Inhibition by Bryostatin 1 of the Phorbol Ester-induced Blockage of Differentiation in Hexamethylene Bisacetamide-treated Friend Erythroleukemia Cells

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INTRODUCTION

Phorbol esters inhibit chemically induced differentiation in Friend erythroleukemia cells. This study examines the effect of the macrocyclic lactone bryostatin 1 on phorbol ester responses in a Friend erythroleukemia cell clone, PS 7. In several biological systems, bryostatin 1 was reported to mimic phorbol ester action, including activation of protein kinase C, but in HL-60 cells it blocked phorbol ester-induced differentiation. We report here that bryostatin 1 blocks phorbol ester action in Friend cells (clone PS 7), a second differentiating system. In this system, in contrast to HL-60 cells, the phorbol esters inhibit rather than induce differentiation. Bryostatin 1 restores the differentiation response (50% effective dose, 15 ± 3.5 nm (SEM)) as well as blocks a second phorbol ester effect, induction of cellular adherence. The inhibition of erythroid differentiation by dexamethasone, a nonphorbol compound whose action presumably is not protein kinase C mediated, is unaffected by bryostatin 1. Although bryostatin 1 inhibits [3H]phorbol 12,13-dibutyrate binding in intact Friend erythroleukemia cell clone PS 7, the mechanism for the antagonism of phorbol ester action by bryostatin 1 in Friend cells cannot be explained by simple competition at the binding site.

ABSTRACT

Phorbol esters inhibit chemically induced differentiation in Friend erythroleukemia cells. This study examines the effect of the macrocyclic lactone bryostatin 1 on phorbol ester responses in a Friend erythroleukemia cell clone, PS 7. In several biological systems, bryostatin 1 was reported to mimic phorbol ester action, including activation of protein kinase C, but in HL-60 cells it blocked phorbol ester-induced differentiation. We report here that bryostatin 1 blocks phorbol ester action in Friend cells (clone PS 7), a second differentiating system. In this system, in contrast to HL-60 cells, the phorbol esters inhibit rather than induce differentiation. Bryostatin 1 restores the differentiation response (50% effective dose, 15 ± 3.5 nm (SEM)) as well as blocks a second phorbol ester effect, induction of cellular adherence. The inhibition of erythroid differentiation by dexamethasone, a nonphorbol compound whose action presumably is not protein kinase C mediated, is unaffected by bryostatin 1. Although bryostatin 1 inhibits [3H]phorbol 12,13-dibutyrate binding in intact Friend erythroleukemia cell clone PS 7, the mechanism for the antagonism of phorbol ester action by bryostatin 1 in Friend cells cannot be explained by simple competition at the binding site.

FELC$ can be induced to differentiate along the erythroid pathway by a variety of cellular agents including HMBA and dimethyl sulfoxide (1, 2). After treatment cells display characteristic morphological changes (1), accumulate globin mRNA (3-5), synthesize α and β globins (6, 7), increase spectrin (8, 9) and heme (10,11) content, and lose the capacity for cell division (3-5), synthesize α and β globins (6, 7), increase spectrin (8, 9) and heme (10,11) content, and lose the capacity for cell division (1, 12). In studies by Rovera et al. (13) and Yamasaki et al. (14, 15), the tumor-promoting phorbol ester PMA and its biologically active congeners were found to be potent inhibitors of chemically induced differentiation in Friend cells.

The biochemical mechanism of action of tumor-promoting phorbol esters involves the binding and activation of the calcium and phospholipid-dependent kinase, PKC (16). Recently, bryostatin 1, as macrocyclic lactone, was shown to activate protein kinase C in vitro (17). The bryostatins represent a group of nonphorbol compounds isolated from the marine bryozoan Bugula neritina (18-20). These compounds were found to exhibit antineoplastic activity when tested with the murine leukemic cell lines P388D1 and L1210 (21).

Although structurally unrelated to the phorbol esters, bryostatin 1 mimics a number of phorbol ester-induced responses in several systems. In studies with PMNs, bryostatin 1 caused the generation of superoxide and the release of specific granules. Furthermore, it inhibited the binding of PDBu to its receptor in PMNs with good affinity. The 50% inhibitory dose of bryostatin 1 was 34 nm compared to 12 nm for PMA (22).

Like phorbol esters, bryostatin 1 induced DNA synthesis in density arrested Swiss 3T3 cell cultures. In this system, Rodriguez-Pena and Rozengurt (23) had observed that PMA pretreatment led to protein kinase C down regulation and desensitization to further mitogenic stimulation by PMA. Consistent with both agents acting through protein kinase C, the mitogenic response to bryostatin 1 was abrogated by pretreatment with PMA, and, conversely, cells desensitized by pretreatment with bryostatin 1 did not respond mitogenically to a subsequent exposure to PMA (24). As seen with PMNs, bryostatin 1 potently inhibited the binding of PDBu to a high affinity receptor in intact Swiss 3T3 cells.

These findings supported the straightforward interpretation that bryostatin 1 and phorbol esters act via the same receptor, protein kinase C. The action of bryostatin in HL-60 cells, a human promyelocytic cell line, was found to be more complex, however. Bryostatin 1 activated partially purified protein kinase C from HL-60 cells in vitro and also bound to this receptor in intact cells. Measurable cytoplasmic protein kinase C activity was decreased in bryostatin-treated HL-60 cells, suggesting the association of the enzyme with the particulate fraction as seen after phorbol ester treatment. Unlike the phorbol esters, however, bryostatin 1 was partially (25) or completely (17) unable to induce a macrophage-like differentiation of HL-60 cells and correspondingly blocked phorbol ester-induced differentiation of HL-60 cells in a dose-dependent fashion.

Similarly, the action of bryostatins in GH4C3 rat pituitary cells was not identical to that of the phorbol esters. Bryostatins 1 and 2 inhibited the binding of [3H]PDBu in intact cells and activated partially purified protein kinase C from the GH4C3 cells. However, the bryostatins were only partial agonists because they enhanced prolactin synthesis to a lesser extent than did phorbol ester and, given in combination, they substantially reduced phorbol ester-induced prolactin release (26).

In this paper we report that, similar to the findings in HL-60 cultures, bryostatin 1 blocks phorbol ester action in Friend cells. The inhibition of Friend cell differentiation by a nonphorbol compound (dexamethasone), which presumably does not act through protein kinase C, is unaffected by bryostatin 1. The action of bryostatin 1 in Friend cells is not a generalized antagonism of inhibitors of differentiation, and its blockage of phorbol ester action cannot be accounted for by simple competition at the receptor, PKC.

MATERIALS AND METHODS

Cell Culture. FELC strain 745A was kindly provided by Dr. Charlotte Friend (Mt. Sinai Medical School, New York, NY). Clones were isolated by inoculating 1 x 10⁶ cells/ml in semisolid medium composed of 0.3% (w/v) Noble agar in α-minimal essential medium (Gibco) supplemented with 100 μg/ml gentamicin and 15% FBS (HyClone). When clones were visible, they were picked and resuspended in 1 ml dimethyl sulfoxide (1,2). After treatment cells display characteristic morphological changes (1), accumulate globin mRNA (3-5), synthesize α and β globins (6, 7), increase spectrin (8, 9) and heme (10,11) content, and lose the capacity for cell division (3-5), synthesize α and β globins (6, 7), increase spectrin (8, 9) and heme (10,11) content, and lose the capacity for cell division (1, 12). In studies by Rovera et al. (13) and Yamasaki et al. (14, 15), the tumor-promoting phorbol ester PMA and its biologically active congeners were found to be potent inhibitors of chemically induced differentiation in Friend cells.

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liquid culture medium in 24-well cluster dishes. Cells were maintained in suspension culture in α-minimal essential medium with 10% FBS in a humidified atmosphere of 5% CO₂ in air. Cultures were routinely transferred to fresh medium twice weekly, at a concentration of 1 × 10⁵ cells/ml. For experimentation, cultures were inoculated at 1 × 10⁴ cells/ml from 1-day-old log growth phase cultures. All studies were done with a clone of cells, designated PS 7, selected for high response to phorbol ester inhibition of differentiation.

Evaluation of Differentiation. Cultures were plated in 96-well culture dishes in the presence of 4 mM HMBA (Sigma). After 5 days the percentage of hemoglobin-containing cells was determined by scoring B+ cells. The benzidine stain was performed on cell suspensions dispersed into multiwell trays as described by Orkin et al. (27). Five to 30 min after the addition of the benzidine reagent, a minimum of 300 cells were scored for each of triplicate samples. Dose-response data were analyzed by the method of McPherson et al. (28) to determine maximal response and ED₅₀. Theoretical curves based on these calculated values were derived by the following relationship (29):

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\text{Response} = \frac{\text{Maximal response} \times [\text{agonist}]}{\text{ED₅₀} + [\text{agonist}]} \]

Adhesion Assay. To determine the effects of compounds on FELC adhesion, cells were plated at 2 × 10⁴ cells/35-mm dish in 2 ml medium plus 10% FBS. Sixteen h later, PDBu and bryostatin 1 were added alone or in combination. After 8 h, nonadherent cells were removed and dishes were rinsed twice with 1-ml volumes of PBS. Cells suspended in the medium and washes were pooled and counted on a Model ZB, Coulter Counter. Adherent cells were detached from culture dishes by repeated pipeting and resuspended in PBS by passing through a 22-gauge needle before counting. The values presented are the means from triplicate dishes.

Binding Assay. Cells were harvested during the exponential phase of growth, washed in PBS, and resuspended at 1 × 10⁶ cells/ml in PBS with bovine serum albumin at 4 mg/ml. [³H]PDBu (10 nM) (specific activity, 30.8 Ci/mmol; New England Nuclear) was incubated with the cells in the presence of various concentrations of bryostatin 1 for 10 min at 37°C. Samples were chilled on ice for 5 min and the reaction was terminated by centrifugation at 10,000 × g for 15 min. One hundred μl of supernatant were removed from each sample and radioactivity was quantitated to determine the concentration of free ligand. After aspirating remaining supernatant, cell pellets were dried and also counted in 2 ml Aquasol (New England Nuclear). Non-specific binding was determined in duplicate for each concentration of bryostatin 1 assayed by including 30 μM nonradioactive PDBu (LC Services).

RESULTS

Inhibition of FELC Differentiation by PDBu. Erythroid differentiation in FELC strain 745A was first assayed without cloning. When cultured with 4 mM HMBA, the proportion of benzidine reactive cells reached a maximum of more than 85% by days 5 and 6. However, inhibition of differentiation by PDBu in the uncloned cell line was variable, with greater than 50% of the population routinely remaining benzidine positive in the presence of 300 nM PDBu. An earlier report by Fibach et al. (30) described the heterogeneity of response to tumor promoter-mediated inhibition of differentiation in Friend cells. Twelve randomly isolated clones were found to vary considerably with respect to phorbol ester sensitivity; the effect of PMA ranged from 0 to >99% inhibition of differentiation.

Based on these findings, FELC strain 745A was cloned as described in “Materials and Methods” and screened for sensitivity to PDBu. A wide response to both HMBA and phorbol ester action was observed among tested clones. Clone PS 7, selected for subsequent studies, consistently yielded ~55% B+ cells in 4 mM HMBA and <8% B+ cells in the presence of HMBA and 200 nM PDBu (Fig. 1, ◦). The experimental PDBu dose-response data show good agreement with the theoretical curve predicted for a response directly coupled with receptor occupancy (29). Fifty % inhibition of the effect of 4 mM HMBA was observed at approximately 19 nM PDBu, very similar to the value of 20 nM reported by Yamasaki et al. (14) for FELC clone DS 19.

Effect of Bryostatin 1 on PDBu Action in FELC Clone PS 7. To examine the effect of bryostatin 1 on phorbol ester action, cells were cultured for 5 days in the presence of 4 mM HMBA, 200 nM PDBu, and varying concentrations of bryostatin 1 from 0-300 nM. In control samples 58.9% of the cells treated with 4 mM HMBA alone were benzidine positive on day 5; cultures treated with HMBA and 200 nM PDBu yielded 4.1% B+ cells (Fig. 2, ◦). Bryostatin 1 restored benzidine staining in the presence of 200 nM PDBu in a dose-dependent fashion, thereby overcoming the phorbol ester-induced inhibition of differentiation. Analysis of the bryostatin 1 dose-response data under these conditions gave a maximal response of 51.1 ± 2.9% B+, and an ED₅₀ of 15.0 ± 3.5 nM, and showed good agreement with the theoretical curve determined from these calculated values.

Interestingly, the calculated maximal responses for bryostatin 1 dose-response studies were consistently lower than the response seen with 4 mM HMBA alone in all replicate experi-
ments. Although bryostatin 1 clearly counteracts the PDBu effect in FELC, it does not restore the percentage of B+ cells to the same level as in control cultures. In HMBA-induced cultures treated with bryostatin 1 only, at concentrations of 1 nM or greater, there was a small but reproducible reduction in percentage of B+ cells relative to control values (Fig. 1, O). Cytotoxicity was not observed and cell cultures reached final cell densities comparable to controls. This observation suggests that bryostatin 1 gives a detectable but limited phorbol ester-like response on Friend cell differentiation, although its major action is phorbol ester antagonistic.

Phorbol esters induce various other responses in Friend cells. Yamasaki et al. (31) characterized the rapid induction of adhesion of suspension cultures of FELC clone DS 19 by PMA. Two PMA-sensitive clones yielded 74 and 93% adherence 4 h after treatment with 100 ng/ml (167 nM) PMA. Phorbol ester treatment of clone PS 7 also induced cell adherence which reached a maximal level 6–10 h after treatment. Treatment with 200 nM PDBu induced 56.1% cell adherence while control cells, treated with vehicle only, remained in suspension (1.9% adherence) (Table 1). Treatment with bryostatin 1 alone had no effect on cellular adherence; however, cells treated with both 200 nM bryostatin 1 and 200 nM PDBu remained nonadherent. Bryostatin 1 thus blocked both phorbol ester-induced responses, cellular adherence, and inhibition of differentiation.

Effect of Bryostatin 1 on Dexamethasone-induced Inhibition of Differentiation in Clone PS 7. The antagonism of the inhibition of differentiation by bryostatin 1 could have been either phorbol ester specific or a more generalized effect. To distinguish between these alternatives, we examined the effect of bryostatin 1 on inhibition induced by a nonphorbol compound whose action presumably would not be PKC mediated. Dexamethasone and other steroids have been shown to inhibit the HMBA-induced differentiation of the Friend cell line DS 19 (32). A similar response was observed in FELC clone PS 7. Cultures were treated with 4 mM HMBA and varying concentrations of dexamethasone for 5 days before staining with benzidine. Differentiation was almost completely inhibited by concentrations of dexamethasone greater than 30 nM, with an EDso of 2.5 nM (data not shown). While control cells treated with HMBA alone gave 54.2 ± 4.1% B+ cells, HMBA-induced cells treated with dexamethasone and bryostatin 1 in combination showed that the inhibition of differentiation by 30 nM dexamethasone (1.4 ± 0.1% B+ cells) was unaffected by bryostatin 1 over the concentration range of 3–300 nM (e.g., 1.1 ± 0.1% B+ cells at 300 nM bryostatin 1). Thus, it appears that the action of bryostatin 1 on PDBu-inhibited inhibition of differentiation is not a generalized effect on cellular differentiation but, rather, may be specific for PKC-mediated events.

Effect of Bryostatin 1 on [3H]PDBu Binding in Clone PS 7. Because bryostatin 1 blocked phorbol ester effects in Friend cells, we examined its ability to inhibit the binding of [3H]PDBu to the phorbol ester receptor. The specific binding of [3H]PDBu has been characterized previously in Friend cells (33, 34). Scatchard analysis revealed one class of specific and saturable [3H]PDBu receptors with high affinity (i.e., Kd = 14.1 ± 4.2 nM; 1.1 × 10^{5} ± 0.22 binding sites/cell) (34). PDBu binding analysis in our FELC clone PS 7 yielded comparable values of 18.1 ± 1.1 for the Kd and 1.4 × 10^{5} ± 0.3 sites/cell. To investigate the action of bryostatin 1 in a binding assay, intact cells were incubated for 15 min at 37°C with 10 nM [3H]PDBu in the presence of varying concentrations of bryostatin 1. Bryostatin 1 was an effective inhibitor of phorbol ester binding in intact PS 7 cells and blocked specific [3H]PDBu binding in a dose-dependent fashion. The percentages of control specific binding in the presence of 2, 20, or 200 nM bryostatin 1 were 72.6 ± 4.4, 45.8 ± 4.4, and 11.9 ± 2.6%, respectively. Thus, at 200 nM, bryostatin 1 inhibited phorbol ester binding greater than 88%. The calculated EDso for the inhibition of PDBu binding by bryostatin 1 was 17.2 ± 5.8 nM from triplicate experiments.

Blockage of Phorbol Ester Action by Bryostatin 1 at Varying Concentrations of PDBu. There are two possible mechanisms which could explain the blockage of phorbol ester action by bryostatin 1. Bryostatin 1 may act directly on PKC at the phorbol ester binding site but cause it to respond in a different manner than do phorbol esters. Alternatively, bryostatin 1 may act at an independent target that PDBu does not recognize but which alters PKC or PKC-mediated responses. In the competitive model, a high concentration of phorbol ester would displace bryostatin 1 from the receptor and would thus reverse the bryostatin effect. In the second model which supposes an independent site of action, bryostatin 1 would act noncompetitively and its effect would not be reversed by a high concentration of PDBu. In studies with FELC clone PS 7, the action of bryostatin 1 on PDBu response was observed even in the presence of 600 nM PDBu (data not shown), suggesting the possible existence of a bryostatin target for which nanomolar quantities of PDBu cannot compete.

To distinguish more clearly between a competitive or noncompetitive model, we assayed bryostatin 1 dose responses at different levels of PDBu, 20 and 200 nM (Fig. 2). The experimental dose-response data are expressed as percentage of benzidine positive cells. In PDBu control cultures (bryostatin 1 = 0 nM), percentage of differentiation was 7.7% at 200 nM PDBu but 27.9% at 20 nM, due to only partial inhibition of differentiation of this concentration of phorbol ester. Bryostatin 1 inhibited PDBu action in a dose-dependent fashion at both concentrations of PDBu. The EDso for bryostatin 1 was 19.3 ± 6.3 nM at 20 nM PDBu and 14.9 ± 3.6 nM at 200 nM PDBu.

A competitive mechanism of action for bryostatin 1 would predict that the dose-response curve at 20 nM PDBu would shift toward lower concentrations of bryostatin 1 relative to the dose-response curve at 200 nM PDBu. The magnitude of the shift in EDso is determined by the relationship 1 + [B]/Kb (35), where [B] = [PDBu] and Kb = binding affinity of PDBu (15 nM in FELC clone PS 7). Thus for a competitive mechanism of action, the EDso for bryostatin 1 at 200 nM PDBu would be expected to be ~6 times greater than the EDso at 20 nM PDBu; for a noncompetitive mechanism, no shift would be observed and EDso would be identical. The EDso for bryostatin 1 calculated from the data in Fig. 2 (19.3 and 14.9 nM) show no indication of the shift expected for a competitive mechanism and suggest that the effect of bryostatin 1 on PDBu action in FELC clone PS 7 is noncompetitive.

DISCUSSION

The studies presented here indicate that the blockage of phorbol ester effects by bryostatin, as first suggested in HL-60 cells, is a more generalized category of behavior. In HL-60 cells, where PMA treatment induces macrophage-like differ-
entiation (36), bryostatin 1 blocked phorbol ester action even though it was able to bind and activate PKC in this cell system (17). Similarly, although bryostatin 1 interacts with the PDBu binding sites in Friend cells, it inhibits rather than mimics phorbol ester effects on erythroid differentiation. The action of bryostatin 1 in Friend cells, however, is not simply at the level of cellular ability to differentiate since the compound is unable to reverse dexamethasone-induced inhibition of differentiation. Thus, the effect of bryostatin 1 on phorbol ester-induced differentiative responses may well be PKC specific.

The dose-response studies with FELC PS 7 in the presence of two different concentrations of PDBu indicate that the inhibition of phorbol ester action by bryostatin 1 observed here is noncompetitive rather than a simple, direct interaction with the phorbol ester binding site. One possible model to explain these findings could be the existence of a unique target for bryostatin 1 action on the PKC pathway. However, in light of recent knowledge about the existence of a family of PKC genes (37, 38) and the complexity of subcellular localization of PKC, a more direct effect on PKC should not be excluded.

Investigation of the anti-phorbol ester action of bryostatin 1 may lend important insight into a general mechanism for the inhibition of PKC responses. For drug selectivity, bryostatin 1 offers another possible level of heterogeneity of response to aid in the search for compounds which may specifically modulate PKC activity. Also, because of its antagonism of phorbol ester effects on cellular differentiation, suggested as a site for promotor action in skin (39), bryostatin 1 may possess anti-promotor activity in vivo. Lastly, bryostatin 1 illustrates the utility of natural product chemistry in the isolation of critical new probes to investigate biological systems.

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