Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1334–1338, March 1986
Cell Biology

Bryostatin, an activator of the calcium phospholipid-dependent protein kinase, blocks phorbol ester-induced differentiation of human promyelocytic leukemia cells HL-60

(phorbol ester/protein kinase C)

ANDREW S. KRAFT*, JEFFREY B. SMITH†, AND ROGER L. BERKOW‡

*Department of Medicine, Division of Hematology/Oncology, †Department of Pharmacology, and ‡Department of Pediatrics, Division of Hematology/Oncology, University of Alabama, Birmingham, AL 35294

Communicated by Elizabeth C. Miller, October 7, 1985

ABSTRACT **Phorbol esters bind to and activate a calcium phospholipid-dependent protein kinase (C kinase). Some researchers believe that activation of C kinase is necessary for the induction of phorbol ester biologic effects. Our research indicates that bryostatin, a macrocyclic lactone that binds to the phorbol ester receptor in human polymorphonuclear leukocytes, also binds to this receptor in the human promyelocytic leukemia cell line, HL-60. Bryostatin activates partially purified C kinase from HL-60 cells *in vitro*, and when applied to HL-60 cells *in vivo*, it decreases measurable cytoplasmic C kinase activity. Unlike the phorbol esters, bryostatin is unable to induce a macrophage-like differentiation of HL-60 cells; however, bryostatin, in a dose-dependent fashion, blocks phorbol ester-induced differentiation of HL-60 cells and, if applied within 48 hr of phorbol esters, halts further differentiation. These results suggest that activation of the C kinase by some agents is not sufficient for induction of HL-60 cell differentiation and imply that some of the biologic effects of phorbol esters may occur through a more complex mechanism than previously thought.**

Considerable evidence suggests that the biochemical mechanism of action of tumor-promoting phorbol esters involves the activation of a calcium phospholipid-dependent protein kinase (C kinase) (1). When cell-free preparations were studied, this enzyme was found to copurify with the cellular phorbol ester receptor (2). Further studies have shown that [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PBt$_2$) binds to homogeneous preparations of C kinase isolated from mouse brain (3–5) and that phorbol esters compete directly with 1,2-diacylglycerols, the natural activators of the C kinase, for binding to this enzyme (6). The addition of phorbol esters to intact cells induces a marked increase in membrane-associated C kinase (7) and in the phosphorylation of specific membrane proteins—e.g., the transferrin and insulin receptors (8, 9). Activation of C kinase by phorbol esters has been implicated in several biologic responses, including platelet and polymorphonuclear leukocyte (PMN) degranulation, Swiss 3T3 cell mitogenesis, and human lymphocyte activation (1, 10–14). Because of the central role of C kinase, other activators of this enzyme should share most, if not all, of the biologic properties of phorbol esters.

To examine this question, recent studies have used the synthetic diacylglycerol, 1-oleoyl-2-acetylglycerol (OAG), which directly competes in intact cells for binding to phorbol ester receptors (15). OAG, added to Swiss 3T3 cells and platelets, induces similar phosphorylation patterns to those caused by phorbol esters (14, 16, 17); it also induces platelet and PMN degranulation, mitogenesis in Swiss 3T3 cells, and the β-adrenergic cyclic nucleotide response (15, 18, 19). Although phorbol esters cause the human promyelocytic cells (HL-60) to differentiate to macrophages (20, 21), it remains unclear whether OAG, applied even at frequent intervals, can induce differentiation of this cell line (6, 22, 23). To test whether another activator of C kinase can induce differentiation of HL-60 cells (24) as can phorbol esters, we examined whether bryostatin, a newly described C kinase stimulator, could cause this cell line to differentiate.

Recently, bryostatin, a macrocyclic lactone with a significantly different structure from phorbol esters (Fig. 1), has been isolated from the marine bryozoan *Bugula neritina* (25–27). Although bryostatin was initially investigated as an antineoplastic agent and was found to have activity against a limited spectrum of mouse tumors (S. Matthew, personal communication), we have shown that, like phorbol esters, bryostatin induces DNA synthesis in Swiss 3T3 cells (28, 41) and activates human PMN (29). Recent studies have demonstrated that bryostatin will compete with [^{3}H]PBt$_2$ in binding to the intact PMN and that it will stimulate the phosphorylation of proteins in the PMN similar to those induced by phorbol esters. In extracts of normal rat kidney cells, bryostatin activates C kinase identically to the activation by phorbol esters (J.B.S. and A.S.K., unpublished results).

In this paper, we report that, despite its ability to activate C kinase in HL-60 cells and to compete with [^{3}H]PBt$_2$ in binding to HL-60 cells, bryostatin does not induce differentiation of this cell line. Further experiments demonstrate that bryostatin in a concentration-dependent fashion blocks phorbol ester-induced differentiation of HL-60 cells. Furthermore, even after 48 hr of treatment with phorbol esters, we have found that bryostatin can halt further cell differentiation, allowing proliferation of previously undifferentiated cells. Our evidence indicates that, although activation of C kinase is sufficient to induce many phorbol ester effects, induction of differentiation in HL-60 cells is more complex and may involve important stereochemical relationships found only in the phorbol ester molecule.

MATERIALS AND METHODS

We procured bryostatin 1 from George Pettit (Arizona State University, Tempe, AZ), phorbol 12-myristate 13-acetate (PMA) from P-L Biochemicals, phosphatidylserine and diolein from Supelco (Bellefonte, PA), and [^{3}H]PBt$_2$ from New England Nuclear (specific activity, 30.8 Ci/mmol; 1 Ci = 37 GBq). We procured the HL-60 cells from R. Cooper

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Abbreviations: C kinase, calcium phospholipid-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; Bt$_2$, phorbol dibutyrate; PMN, polymorphonuclear leukocyte; OAG, 1-oleoyl-2-acetylglycerol.

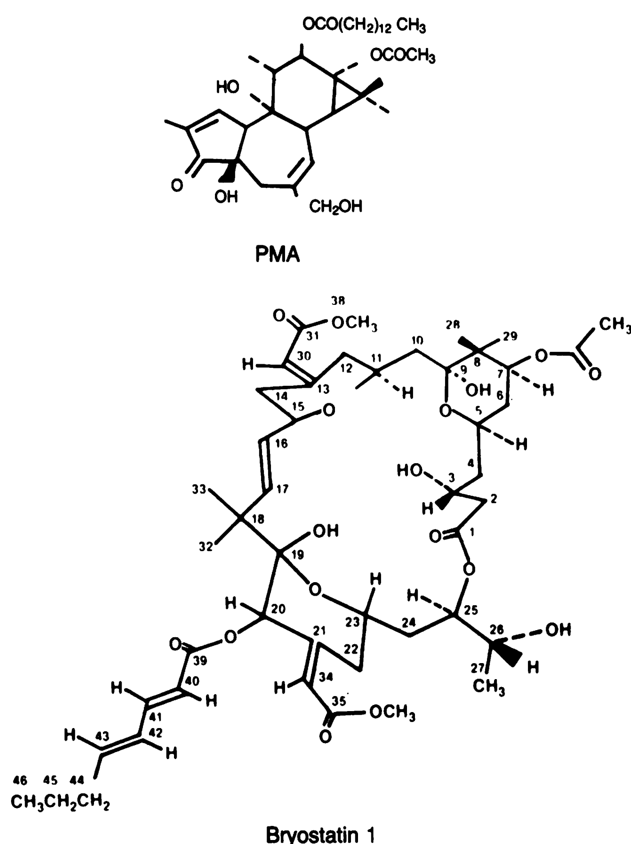

FIG. 1. Structure of bryostatin and PMA. Both PMA and bryostatin were stored in dimethyl sulfoxide at −70°C. Final concentration of dimethyl sulfoxide was always <0.01% and did not affect the assays.

(University of Pennsylvania) and, as described by others, maintained them in RPMI 1640 medium, 10% fetal calf serum, and antibiotics (21). We studied the cells in the exponential phase of their growth.

Competitive binding of [^{3}H]PBt$_2$ to HL-60 cells was carried out by a modification of Lehrer's method (30).

Human PMNs were isolated from the blood of normal volunteers, and superoxide release was assessed by measuring the superoxide dismutase inhibitable reduction of ferricytochrome *c* (31).

As described (7), we assayed C kinase activity in cell extracts, using supernatants of HL-60 cells that had been homogenized and centrifuged at 100,000 × *g*. Fifty micrograms of supernatant protein was assayed for C kinase activity in a 250-μl reaction mixture containing 20 mM Tris·HCl (pH 7.5), 1.0 mM CaCl$_2$, 10 mM MgCl$_2$, 100 μM [γ-^{32}P]ATP (60 cpm/pmol), 25 μg of histone H1, and 16 μg of leupeptin per ml. The amount of phosphatidylserine and/or diolein added to stimulate C kinase activity depended on the experimental design. Small amounts of phosphatidylserine alone (6 μg/ml) were added when additional stimulation by PMA or bryostatin was to be studied. After 3 min at 30°C, the assay was terminated by the addition of 1 ml of ice-cold 20% trichloroacetic acid, filtered over a Millipore filter (0.45 μm), and counted using Filtron-X scintillation fluor.

RESULTS

Bryostatin Does Not Induce Differentiation of HL-60 Cells. HL-60 cells differentiate along different pathways to either mature granulocytic cells when treated with dimethyl sulfoxide, vitamin D, or retinoic acid or to a macrophage-like cell when incubated with phorbol esters. In Table 1, we compare the ability of the phorbol ester PMA and bryostatin to induce differentiation of HL-60 cells. Phorbol ester-induced differentiation is associated with an increase in cytoplasmic staining for nonspecific esterase, a decrease in surface in transferrin receptors as measured by the OKT-9 monoclonal antibody, and an increase in expression of C3bi receptors as shown by increased staining with OKM-1. Also, the phorbol ester-treated cells adhered to the plastic dish, slowed their proliferative rate, and, on Wright's stain, assumed a macrophage-like morphology. In these studies, 10 nM PMA was used, a concentration that we found to be the most potent inducer of differentiation. As previously demonstrated, bryostatin, over a concentration range of 1 nM to 1 μM, activates human PMNs. Within this concentration range bryostatin does not induce differentiation of HL-60 cells. The phorbol ester-induced differentiation was seen in >90% of the cells by 72–96 hr, with no further changes through 7 days. Similar observation of bryostatin-treated cells showed no morphologic changes as late as day 14.

To assess whether degradation of bryostatin caused its inability to induce HL-60 maturation, we performed another experiment. We incubated HL-60 cells with 1 μM bryostatin, 0.1 μM PMA, or a standard culture medium for 72 hr at 37°C in 5% CO$_2$/95% air, harvested and centrifuged the cells, and added a 1:10 dilution of the supernatants to stimulate PMN oxidative burst (see *Materials and Methods*). Previously, we showed that both PMA and bryostatin stimulate superoxide release in human PMN (29). In these studies, we found no diminution of this activity by either bryostatin or PMA after the 72-hr incubation with HL-60 cells at 37°C. Under these

Table 1. Effect of bryostatin or PMA on HL-60 cell differentiation

	Adherence to culture plate	Cell growth, % of control	NSE staining	Morphology	Surface receptors: OKT-9, % positive	Surface receptors: OKM-1, % positive
Control	Negative	100	+	98% undifferentiated	92	15
PMA (10 nM)	Positive	15	++++	95% macrophage-like	15	95
Bryostatin (10 pM to 1 μM)	Negative	96	+	98% undifferentiated	85	20
PMA (10 nM) bryostatin (1 μM)	Negative	91	+	98% undifferentiated	86	10

HL-60 cells growing in RPMI 1640 medium/10% fetal calf serum and antibiotics were plated at 1 × 10^4 cells per ml in 24-well tissue culture dishes with the stated concentration of bryostatin, PMA, or no stimulant. The plates were incubated at 37°C in 5% CO$_2$/95% air for 5 days. Adherence to the culture dish was determined by visual inspection; cell growth was based on cell counts as compared to control. Nonspecific esterase (NSE) staining was performed with a Sigma kit. +, Fine granular pattern not distinguished from background; ++++, numerous large darkly staining granules in the cell cytoplasm. Morphology was based on appearance of Wright's-stained cytocentrifuge preparations. Transferrin receptors were assessed with the OKT-9 monoclonal antibody.

conditions, a 25% decrease in the activity of either bryostatin or PMA would have been detectable. The supernatant from untreated HL-60 cells did not stimulate the PMN oxidative burst, thus suggesting that sufficient bryostatin and PMA activity was retained during the incubation with HL-60 cells to induce biochemical alterations.

Bryostatin Is a Competitive Inhibitor of Phorbol Ester-Induced Differentiation. We conducted experiments to assess whether bryostatin would prevent phorbol ester-induced differentiation of HL-60 cells. When 1 μM bryostatin was added to HL-60 cells simultaneously with 10 nM PMA, no differentiation, as measured by the lack of morphologic and biochemical change, was observed for up to 7 days (Table 1). Next, observing that PMA-induced differentiation causes HL-60 cells to stop proliferating, we assessed the ability of various bryostatin concentrations to inhibit phorbol ester-mediated differentiation. As shown in Fig. 2, when PMA and high concentrations of bryostatin (10 nM to 1 μM) are added together, cell growth proceeds as in the untreated cells. However, at lower bryostatin concentrations, the HL-60 cells are stimulated to differentiate by PMA, and cell growth is inhibited. Fig. 3 shows that when 1 μM bryostatin is added within 7 hr of 10 nM PMA, differentiation of most of the cells does not occur. Even when bryostatin is added at later times, 24–48 hr after PMA is added to the culture, some HL-60 cell proliferation resumes. These proliferating cells resemble control untreated cells. Our data suggest that bryostatin blocks phorbol ester-induced differentiation of HL-60 cells in a dose-dependent fashion and, when added after phorbol ester, halts further differentiation of the HL-60 cells and allows uncommitted cells to begin proliferating.

Bryostatin Binds to the Phorbol Ester Receptor and Activates C Kinase in HL-60 Cells. Because bryostatin blocks PMA effects, we examined whether bryostatin would bind to the phorbol ester receptor in HL-60 cells. This was investigated by incubating HL-60 cell for 30 min at 25°C with 10 nM [^{3}H]PBt$_2$ in the presence of various concentrations of bryostatin or PMA. Nonspecific binding is determined by the addition of a 1000-fold excess of nonradioactive PBt$_2$. The reaction is stopped by rapid filtration, and the [^{3}H]PBt$_2$ bound to 1.25 × 10^6 cells is determined. In a representative experiment, Fig. 4 shows that bryostatin competitively displaces [^{3}H]PBt$_2$ from HL-60 cells. Maximal displacement was seen at concentrations >50 nM and was noted to occur rapidly, being maximal by 10 min. When compared to the displacement by PMA, bryostatin was noted to have a higher ID$_{50}$, suggesting that it has a lower binding affinity to the receptor than does PMA.

To further examine the relationship of bryostatin to the phorbol ester receptor in HL-60 differentiation, we assessed the ability of bryostatin to activate C kinase in HL-60 supernatant *in vitro*. Fig. 5 reveals that over a similar concentration range to phorbol esters, bryostatin activates C kinase from supernatants of HL-60 cells, which had been

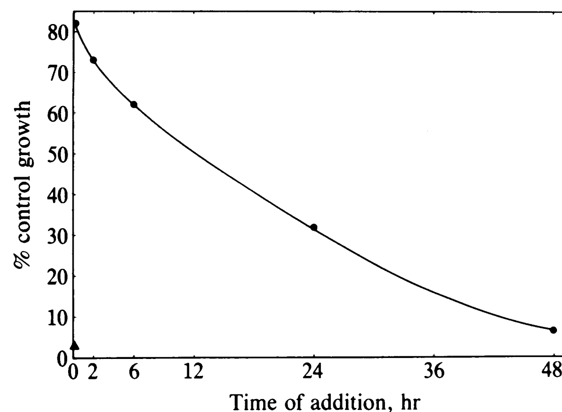

FIG. 3. Effect of time of addition of bryostatin on PMA-induced differentiation. Cells were grown as described in Fig. 2. Control cells were treated with vehicle alone. All other cells received PMA (10 nM) alone (▲), or PMA (10 nM) and, at various time points after the addition of PMA, bryostatin (1 μM) (●). Cells were counted at 120 hr, as described in Fig. 2. Each treatment was done in triplicate, and cell counts varied by <10%. Results are expressed as a percentage of control growth.

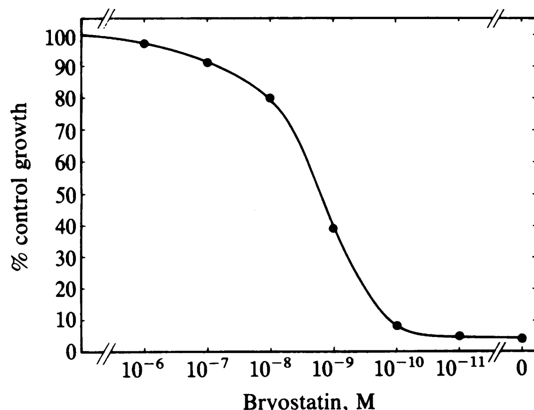

FIG. 2. Effect of various concentrations of bryostatin on PMA-induced cell differentiation. HL-60 cells (1 × 10^5) were plated in a 24-well tissue culture dish and grown as described. Control cells were treated with vehicle alone. All other cells were incubated with PMA (10 nM) with or without various concentrations of bryostatin. At 120 hr after treatment, cells growing in medium and attached to the plastic were counted. Each treatment was done in triplicate, and cell counts varied by <10%. Results are expressed as a percentage of control growth.

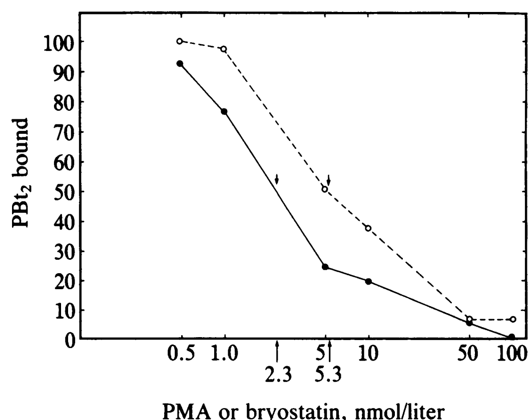

FIG. 4. Competitive displacement of PBt$_2$ from HL-60 cells by bryostatin or PMA. HL-60 cells were harvested during the exponential phase of growth and resuspended (2.5 × 10^6 cells per ml) in phosphate-buffered saline with bovine serum albumin at 4 mg/ml. [^{3}H]PBt$_2$ (10 nM) was incubated with the HL-60 cells in the presence of various concentrations of bryostatin (○) or PMA (●). The incubation was allowed to proceed for 30 min at 25°C. The reaction was terminated by rapid filtration over glass fiber filters, and the cells were washed twice with ice-cold buffer to remove all unbound material. The filters were assayed in 10 ml of Aquasol (New England Nuclear). Nonspecific binding was determined by the addition of a 1000-fold excess of nonradioactivate PBt$_2$ and the value obtained was always <10% of total binding. The values shown represent the percentage of PBt$_2$ bound to cells treated with bryostatin or PMA, compared with cells treated with vehicle alone. Control binding was 6838 ± 812 cpm per 1.25 × 10^6 cells, and nonspecific binding was 567 ± 139 cpm per 1.25 × 10^6 cells. Results are the mean of triplicate samples from a representative experiment. Repeat experiments gave values within 15% when corrected for cell numbers.

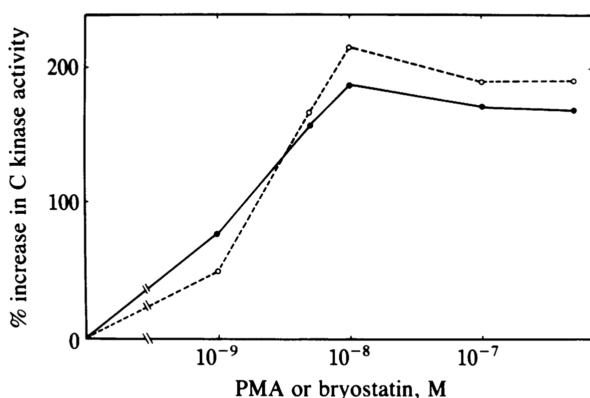

FIG. 5. Effect of bryostatin and PMA on C kinase activity *in vitro*. HL-60 cells were grown as described in Table 1. The cells were washed twice, collected, and disrupted by Dounce homogenization, and a supernatant centrifuged at 100,000 × *g* was prepared (7). Supernatant protein (50 μg) was assayed for C kinase activity in a reaction mixture containing 6 μg of phosphatidylserine in the presence or absence of various concentrations of bryostatin (○) or PMA (●). Experiments carried out in an identical fashion but in the absence of phospholipids were used as background. Background was subtracted from all tubes prior to calculation of results. Results are expressed as percentage of C kinase activity in tubes containing phosphatidylserine plus bryostatin or phosphatidylserine plus PMA compared with that found in tubes containing phosphatidylserine alone. Control tubes without bryostatin or phorbol ester had ≈24,000 cpm per mg of protein per min incorporated.

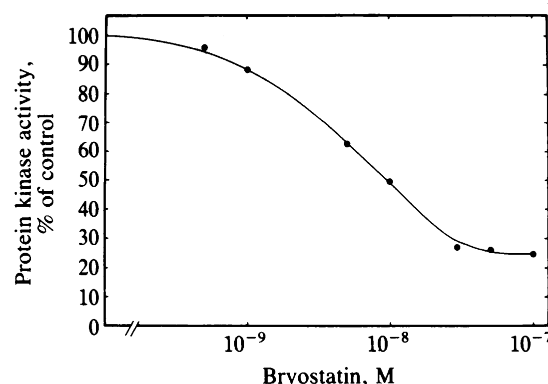

FIG. 6. Effect of treatment of HL-60 cells with increasing concentrations of bryostatin on cytosolic C kinase. HL-60 cells (3 × 10^7) were treated for 30 min at 37°C with the indicated concentration of bryostatin. Supernatants (100,000 × *g*) were prepared as described in Fig. 5, and aliquots (50 μg of protein) were assayed for C kinase activity. Phosphatidylserine (24 μg/ml) and diolein (0.8 μg/ml) were added to the reaction mixture to maximally stimulate kinase activity (7). Results are expressed as the C kinase activity found in the cytosol of cells treated with bryostatin, compared with that found in cells treated with vehicle alone. Control enzyme incorporated ≈45,000 cpm per mg of protein per min.

homogenized and centrifuged at 100,000 × *g*, as demonstrated by an increase in phosphate incorporation into histone H1. Like that of phorbol esters, the extent of this activation is dependent on the concentration of phosphatidylserine in the reaction mixture. At a low phosphatidylserine concentration, such as 6 μg/ml (Fig. 5), bryostatin activates C kinase, but at high concentrations, such as 80 μg/ml, no further stimulation of C kinase activity occurs.

Given the ability of bryostatin to activate C kinase *in vitro*, one would expect this compound to activate C kinase in intact HL-60 cells. We and others have shown that treatment of various cell lines, including mouse thymoma, embryonal carcinoma cells, and human PMNs, with phorbol esters induces a rapid decrease in measurable cytosolic C kinase (32). This decrease is associated with a concomitant increase in membrane-bound C kinase. When HL-60 cells were treated with bryostatin, measurable C kinase activity decreased markedly (Fig. 6). This decrease occurs in a concentration-dependent manner and parallels that induced by phorbol esters, as described (32). Concomitant with this decrease is an increase in C kinase activity associated with the particulate fraction (data not shown). This fact suggests that bryostatin enters the HL-60 cells and activates the C kinase, causing the association of this enzyme with the particulate fraction.

DISCUSSION

Our results indicate that bryostatin binds to the phorbol ester receptor on the HL-60 cells, decreases measurable C kinase from the cytosol, and activates C kinase from HL-60 cell supernatant. Despite the ability of bryostatin to bind and activate this enzyme, which is believed to be the cellular receptor for the phorbol esters (1, 2, 33), it cannot induce differentiation of the HL-60 cells. This inability cannot be explained by degradation of bryostatin, since we have demonstrated that bryostatin is stable in the medium for 72 hr, the time needed to induce differentiation. Furthermore, bryostatin, in a concentration-dependent fashion, blocks phorbol ester-induced HL-60 differentiation. This indicates that although bryostatin is an agonist of C kinase activity, it is an antagonist for phorbol ester-induced differentiation of the HL-60 cell line. These results suggest, therefore, that C kinase activation by certain agents is insufficient to cause phorbol ester-induced differentiation of HL-60 cells. Whereas C kinase may be essential for the differentiation event, the phorbol ester chemical structure may be necessary to induce other events equally essential to the differentiation process.

Additional evidence to support the hypothesis that C kinase activation is not solely responsible for HL-60 differentiation comes from the data presented in Fig. 3. It is shown that when bryostatin is added to HL-60 cells at various times after the addition of phorbol esters, further differentiation stops and proliferation resumes. Most likely, this occurs because of competitive displacement of phorbol esters by bryostatin from the C kinase of the HL-60 cells with proliferation of a previously uncommitted cell population. Supporting data for this hypothesis come from the observation that removal of PBt_2 from HL-60 cells by washing 24 hr after treatment allowed 23% of the cells to continue to grow and divide (34). Of note is the fact that OAG, which activates C kinase but may not induce HL-60 differentiation, is unable to block phorbol ester-induced differentiation (23).

Why would an activator of C kinase, which is believed to be central in phorbol ester-induced differentiation of HL-60 cells, act as an antagonist of this event? Although the gross structures of bryostatin and PMA are quite different, as noted in Fig. 1, some structural similarity is seen. Both six-member rings of bryostatin have side chains that show similarities to the PMA molecule. However, stereochemical similarities have not been observed in other agents, such as alkaloids and aplysiatoxins, which also activate C kinase and differentiation HL-60 cells (35, 36). Thus, it is possible that special spatial restrictions prevent bryostatin from inducing some events necessary for HL-60 differentiation. Conversely, bryostatin may activate a biochemical pathway, in addition to C kinase in HL-60 cells, that inhibits the differentiation of the HL-60 cells.

An additional explanation for the results is that bryostatin may stimulate the C kinase to phosphorylate a different set of proteins than those that are induced by phorbol esters. In human PMNs, however, bryostatin and PMA induce phosphorylation of almost identical proteins (29). Furthermore, in platelets, other activators of C kinase, such as teleocidin and

diacylglycerols, induce protein phosphorylation patterns similar if not identical to those of phorbol esters (35, 37). In HL-60 cells, however, diacylglycerols have been reported to phosphorylate only 10 of the 14 proteins phosphorylated by PMA (22, 23). This raises the possibility that different activators of C kinase may stimulate the phosphorylation of somewhat different proteins, thus leading to the effects seen with bryostatin. In addition, bryostatin may activate C kinase but not cause changes in lipid metabolism as do phorbol esters (38, 39). Furthermore, a recent study of HL-60 cells indicates that OAG does not induce some phospholipid changes that are induced by PMA (23).

Activation of C kinase plays a role in the response of both normal and transformed cells to various mitogenic agents (15, 40). Bryostatin, a weak antineoplastic agent, both activates C kinase and functions as a competitive antagonist for the action of tumor-promoting phorbol esters in HL-60 cells. These results may suggest that the antineoplastic activity of bryostatin is related to its modulation of C kinase and its capability of inhibiting the mitogenic response. Further investigation into the mechanism of bryostatin action may clarify the role of C kinase in modulating growth and differentiation.

Note Added in Proof. Recently, Yamamoto *et al.* (42) have also demonstrated that OAG does not differentiate HL-60 cells.

We thank Mr. Robert Dodson for technical help and Dr. R. Whitley for helpful advice. This work was supported in part by a grant from the Milheim Foundation and by National Institutes of Health Grant AI 22098.

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