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Special Lecture

Protein Kinase C as the Receptor for the Phorbol Ester Tumor Promoters: Sixth Rhoads Memorial Award Lecture[1]

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The focus of my research has been on understanding the initial events in phorbol ester action. The phorbol esters are natural products derived from *Croton tiglium*, the source of croton oil, and from other plants of the family Euphorbiaceae (1). The phorbol esters initially became the object of intense research interest on the basis of their potent activity as mouse skin tumor promoters (2).

The behavior of tumor promoters has been characterized in detail in the mouse skin system. Briefly, tumor promoters are compounds which by themselves are not carcinogenic or indeed mutagenic but which if administered chronically after exposure of an animal to a subeffective dose of a carcinogen are then able to lead to the rapid appearance of skin tumors. Whereas the action of the carcinogen is irreversible, as shown by the fact that one can delay the promotion treatment by up to 1 year after application of the carcinogen, referred to as an initiator, the action of the tumor promoters is reversible. Thus, if the chronic tumor promoter treatments precede rather than follow the treatment with the initiator, tumors do not arise.

Although tumor promoting activity provided the initial motivation for studying the mechanism of the phorbol esters, as people began to examine the effects of the phorbol esters in different *in vitro* systems, it turned out that the phorbol esters had profound effects on a wide variety of biological systems (3–5). These effects included (*a*) induction or inhibition of differentiation, (*b*) the partial mimicry in normal cells of the transformed phenotype, (*c*) stimulation of secretory responses in a variety of cell types such as platelets and neutrophils, (*d*) modulation of many membrane activities, and (*e*) generation of active oxygen species. The range of these effects suggested early on that the phorbol esters might be functioning through stimulation of some central regulatory pathway within cells.

Two indirect arguments had suggested that the phorbol esters might bind to specific receptors: (*a*) the compounds had extraordinarily high potency, being active at nanomolar concentrations; (*b*) small structural changes in the molecule led to marked differences in activity. For example, elimination of the hydroxyl group at position 20 of phorbol led to complete loss of activity, and methylation of the hydroxyl group at position 4 led to a several hundred-fold loss in potency. Initial efforts to demonstrate specific phorbol ester binding to cells were frustrated, however, because investigators used the biologically most active phorbol ester, phorbol 12-myristate 13-acetate. The myristate side chain makes the molecule highly lipophilic, and specific binding is therefore obscured by very high nonspecific uptake. On the basis of structure-activity analysis which we had carried out *in vitro* together with published data concerning the lipophilicity of different phorbol esters as a function of biological potency, we predicted that a different derivative, phorbol 12,13-dibutyrate, would be optimized for specific binding activity relative to nonspecific uptake due to lipophilicity (Fig. 1) (6). We therefore prepared radioactively labeled phorbol 12,13-dibutyrate; using this derivative, we and subsequently many other laboratories were indeed able to demonstrate the existence of specific phorbol ester receptors in a variety of cells and tissue preparations (7). An alternative approach developed by Curt Ashendel, in Dr. Boutwell's laboratory at the time, also proved successful. This approach used radioactive phorbol 12-myristate 13-acetate but reduced the nonspecific binding of the phorbol 12-myristate 13-acetate by means of washing with cold acetone (8).

As we and others proceeded to characterize what we referred to as the phorbol ester receptor, marked similarities began to emerge with that of an enzymatic activity called protein kinase C being characterized by Nishizuka *et al.* at Kobe University in Japan (9, 10). In both cases, activity was highest in brain, high in lung and in neutrophils, and lower although present in other tissues. The absolute amounts in brain were similar. In both cases there was high conservation of activity over evolution. Phorbol ester binding was demonstrated in nematodes, and protein kinase C was found in earthworms. There was sensitivity to very low concentrations of calcium and, as will be described later, evidence for association with specific phospholipids.

It is now clear that the phorbol ester binding assays and the protein kinase C enzymatic assays were measuring different functional activities of the same protein: (*a*) Nishizuka's group demonstrated that under appropriate conditions with limiting amounts of calcium and phospholipid, the required cofactors for protein kinase C, the phorbol esters were able to stimulate protein kinase C activity (11); (*b*) several laboratories, including those of Dr. Niedel at Duke (12), Dr. Sando at the University of Virginia (13), Drs. Ashendel and Boutwell at McArdle Laboratory (14), and our own group (15) demonstrated that protein kinase C enzymatic activity and phorbol ester binding activity copurified, ultimately to homogeneity.

The identification of protein kinase C as the major target for the phorbol esters was very exciting because it suggested that the phorbol esters were acting on one of the major signal transduction mechanisms within cells. For a large class of hormones and other cellular effectors, the initial action of the hormones upon occupying their individual extracellular receptors is to activate phospholipase C in what is now thought to be a GTP binding protein mediated process (16, 17). Phospholipase C degrades a specific phospholipid, phosphatidylinositol 4,5-bisphosphate, to generate two products, inositol 1,4,5-trisphosphate and diacylglycerol. Nishizuka *et al.* demonstrated that diacylglycerols, like the phorbol esters, are able to activate protein kinase C. Inositol 1,4,5-trisphosphate, in turn, activates the other arm of the signal transduction pathway, elevating

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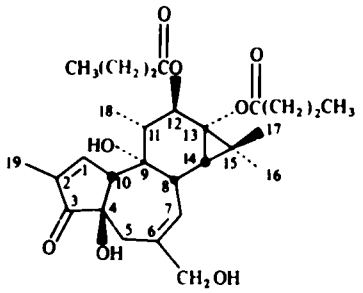

Fig. 1. Structure of phorbol 12,13-dibutyrate.

intracellular calcium and thereby activating calcium dependent protein kinases.

The demonstration that protein kinase C was the major receptor for the phorbol esters helped rationalize the broad range of biological activities that had been observed previously for the phorbol esters. In addition, because the phorbol esters are much better behaved as pharmacological agents than are the diglycerides, it suggested that the phorbol esters could be very useful probes for identifying the physiological systems in which protein kinase C was involved. Finally, the detailed understanding of the interaction of phorbol esters with the regulatory domain on protein kinase C might permit the development of agents which interfere with protein kinase C activity, thereby functioning as antipromoters or as inhibitors of physiological effectors working through this pathway.

From the perspective of cancer researchers, the protein kinase C pathway is important because, in addition to the phorbol ester tumor promoters, a number of oncogenes and transforming gene products are thought to stimulate this pathway (18–20). *sis* and *erb B* are thought to enhance phosphatidylinositol turnover; *ras* has been shown to cause elevated levels of diacylglycerol, and *src* and the polyoma middle T-antigen stimulate phosphatidylinositol kinase, leading to elevated levels of substrate for phospholipase C. At the other end of the pathway, protein kinase C is of interest because it phosphorylates a number of growth factor receptors such as the receptor for epidermal growth factor (21). Secondly, protein kinase C modulates the activity of other signal transduction systems. For example, Grimm and Marks (22) and Mufson *et al.* (23) have shown that phorbol ester treatment blocks the elevation in cyclic AMP in mouse skin induced by treatment with isoproterenol. Thirdly, protein kinase C exerts feedback control at several points in the phosphatidylinositol turnover pathway (24, 25). It affects, directly or indirectly, the activity of phosphatidylinositol kinase, of phospholipase C, and of inositol trisphosphate 5′-phosphomonoesterase, an enzyme which degrades the inositol 1,4,5-trisphosphate. Finally, protein kinase C has been implicated in the expression of a number of oncogenes including *myc* and *fos* (26, 27).

We had predicted the existence of an endogenous analogue of the phorbol esters based on the high evolutionary conservation of the phorbol ester binding activity (28). The demonstration by Nishizuka *et al.* that diacylglycerol, like the phorbol esters, can stimulate protein kinase C suggested that diacylglycerol might in fact be the endogenous phorbol ester analogue. To explore this question, we examined the ability of diacylglycerol to compete with phorbol ester for binding (29). We found that increasing concentrations of the diacylglycerol derivative 1,2-diolein were able to reduce phorbol ester binding affinity with no change in B_{max} (Fig. 2). This behavior was consistent with the diacylglycerol acting as a competitive inhibitor of phorbol ester binding. An alternative interpretation, however,

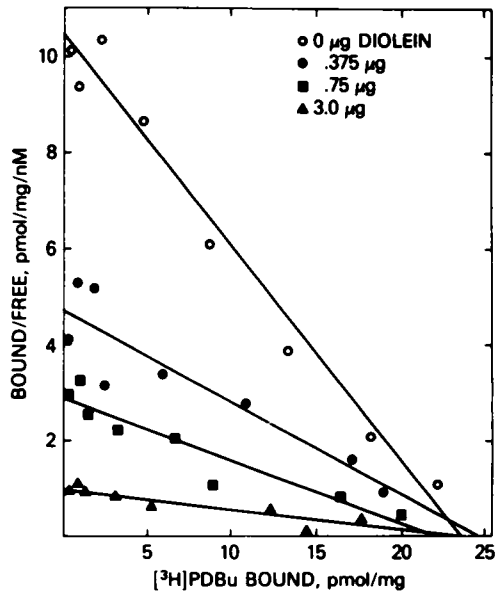

Fig. 2. Inhibition of [20-^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding to protein kinase C in the presence of phosphatidylserine and increasing concentrations of 1,2-diolein (from Ref. 29; reprinted with permission).

was that diacylglycerol was simply functioning to perturb the lipid environment of the enzyme, thereby altering the phorbol ester binding affinity. The actual receptor is a complex between protein kinase C and phospholipid, and, as described below, binding affinity depends on the lipid component of the complex as well as on protein kinase C.

There are three tests which must be met if diacylglycerols actually inhibit the phorbol ester binding through a competitive mechanism. (*a*) There is a specific relationship that must exist between the apparent binding affinity of phorbol 12,13-dibutyrate for the receptor and the concentration of inhibitor present in the assay. This relationship in fact was found (29). (*b*) A competitive inhibitor affects only the rate of association of a ligand with its receptor; it does not affect the rate of ligand release. For diacylglycerol this condition again was fulfilled (30). Had the diacylglycerol been perturbing the lipid environment, it most probably would have affected both the on and off rates for the binding of phorbol ester to the receptor. (*c*) A competitive inhibitor should bind with 1:1 stoichiometry. These experiments were technically quite difficult because the issue essentially was to measure specific binding of a highly lipophilic compound. Nonetheless, using independent approaches, both we (31) and Dr. Robert Bell (32) were able to demonstrate that the stoichiometry of interaction was 1:1.

Although the binding data indicate that the diacylglycerols interact like the phorbol esters, this does not mean that diacylglycerols are as potent as the phorbol esters. We compared three pairs of phorbol esters and analogously substituted diacylglycerols. We found that glycerol 1-myristate 2-acetate was some 30,000-fold less active than was the potent phorbol ester phorbol 12-myristate 13-acetate (33). The differences were much smaller, however, for the other two pairs of compounds we examined, the di-C-12 and di-C-18-substituted derivatives (34). This variation implies that the phorbol esters and diacylglycerols have different structure-activity requirements for their side chains.

The *in vitro* evidence that the diacylglycerols bind to protein kinase C in a fashion similar to that of the phorbol esters suggests that methods which elevate diacylglycerol levels in cells will lead to phorbol ester-like effects. Three approaches

have been used to test this prediction: (*a*) treatment of cells with diacylglycerols which are more hydrophilic than physiologically present diacylglycerols but still sufficiently hydrophobic to partition into the cell membrane (33, 35, 36); (*b*) treatment of cells with bacterial phospholipase C to degrade phospholipids on the outer surface of the plasma membrane to diacylglycerol (37, 38); (*c*) treatment with agents which modulate diglyceride metabolism, either inhibitors of diglyceride kinase, blocking diglyceride breakdown (39), or inhibitors of triglyceride synthesis, leading to diglyceride accumulation (40). The conclusion from all three approaches is that the diglycerides mimic many, but perhaps not all (41), of the responses seen with the phorbol esters. The significance of those differences which have been observed is hard to interpret because the diglycerides are rapidly metabolized, and it is not certain that physiologically relevant concentrations of diglycerides will have entered the cells. Because of both their greater stability and their greater potency, the phorbol esters are thus better diglycerides than are the endogenous compounds.

Although the biochemical evidence that protein kinase C is the major phorbol ester receptor is very strong, the behavior of the phorbol esters in biological systems clearly indicates that all of its activities cannot be explained by a single, homogeneous class of well behaved receptors. The biological evidence for heterogeneity is of four types (42). (*a*) Different phorbol derivatives may induce different spectra of biological responses. Thus phorbol 12-myristate 13-acetate and the related diterpene mezerein are similar within a factor of 2 in inflammatory potency. In contrast, whereas phorbol 12-myristate 13-acetate is a potent, complete tumor promoter, mezerein is very weak as a complete tumor promoter although it has potent activity as a so-called second stage tumor promoter. (*b*) For a single derivative, different concentrations of the same derivative may be required to induce different responses. (*c*) There are several reports, although not very many, in which the activity of one phorbol ester such as phorbol 12-myristate 13-acetate is antagonized by another, typically more hydrophilic, phorbol ester. (*d*) There are a number of instances of biphasic dose-response curves, for which both the ascending and the descending phases occur at nanomolar concentrations, consistent with specific rather than nonspecific phorbol ester actions.

Binding analysis likewise supports the concept of phorbol ester receptor heterogeneity (43). Dunn, when he was in my laboratory, compared the binding activity to mouse skin particulate preparations of phorbol 12,13-dibutyrate, the usual ligand used in binding assays, and of 12-deoxyphorbol 13-isobutyrate, a phorbol derivative of interest because it had been reported by Hecker *et al.* to be inflammatory but nonpromoting. Both derivatives yielded curved Scatchard plots, indicative of heterogeneity in binding affinity (Fig. 3). Moreover, the total number of sites detected by phorbol 12,13-dibutyrate and by 12-deoxyphorbol 13-isobutyrate in the mouse skin particulate preparations were different. These results suggested that there was a subclass of binding sites which was selective for phorbol 12,13-dibutyrate but not for the 12-deoxyphorbol 13-isobutyrate. This evidence for heterogeneity makes it important to determine what fraction of the phorbol ester responses really involve the major phorbol ester receptor, protein kinase C.

Several different approaches have proved valuable. For a number of biological responses, it has been possible to demonstrate good correlation between the binding affinities of phorbol esters to their major receptor and the biological response. Early data which we obtained on stimulation of deoxyglucose uptake

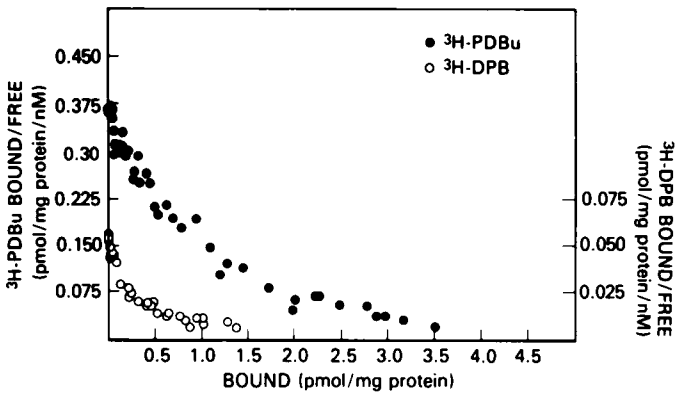

Fig. 3. Binding of [20-^{3}H]phorbol 12,13-dibutyrate (^{3}H-PDBu) and [20-^{3}H]-12-deoxyphorbol 13-isobutyrate (^{3}H-DPB) to mouse skin particulate preparations (from Ref. 43; reprinted with permission).

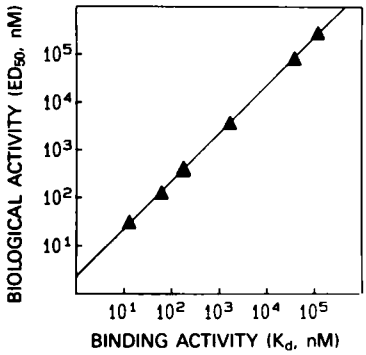

Fig. 4. Correlation between stimulation of 2-deoxyglucose uptake and [20-^{3}H]-phorbol 12,13-dibutyrate binding by chicken embryo fibroblasts (from Ref. 6; reprinted with permission). ED_{50}, 50% effective dose.

in the chick embryo fibroblast system are illustrated in Fig. 4 (6).

A second approach, pioneered by Rozengurt *et al.* (44) at the Imperial Cancer Research Fund, was to show that for appropriate cell types, such as confluent Swiss 3T3 cells, one can abrogate responsiveness to the phorbol esters by prolonged treatment with the phorbol ester, leading to down-regulation. In parallel with the loss of responsiveness is the loss of protein kinase C activity.

A third general approach has been to demonstrate *in vitro* that one can reconstitute with purified protein kinase C a response seen in the intact cells upon phorbol ester treatment. In collaboration with Dr. Tauber's laboratory at Boston University we have been studying the human neutrophil both as a model of stimulus-response coupling for the phorbol esters and as a physiological source of active oxygen. In intact cells, phorbol esters lead to activation of the NADPH oxidase, the enzyme system responsible for the oxidative burst. *In vitro*, phorbol esters similarly activate the oxidase in a reconstituted system which includes a plasma membrane fraction, a cytosolic fraction, ATP, phospholipid, and NADPH. We were able to demonstrate that the requirement for the cytosolic fraction in the reconstituted system can be replaced with purified protein kinase C (45).

An extension of the approach of *in vitro* reconstitution to demonstrate a role for protein kinase C in a specific phorbol ester response is that of *in vivo* reconstitution, using microinjection. A system which we have used is the one described above of Swiss 3T3 cells which are depleted of protein kinase C by chronic phorbol ester treatment and which no longer respond mitogenically to the phorbol esters. As expected, in control

cells neither injection buffer nor purified protein kinase C by itself stimulated mitogenesis. Likewise, in the pretreated cells in the absence of phorbol ester, neither injection buffer nor purified enzyme was mitogenic. However, microinjection of the purified enzyme together with phorbol 12,13-dibutyrate treatment restored mitogenic response to the down-regulated cells (46).

The combination of these approaches in a variety of systems strongly supports the conclusion that most phorbol ester responses are mediated through protein kinase C. We and others have therefore been quite interested in mechanisms for generating heterogeneity within the protein kinase C system itself. As described by Housey *et al.* (47), Knopf *et al.* (48), and others (49), cloning studies now clearly indicate that protein kinase C is not a single enzyme but, rather, a family of related isozymes. Heterogeneity in the behavior of this family of isozymes is one attractive mechanism for heterogeneity in the protein kinase C system. It is not, however, the only mechanism.

In order to display phorbol ester binding activity, protein kinase C must be complexed with phospholipid. The nature of the phospholipid plays an important part in determining the binding characteristics of this complex. Dr. Konig from my laboratory complexed protein kinase C with a variety of phospholipids or phospholipid mixtures and then measured the binding affinity of phorbol 12,13-dibutyrate for the complexes (50). He found a 30-fold difference in binding affinity of phorbol 12,13-dibutyrate for the receptor complexed with phosphatidylserine as compared to the complex with a mixture of phospholipids corresponding to that of the RBC.

The lipid domain of the complex modulates not only the binding affinity of the receptor but also the coupling between phorbol ester binding and enzymatic activation. Nakadate in my laboratory showed that increasing concentrations of the lipid analogue palmitoylcarnitine suppressed activity of the enzyme in the presence of calcium and phosphatidylserine, whereas it enhanced the activity of the enzyme observed in the presence of increasing concentrations of the phorbol ester phorbol 12,13-dibutyrate (51).

A third mechanism for generating heterogeneity from protein kinase C is through differences in intracellular location. Anderson, Kraft, and Sando at the National Cancer Institute were the first to show that treatment of cells with phorbol ester led to translocation of cytosolic protein kinase C to the membrane, where it could complex with phosphatidylserine and be activated (52, 53). The subcellular location of the phorbol ester within the cell should dictate the subcellular location of the activated protein kinase C and, by extension, what substrates will be accessible to the activated enzyme.

Nishizuka *et al.* (9, 10) demonstrated that treatment of protein kinase C with low concentrations of protease generated a catalytic fragment which no longer required calcium and phospholipid for activity. Because this fragment is lacking the phospholipid-binding regulatory domain, it does not interact well with membranes and would be expected to be located in the cytosol in contrast to the membrane location of the activated, intact protein kinase C. The catalytic fragment would thus be in contact with a different distribution of substrates than would the intact enzyme. Nishizuka *et al.* reported that activation of the enzyme with phorbol esters, phospholipids, and calcium enhanced its susceptibility to proteolysis *in vitro*. Several groups have demonstrated in intact cells that phorbol ester treatment enhances the rate of formation of the catalytic fragment (54, 55). The other product of breakdown, the regulatory domain, turns out to be highly resistant to proteolysis (56). The phorbol

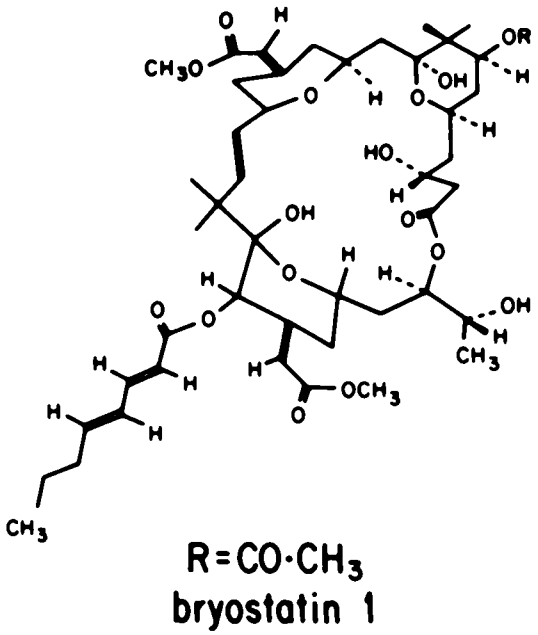

Fig. 5. Structure of bryostatin 1.

ester binding assay thus detects both the intact phorbol ester receptor as well as the cleaved regulatory domain, a pseudoreceptor. The understanding of heterogeneity in the protein kinase C pathway is of great importance because it may permit selective inhibition of a subclass of phorbol ester responses.

A group of compounds which have proved to be particularly interesting in this regard are the bryostatins. The bryostatins are macrocyclic lactones, isolated from marine bryozoans by Pettit *et al.* (57) at Arizona State University (Fig. 5). The bryozoans are colonial plankton feeders known as false corals or as sea mosses.

Initial studies by Pettit, Smith, and Kraft suggested that the bryostatins were simply another structural class of protein kinase C activator. Thus, the bryostatins activated protein kinase C, inhibited phorbol ester binding, and induced several biological responses in a comparable fashion to the phorbol esters (58, 59). An important finding by Kraft, therefore, was that bryostatin 1 in the HL-60 promyelocytic leukemia cell line failed to induce differentiation, a well-studied response to the phorbol esters in this system (60). Moreover, if administered in conjunction with phorbol ester, bryostatin 1 blocked the differentiation induced by the phorbol ester in these cells. We therefore, in collaboration with Pettit, have been exploring the biological actions of these compounds in detail.

The first question we sought to examine was whether the bryostatins blocked differentiation responses *per se* or whether they were simply inhibiting the response to the phorbol esters in certain systems. To study this question, Dell'Aquila (61) in my laboratory characterized the activity of bryostatin 1 in the Friend erythroleukemia cell system. This system is of interest because it responds to the phorbol esters in the opposite way from HL-60, namely that differentiation can be induced by the inducer hexamethylene bisacetamide and suppressed by treatment with phorbol 12,13-dibutyrate. Dell'Aquila therefore treated the cells with inducer and phorbol 12,13-dibutyrate together with increasing concentrations of bryostatin 1 to determine whether or not bryostatin 1 could, in this case, restore the differentiation response. Indeed, bryostatin 1 at nanomolar concentrations was able to restore differentiation, arguing that the bryostatins act to inhibit the phorbol ester pathway itself.

A second highly informative system proved to be that of primary mouse epidermal cells (62). Previous studies had shown in separate cell types that the bryostatins stimulated proliferation but inhibited differentiation. Dr. Sako in my laboratory chose the epidermal cell system because, in this system, subpopulations of cells are both induced to proliferate and to differentiate in response to phorbol ester treatment (63). At nanomolar concentrations, bryostatin 1 induced ornithine decarboxylase, a marker of proliferation in the keratinocytes. The level of induction was not, however, as great as that seen upon phorbol 12,13-dibutyrate treatment. In contrast to the induction of ornithine decarboxylase, epidermal transglutaminase, a marker of differentiation in these cells, was not induced by bryostatin 1. Administered together with phorbol 12,13-dibutyrate, bryostatin 1 blocked the response to the phorbol ester. Similar results were obtained for cornified envelope formation, a second marker of differentiation. Cornified envelope formation was stimulated by phorbol 12,13-dibutyrate and was not stimulated by bryostatin 1. Treatment with phorbol ester and bryostatin 1 in combination blocked the stimulation induced by the phorbol ester alone.

Although these results could indicate that the bryostatins differently affect proliferative and differentiated responses within the same cell type, experiments by Pasti in the laboratory suggested that the time course of the response may be a critical variable (64). Pasti examined the ability of phorbol 12,13-dibutyrate and bryostatin 1 to inhibit cell-cell communication in keratinocytes as quantitated by microinjection of Lucifer yellow dye into single cells and measurement of the number of cells into which it transferred. Using keratinocytes shifted from low (0.05 mM) to high (1.2 mM) calcium medium, Pasti showed that phorbol 12,13-dibutyrate rapidly suppressed dye transfer and that this suppression was maintained for 8–24 h. Comparison at later times was obscured somewhat because of the decrease in the level of dye transfer as a function of time after calcium shift for the control cells. Bryostatin 1 treatment, like phorbol ester treatment, caused the rapid suppression of dye transfer in these cells. In contrast to the action of the phorbol esters, however, the action of bryostatin 1 was transient, with activity returning to control levels between 2 and 4 h. For comparison, induction of ornithine decarboxylase was measured after 3 h of treatment, epidermal transglutaminase after 9 h, and cornified envelope formation after 24–72 h.

A mechanism which may partially explain this more transient action of the bryostatins is that of accelerated degradation of protein kinase C. Rivedal quantitated protein kinase C by immunoblotting after treatment of the keratinocytes with bryostatin 1 or phorbol 12,13-dibutyrate. After 4 h of bryostatin 1 treatment protein kinase C was largely lost whereas it showed little degradation after phorbol ester treatment.

Although a shorter duration of action for bryostatin 1 can account for some of the differences between the activities of bryostatin 1 and the phorbol esters, it cannot fully account for the differences. In other systems, different end points appear to differ intrinsically in their response to the bryostatins. Dell'Aquila, in my laboratory, showed that in the case of arachidonic acid release from C3H 10T½ cells the phorbol esters were able to cause rapid release, observable within 30 min. In contrast, very little release was seen in response to bryostatin 1; and, as in other systems, bryostatin 1 together with phorbol 12,13-dibutyrate blocked the effect of the phorbol 12,13-dibutyrate. This failure of the C3H 10T½ cells to respond cannot be due to a very short duration of bryostatin action because, for a different response, that of epidermal growth factor binding, both the phorbol esters and the bryostatins show a long duration of action.

The inhibition of phorbol ester effects by the bryostatins could reflect either the interaction of bryostatin at protein kinase C, *e.g.*, making it more labile, or else an action of bryostatin at some other target which feeds back on the protein kinase C pathway. To distinguish these possibilities, Dell'Aquila (61) examined the ability of bryostatin 1 to restore differentiation in the Friend erythroleukemia cell system as a function of phorbol ester concentration. The concept was that if the effect of the bryostatin to inhibit the phorbol ester response was mediated through the usual phorbol ester binding site, then higher concentrations of phorbol ester should be able to compete with bryostatin; experimentally, at a high phorbol ester concentration the dose-response curve for bryostatin inhibition should thus be shifted to higher concentrations. This proved not to be the case; a similar concentration of bryostatin 1 was required to inhibit the phorbol 12,13-dibutyrate effect at either 20 or 200 nM phorbol 12,13-dibutyrate. These data suggest that the bryostatins may be acting at a second target in addition to the typical high affinity phorbol ester binding site of reconstituted protein kinase C.

Further evidence supporting the possibility of an additional target for the bryostatins is data obtained by Warren in my laboratory examining phosphorylation in the HL-60 cell system (65). Warren compared the pattern of phosphorylation induced in response to either phorbol 12,13-dibutyrate or bryostatin 1. He found that bryostatin 1 induced all of the same substrates to be phosphorylated as seen in response to the usual concentrations of phorbol 12,13-dibutyrate. In addition, however, he identified a family of several proteins with molecular weights of approximately 70,000 which were selectively phosphorylated in response to bryostatin 1 treatment but which were not phosphorylated in response to the phorbol 12,13-dibutyrate.

In order to explore the possibility of targets for bryostatin 1 in addition to protein kinase C, we have worked collaboratively with Pettit and his associates on preparation of radioactively labeled bryostatin 4. In our initial studies, we have used the radioactively labeled bryostatin 4 to examine its interaction with protein kinase C. We find substantial differences in some of the binding characteristics. In particular, radioactive phorbol 12-myristate 13-acetate is very rapidly lost from the enzyme after addition of nonradioactive phorbol ester. In contrast, a major portion of the bound bryostatin 4 is released with a very slow half-time of several hours. This slow off-rate of bryostatin 4 from protein kinase C suggests that treatment with the bryostatins may anchor protein kinase C at the first location within the cell at which it recognizes the bryostatin. In contrast, the phorbol esters may permit redistribution of protein kinase C as a function of time. We refer to this model as the glue trap model.

Yuspa and Hennings, in the Laboratory of Cellular Carcinogenesis and Tumor Promotion, have presented strong evidence that induction of terminal differentiation of normal keratinocytes may play a crucial role in mouse skin tumor promotion (63). The failure of the bryostatins to induce markers of terminal differentiation in mouse primary epidermal cells, whether due to effects on differentiation *per se* or due to a transient duration of action, suggests that the bryostatins should lack tumor promoting activity and might in fact be antipromoters. Both *in vitro* and *in vivo* experiments support this prediction.

Yuspa and Hennings have established a reconstitution system in which they can disperse a small number of putative initiated

cells on a lawn of keratinocytes and get stimulation of the outgrowth of the initiated cells in the presence of phorbol esters. Bryostatin 1 by itself was unable to stimulate such outgrowth. In the presence of bryostatin 1, moreover, the stimulation of outgrowth by phorbol ester was suppressed. The results with Sencar mice support the *in vitro* findings (66). Bryostatin 1 was basically inactive as a complete tumor promoter and very much weaker than the control mezerein as a second stage tumor promoter. If bryostatin 1 and phorbol 12-myristate 13-acetate were administered simultaneously in a complete tumor promotion protocol, bryostatin 1 substantially suppressed the promotion by the phorbol ester. In this experiment, bryostatin 1 and phorbol 12-myristate 13-acetate were administered simultaneously. On the basis of our current understanding of biological responses to the bryostatins in the keratinocyte system, we predict that a more effective protocol might have been to administer the bryostatin 1 several hours before the phorbol ester treatment. Such experiments are now in progress.

The studies with the bryostatins emphasize that different structural classes of protein kinase C activators may yield important new insights into protein kinase C function. In order to fully utilize this approach, it would be highly desirable if one could have synthetic protein kinase C activators the structure of which could be modified more readily than those of many of the natural products. We have collaborated with Dr. Wender's laboratory at the Stanford University Department of Chemistry with the long-term objective of developing such classes of synthetic activators (67). The strategy has been to take advantage of the structurally distinct classes of natural activators of protein kinase C to identify by computer modeling the critical features required for protein kinase C activation and then to incorporate these features into *de novo* synthesized compounds. Comparison of different classes of phorbol esters and related diterpenes, as well as of the indole alkaloids, suggests that the functional groups which are isosteric between these classes are the hydroxyl groups in positions 4, 9, and 20 of phorbol as well as of a hydrophobic domain the spatial orientation of which may be indicated by the fixed positions of the hydrophobic domains in the indole alkaloids and gnidimacrin (Fig. 6). As an initial test of this modeling approach, Wender's group has synthesized two simple ring systems which possess the appropriate hydrophobicity and isosteric functional groups.

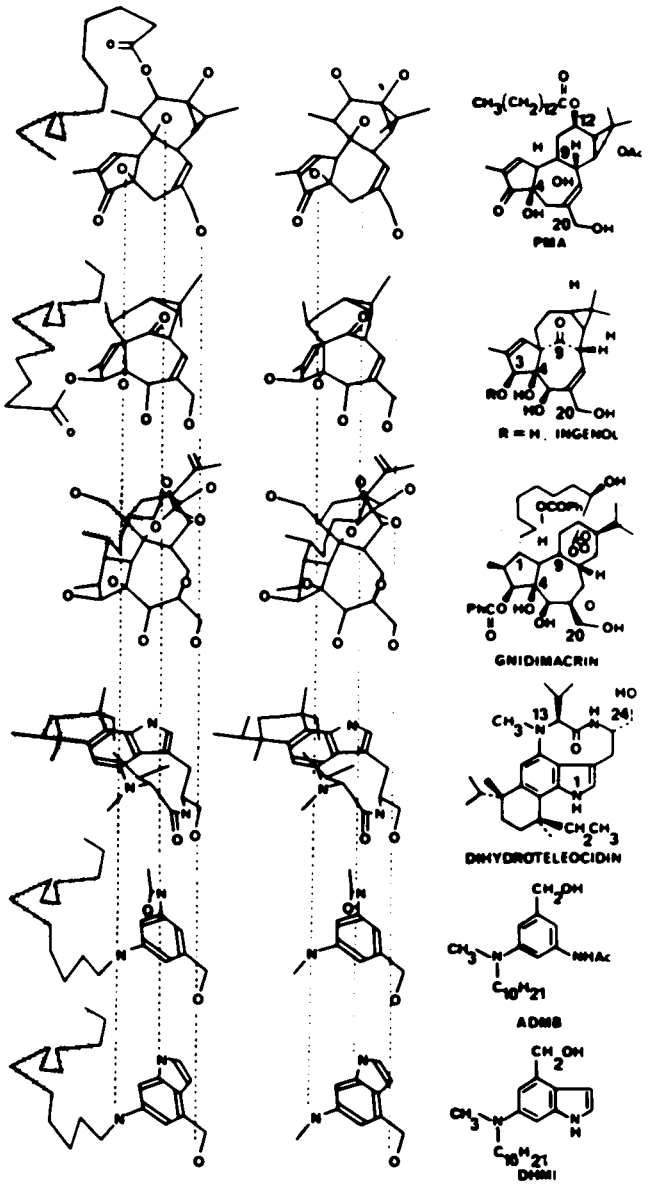

Fig. 6. Comparison of classes of protein kinase C activators. *PMA*, phorbol 12-myristate 13-acetate; *ADMB*, 3-(*N*-acetylamino)-5-(*N*-decyl-*N*-methylamino)benzyl alcohol; *DHMI*, 6-(*N*-decyl-*N*-methylamino)-4-hydroxymethylindole (from Ref. 67; reprinted with permission).

We have analyzed these derivatives for binding activity and for biological response. We showed that 6-(*N*-decylamino)-4-hydroxymethylindole, one of the synthetic derivatives, was in fact able to inhibit phorbol ester binding competitively. Likewise, if added to Swiss 3T3 cells, the compound was able to inhibit the binding of epidermal growth factor, a typical phorbol ester response. Comparison of the activity of this first generation of synthetic compounds indicated that they were approximately 10- to 50-fold less active than the endogenous activators of protein kinase C, the diacylglycerols. As already discussed, the phorbol esters are much more potent compounds than are the endogenous analogues, and the synthetic derivatives are correspondingly less potent. Nonetheless, the activity of the compounds provides strong encouragement that the modeling is on the right track and may ultimately yield potent synthetic probes of the protein kinase C pathway.

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