38], although not in bacteria [19]. For comparison, receptors for epidermal growth factor [39] or the opiates [40] are only present in vertebrates. The high evolutionary conservation of the phorbol ester receptors suggests that invertebrate systems may be suitable for examining certain aspects of phorbol ester action. The nematode *Caenorhabditis elegans*, for example, is widely used for genetic analysis. A second implication of the evolutionary conservation is that some endogenous ligand must exist to interact at the phorbol ester binding site and to have prevented its divergence over the course of evolution. The identity of at least one class of such endogenous compounds will be discussed below.

Because of the variety of cell types responsive to the phorbol esters *in vitro*, it seemed improbable that phorbol ester receptors would be limited to skin. Indeed, specific phorbol ester binding was observed in all tissues with the exception of red blood cells [23, 41, 42]. Brain was highest in binding; spleen was second highest. The absolute level of binding in brain, 30 pmoles/mg protein, indicates a receptor density of several million sites per cell. For a protein of 80,000 MW, the receptor would thus constitute 0.2% of the particulate protein. These levels are higher than that for any neurotransmitter receptor ($\leq$2 pmoles/mg) or for most hormone receptors. We had therefore suggested that the phorbol esters in fact bound to a modulatory site on an enzyme or transport protein [43]. Other implications of the high level of phorbol ester receptors observed in brain were (a) that the receptor may have been identified by neurochemists studying it on account of some other activity which it possessed and (b) that, since non-specific binding was similar for most tissues, the proportion of total binding that was specific would be greatest in brain, facilitating analysis. Much of the biochemical analysis of binding has therefore been carried out using the brain receptor. It should be noted, however, that the structure–activity and absolute potencies of phorbol ester binding to these receptors in brain are somewhat different from those for most other cells and tissues [23]. The identity of the receptors in brain and other tissues will therefore need to be confirmed.

Studies using degradative enzymes indicated that phorbol ester binding was sensitive both to proteases and to phospholipase A_2 [43]. To obtain more information about the nature of the receptor, we synthesized a photoactivatable phorbol ester derivative, phorbol 12-p-azidobenzoate 13-benzoate (PaBB) [44] (Fig. 4). In the dark, this derivative bound to brain membranes with high affinity (0.8 nM) and in a reversible fashion. When PaBB was incubated with brain membranes in the dark and then u.v.-irradiated, the specific binding became irreversible with good efficiency (35–45%). Although the specific, irreversible binding could be inhibited by pretreatment of the membranes with protease, virtually all of the specific, covalent adducts were in fact to two classes of phospholipids, phosphatidylserine and phosphatidylethanolamine. The photoaffinity studies thus suggested that specific phospholipids were associated with the phorbol ester receptor.

The characterization of the phorbol ester receptors indicated marked similarities to an enzyme, the Ca^{2+}-phospholipid dependent protein kinase or protein kinase C, first described for brain by Nishizuka and co-workers in 1977 [45, 46]. Resemblances included:

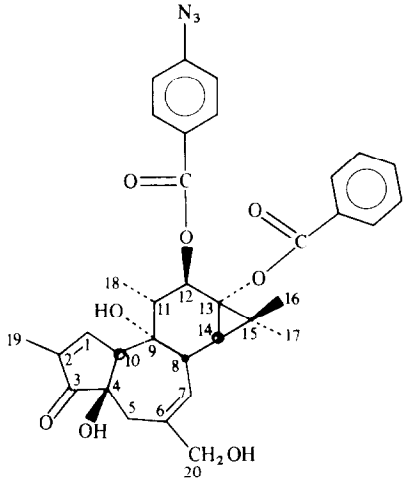


Fig. 4. Structure of the photoactivatable phorbol ester derivative phorbol 12-p-azidobenzoate 13-benzoate.

(1) degree of evolutionary conservation [47]; (2) tissue distribution [47]; (3) absolute levels in brain [48]; (4) lipid specificity [49]; and (5) responsiveness to μM concentrations of Ca^{2+} [49]. Protein kinase C is present both in membranes and cytosol. The cytosolic form exists as an apo-enzyme, with an absolute requirement for Ca^{2+} and phospholipid, preferably phosphatidylserine, for activity. In the presence of a limiting concentration of Ca^{2+}, protein kinase C can be stimulated by diacylglycerols, e.g. diolein, which appear to function by shifting the Ca^{2+}-dose–response curve for activation of the enzyme to lower Ca^{2+} concentrations [50–52]. Castagna, Nishizuka and co-workers recently reported that, under conditions of limiting Ca^{2+}, phorbol esters at nM concentrations can also stimulate protein kinase C [53]. This report was striking in that only one previous example [54] of an effect of the phorbol esters at nanomolar concentrations in a sub-cellular system had been reported.

On the other hand, there were a number of significant discrepancies between the properties of the protein kinase C and the phorbol ester receptor. For example, whereas much of the protein kinase C was in the cytosol, the phorbol ester binding activity was particulate [25]. Likewise, although PMA was reported to activate protein kinase C with an ED_{50} of 1.5 nM [53], binding to the brain membrane receptor showed 20-fold higher affinity (66 pM) [43].

A possible explanation for the apparent difference in subcellular localization of protein kinase C and the phorbol ester receptor was that the kinase measured in cytosol was an apo-enzyme, which was reconstituted into phospholipid to yield active enzyme. We therefore re-examined cytosol for phorbol ester binding in the presence of added phospholipid [22, 55]. Under such conditions, specific [^{3}H]PDBu binding could be detected with a specific activity, 20–40 pmoles/mg protein, similar to that for the particulate fraction. Scatchard analysis was consistent with a single class of binding sites having an affinity of 3.1 nM. Since the cytosolic binding activity requires phospholipid for reconstitution, we refer to it as a "phorbol ester apo-receptor".

Different phospholipids varied in their ability to reconstitute specific [^{3}H]PDBu binding activity [55 and manuscript in preparation]. Using partially purified apo-receptor to reduce contamination by lipids retained in the cytosol fraction, we found that phosphatidylserine was most effective at reconstitution, with an ED_{50} of 7 μg/ml. The negatively charged phospholipids phosphatidylinositol and phosphatidic acid were also active, with similar ED_{50} values, whereas the uncharged phospholipids phosphatidylethanolamine and phosphatidylcholine failed to reconstitute activity.

Fractionation of the cytosolic apo-receptor from brain [55, 56] and mouse thymoma cells [57] indicated co-elution with protein kinase C, consistent with the two activities being the same.

Although the relationship of the cytosolic apo-receptor to the membrane receptor remains to be conclusively determined, the two activities probably represent different states of the same protein. We have found that in rat pituitary cells the distribution of cellular binding recovered in the membrane versus cytosolic fractions depends on the lysis conditions. More is associated with the membranes in the presence of Ca^{2+}; more is found in the cytosol if the cells are lysed in the presence of EGTA. Under both conditions, total activity recovered in lysates is similar to that for the intact cells. For rat brain, likewise, a shift in phorbol ester binding and of protein kinase C activity [56] between cytosol and membranes as a function of free Ca^{2+} has been observed.

A second treatment leading to a shift in distribution (as determined in cells lysed in the presence of EGTA) is exposure of cells to phorbol esters. Such treatment of intact EL4 mouse thymoma and parietal yolk sac cells causes virtually complete transfer of protein kinase C to the particulate fraction [58, 59]. In rat pituitary cells, transfer, measured for [^{3}H]PDBu binding, still occurs but is less complete (manuscript in preparation).

We have carried out limited structure–activity comparison between mouse brain apo-receptor reconstituted into phosphatidylserine and the membrane receptor [55]. Among active derivatives, affinities for the apo-receptor ranged from 0.041 nM for PMA to 440 nM for PDA (Fig. 5). These values were all within a factor of 3 of those previously determined for the membrane receptor.

This agreement may reflect a similarity in lipid microenvironments for the protein under the two conditions. In a different system, namely that of GH_4C_1 rat pituitary cell membranes, the affinity of the membrane receptor for [^{3}H]PDBu is markedly less (30–40 nM) than is found for mouse brain membrane binding. Addition of a large excess of phosphatidylserine to the GH_4C_1 membranes increased the affinity (K_d = approximately 5 nM) to a value more like that of the reconstituted cytosolic apo-receptor. Conversely, reconstitution of the brain apo-receptor using a phospholipid mixture resembling that of human red blood cells yielded an affinity for [^{3}H]PDBu of approximately 20 nM, a value typical for intact cells and membranes but lower than that for the apo-receptor in phosphatidylserine.

The similarity in the activation of protein kinase

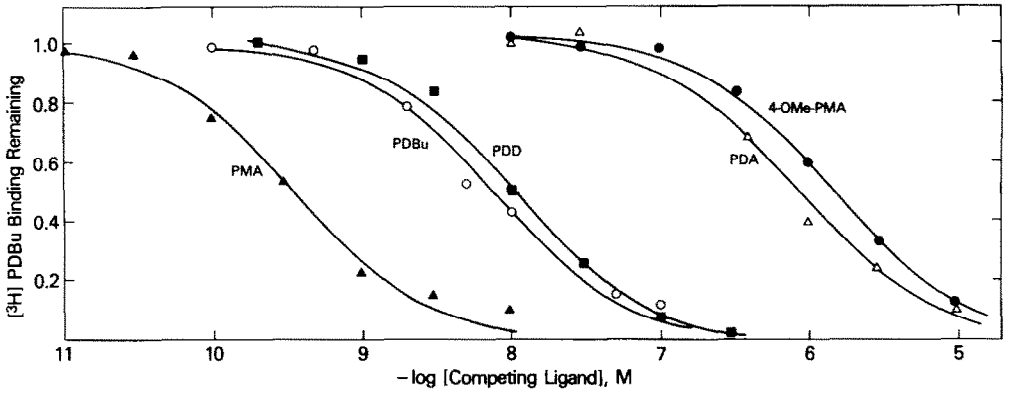


Fig. 5. Inhibition of [^{3}H]PDBU binding to the reconstituted mouse brain cytosolic apo-receptor by non-radioactive phorbol esters. The competition assays were carried out as described [55]. K_i values were as follows: PMA, 0.04 nM; PDBu, 4.7 nM; PDD (phorbol 12,13-didecanoate), 7.7 nM; PDA, (phorbol 12,13-diacetate), 440 nM; 4-OMe-PMA (4-O-methylphorbol 12-myristate 13-acetate), 510 nM.

C by diacylglycerol and by phorbol esters suggested that diacylglycerol was a possible candidate for the predicted endogenous analog of the phorbol esters. To test this hypothesis, we examined the ability of the diacylglycerol derivative diolein to inhibit [^{3}H] PDBu binding to the mouse brain apo-receptor reconstituted into phosphatidylserine [N. A. Sharkey, K. L. Leach and P. M. Blumberg, manuscript submitted]. Diolein inhibited binding greater than 90%. Scatchard analysis indicated that the decreased binding reflected an altered affinity for [^{3}H]PDBu rather than a change in maximal binding. If the change in binding affinity reflected competition at the binding site itself, rather than some non-specific disruption of the phospholipid environment into which the apo-receptor was reconstituted, then [^{3}H]PDBu binding affinity as a function of diolein concentration should fit the relationship $K_a = K_d(1 + I/K_I)$, where K_a = dissociation constant for [^{3}H]PDBu in the presence of diolein, K_d = dissociation constant for [^{3}H]PDBu in the absence of diolein, I = concentration of diolein, and K_I = apparent dissociation constant for diolein. This relationship was in fact found, yielding values of K_d and K_I of 3.69 nM and 3.5 μg/ml, respectively. These values agree well with the K_d for [^{3}H]PDBu determined by Scatchard analysis of 3.1 nM and of the K_I, determined by competition at a fixed [^{3}H]PDBu concentration, of 3.6 μg/ml.

The K_I for diolein could represent either its concentration in aqueous solution or else its concentration in the phosphatidylserine lipid phase into which the apo-receptor is reconstituted. The latter result is the predicted one, since diolein should be insoluble in aqueous solution and thus be present dissolved in the phosphatidylserine. To distinguish the two possibilities experimentally, the K_I for diolein was determined as a function of the amount of phosphatidylserine used for reconstitution of the apo-receptor. Over a 400-fold range in phosphatidylserine concentration, the K_I for diolein showed little variation expressed relative to phosphatidylserine (0.35–1.1% of the phospholipid) and a corresponding marked variation expressed relative to volume (6.9–.05 μg/ml). For [^{3}H]PDBu, in contrast, the K_I expressed relative to its aqueous concentration showed little variation with phosphatidylserine concentration, which is once again the expected result since only a few percent of this more hydrophilic ligand partitions into the liposomes under these conditions.

Although diolein acts as a competitive inhibitor of [^{3}H]PDBu binding, it is much less potent. Expressing the K_d for [^{3}H]PDBu in terms of its actual concentration in the liposomes, one obtains a difference in affinities of 6×10^4. Despite this difference in absolute potency, the effective concentrations of diacylglycerols do not appear to be unphysiological. Evidence at least in the platelet system suggests their functional role as endogenous activators of protein kinase C [60–62].

Biological evidence for heterogeneity of phorbol ester receptors is suggested by different dose response curves and structure–activity requirements for different biological responses, at least in certain systems. The subdivision of tumor promotion into first and second stage promotion has been described above. Further examples are cited in Ref. [22]. In addition, in certain systems the dose response curves themselves are unusual, attaining a maximum response and then decreasing at higher ligand concentrations, which are however still below those concentrations at which toxicity due to non-specific perturbation of membranes might be expected [63–65].

Direct evidence for heterogeneity of high affinity phorbol ester binding has been obtained for mouse skin particulate preparations [66], for NRK cells, and, in unconfirmed studies, for myeloid cells [67]. Preliminary reports also suggest the possible existence of distinct nuclear binding activity [68]. The origin of the heterogeneity in phorbol ester binding remains to be determined. It could reflect multiple targets for the phorbol esters in addition to protein kinase C. Alternatively, it could reflect the protein kinase C being differentially modified, whether by proteolysis, phosphorylation, or different lipid environments. Modulation of receptor affinity by varying the phospholipid composition or the diacylglycerol content in the phospholipids has been descri-

bed above. By maintaining heterogeneity of the lipid environment used for reconstitution, curved Scatchard plots can be obtained. Figure 6 compares Scatchard plots of [^{3}H]PDBu binding data for receptors reconstituted into liposomes of phosphatidylserine, 1.8% diolein in phosphatidylserine, or a 1:1 mixture of the two types of liposomes. The 1:1 mixture yielded a curved Scatchard plot fitting that predicted for reconstitution into the two distinct liposome populations.

Nishizuka and co-workers have postulated that protein kinase C functions as a mediator of signal transmission for those ligands whose action is coupled to enhanced phosphatidylinositol turnover [50, 51]. Michell and others [69, 70] have reviewed the evidence that enhanced turnover of phosphatidylinositol and of phosphatidylinositol mono- and diphosphates acts as a second messenger for a variety of neurotransmitters, hormones, and cellular effectors. Examples include some muscarinic cholinergic, α_1-adrenergic, vasopressin (V_1), histaminergic (H_1), and 5-hydroxytryptaminergic (5-HT_1) receptors, as well as those for substance P, bradykinin, thyrotropin releasing hormone, F-met-leu-phe, and thrombin. The turnover of phosphatidylinositol is associated with transient formation of diacylglycerol and enhanced intracellular Ca^{2+}. *In vitro*, these agents function as activators of protein kinase C. *In vivo*, experiments using platelets implicate protein kinase C in message transduction for thrombin [60–62]. The same work suggests that protein kinase C is not the only effector for the pathway, however, since phosphorylation of myosin light chain in response to thrombin depends on Ca^{2+} but is not induced by exogenous diacylglycerol or by phorbol esters.

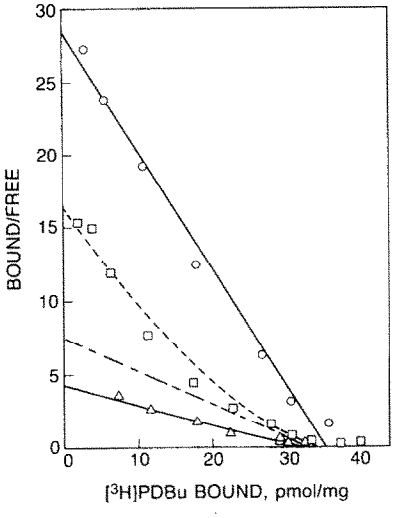


Fig. 6. Heterogeneity in [^{3}H]PDBu binding to the mouse brain cytosolic apo-receptor reconstituted into a heterogeneous lipid environment. The cytosolic apo-receptor was reconstituted into liposomes of (○) phosphatidylserine, (△) 1.8% diolein in phosphatidylserine, or (□) a 1:1 mixture of the above liposomes. Specific binding was assayed as described [55] in the presence of phosphatidylserine at 40 μg/ml, 0.1 mM Ca^{2+}, and no Mg^{2+}. Precipitation of receptors was with polyethylene glycol at 12%. The binding data were plotted by the method of Scatchard. The predicted results for binding to the 1:1 liposome mixture were calculated assuming either no (– – –) or complete (— — —) exchange of diolein between liposomes.

The emerging understanding of the mechanism of phorbol ester action suggests marked analogies between the phorbol esters and cholera toxin (see [71] for review on cholera toxin). Both agents penetrate the plasma membrane to chronically activate an intracellular component of a message transduction pathway common to an extensive series of hormones and cellular effectors. A difference, however, is in the step along the pathway at which activation occurs. In the case of cholera toxin the formation of cAMP, the activator for cAMP dependent protein kinase, is enhanced. The phorbol esters, in contrast, are themselves analogs of an activator for protein kinase C.

The phorbol esters and Rous sarcoma virus induce similar although distinguishable changes in the phenotype of chicken embryo fibroblasts [72–74]. The finding that protein kinase C is the major phorbol ester receptor and that the transforming gene of Rous sarcoma virus is a tyrosine kinase [75] further enhances this parallelism. Whether a sequence coding for the catalytically active fragment of the protein kinase C would function as an oncogene remains to be determined.

That people should have receptors for a plant toxin such as the phorbol esters at first sight appears puzzling. A possible rationale is that many of the Euphorbias, which produce the phorbol esters, grow in arid regions. The acute toxicity of the phorbol esters, irritation to mucus membranes, would discourage predation by herbivores. The chronic toxicity, viz. tumor promotion, would be incidental.

Although the phorbol esters appear to be diacylglycerol analogs, it does not follow that the two classes of compounds should function identically. First, whereas the phorbol esters are relatively stable, diacylglycerols are rapidly metabolized. The phorbol esters should therefore lead to abnormal, chronic stimulation of protein kinase C. Secondly, the hormonally-activated phosphatidylinositol turnover should generate diacylglycerol at the plasma membrane. The phorbol esters, in contrast, should be able to equilibrate with internal membranes in the cell. They may therefore cause an abnormal distribution of activated kinase. The altered distribution of kinase in turn may result in an altered pattern of phosphorylation. Thirdly, protein kinase C can be cleaved proteolytically to a 51,000 MW active fragment which no longer requires phospholipids, Ca^{2+}, or diacylglycerols for activation and which no longer binds to membranes [45, 76]. The susceptibility to cleavage of protein kinase C is enhanced when the enzyme is in the activated state [76]. Chronic activation of kinase C by phorbol esters may therefore lead to enhanced conversion to the constitutive, cytosolic form and again lead to an altered pattern of phosphorylation.

The studies on phorbol ester receptors are yielding important insights into the initial steps in the mechanism of tumor promoter action. Since the phorbol esters bind at a modulatory rather than a catalytic site on their receptor, it should be possible to develop

antagonists which bind to this site but fail to activate the kinase. With the exception of teleocidin and lyngbyatoxin, tumor promoters structurally unrelated to the phorbol esters fail to compete for binding. Binding similarly is not inhibited by inhibitors of tumor promotion. Experiments can now be done to determine whether the activities of these agents feed into the biochemical cascade triggered by the phorbol esters at the subsequent steps of protein kinase C activation or phosphorylation of specific substrates. It is important to emphasize, however, that further understanding of the nature of the heterogeneity of binding and biological response to the phorbol esters will be essential to evaluate whether protein kinase C is the phorbol ester receptor or only the quantitatively most abundant one.

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