Differential Effects of Bryostatin 1 and Phorbol Ester on Human Breast Cancer Cell Lines

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ABSTRACT

The effects of the protein kinase C (PKC) activators, phorbol ester 12-O-tetradecanoyl-13-phorbol acetate (TPA) and the marine natural product, bryostatin 1, on the growth and morphology of human breast cancer cell lines were examined. TPA (1 to 100 nM) inhibited growth of four of six cell lines by up to 75% in 5-day cultures. Bryostatin 1 inhibited growth of only MCF-7 cells and only at a high dose (100 nM). However, bryostatin 1 completely antagonized the growth inhibition and morphological changes induced by TPA in MCF-7 cells. The divergent effects of these two agents are associated with differing effects on PKC activity and isoform expression in MCF-7 cells. TPA induced rapid translocation of the PKC-α isozyme and PKC activity to the membrane fraction of MCF-7 cells. In contrast, bryostatin 1 treatment resulted in the loss of the PKC-α isozyme and PKC activity from both cytosolic and membrane compartments within 10 min of treatment. In coincubation assays the bryostatin 1 effect was dominant over that of TPA. Similar effects on PKC-α isoform and PKC activity were seen in a second cell line whose growth was inhibited by TPA but not by bryostatin 1, MDA-MB-468. In contrast, in the T47D cell line, where TPA was not growth inhibitory, TPA failed to induce translocation of PKC-α to the cell membrane. Bryostatin, however, still caused loss of PKC-α isoform and PKC activity from cytosolic and membrane fractions. Thus, differential actions of bryostatin 1 and TPA on PKC activity and α isoform levels in the membrane-associated fraction of MCF-7 and MDA-MB-468 cells may account for the divergent effects of these two agents on cell growth and morphology. These results suggest that the PKC-α isoform may specifically play a role in inhibiting growth of human breast cancer cells.

INTRODUCTION

Tumor-promoting phorbol esters such as TPA can elicit pleiotropic responses in cultured cells, including either stimulatory or inhibitory effects on growth and differentiation (1, 2). The biological effects of phorbol esters are thought to be mediated by interaction of these agents with PKC (3, 4). PKC plays a role in the transmembrane signalling of a variety of extracellular stimuli, ranging from serotonin release in platelets to lipogenesis in adipocytes (5–7). The binding of certain extracellular ligands to their receptors results in the cleavage of phosphatidylinositol 4,5-bisphosphate to yield the two second messengers, inositol 1,4,5-bisphosphate and DAG. DAG then binds to PKC, resulting in its translocation within the cell to the membrane and activation of its serine-threonine protein kinase function. Signal transduction is then thought to proceed via phosphorylation of cellular targets (8). Bryostatin 1, a macrocyclic lactone isolated from the marine bryozoan Bugula neritina, is also a potent activator of PKC (9). Bryostatin 1 has been found to have effects both agonistic and antagonistic to those of TPA (10–12). Both TPA and bryostatin 1 can substitute for DAG as PKC activators through their ability to bind to PKC and elicit subsequent subcellular translocation activation (13).

Several lines of evidence suggest a role for PKC in human breast carcinogenesis. PKC activity has been shown to be significantly elevated in primary human breast cancer specimens compared with surrounding normal breast tissue (14). Furthermore, in human breast cancer cell lines including MCF-7, treatment with TPA results in growth inhibition by blocking cell entry into the G1 phase and acquisition of a more differentiated phenotype. These effects are reversible on removal of TPA from the medium whereupon cell growth is resumed. Thus, a negative regulatory role for PKC in MCF-7 cell proliferation has been suggested (15, 16).

From a chemotherapeutic perspective bryostatin 1 is a particularly attractive compound as it possesses antineoplastic activity against a variety of leukemias in vitro (17), stimulates proliferation of normal human hematopoietic progenitors (18), and lacks the tumor-promoting properties of TPA (19). Given these properties, bryostatin 1 may have a role in the treatment of human malignancies. A puzzling aspect of the biological activity of bryostatin 1 is that in some systems it can mimic and in others antagonize the effects of TPA (10–12). The molecular mechanisms responsible for the differential effects of bryostatin 1 and TPA are unclear. As PKC consists of a family of several closely related enzyme isoforms with distinct tissue-specific patterns of expression (20–22), one possible explanation for the divergent effects may be that the two agents differentially activate selective isoforms.

The purpose of the work described here was to determine if bryostatin 1 could inhibit the growth of human breast cancer cell lines in vitro and to compare its effects with those of the phorbol ester TPA in order to further define the role of PKC in breast cancer cell growth. We report that TPA but not bryostatin 1 inhibits the growth of several human breast cancer cell lines, including MCF-7. We tested whether the divergent actions of bryostatin 1 and TPA could be explained by differential effects on PKC isoforms. In MCF-7 and MDA-MB-468 cells, where TPA is growth inhibitory, TPA treatment led to rapid translocation of the PKC-α isoform to the membrane-associated fraction, whereas bryostatin 1 treatment resulted in the loss of PKC-α from both cytosolic and membrane compartments. However, in T47D cells, the lack of TPA-induced growth inhibition was associated with a failure to translocate PKC-α to the membrane fraction of treated cells. Bryostatin 1 treatment, however, still produced rapid loss of membrane-associated PKC-α. These data suggest that the ability of TPA to translocate PKC-α to the membrane-associated fraction is a critical event in TPA-mediated growth inhibition for certain breast cancer cell lines. These findings could explain the different effects of these two agents on the growth of a number of human breast cancer cell lines.
MATERIALS AND METHODS

Cell Lines and Culture. MCF-7, ZR-75-1, MDA-MB-231, HaS578t, and T47D cells were obtained from the laboratory of Dr. Marc Lippman, Vincent Lombardi Cancer Center (Washington, DC), and MDA-MB-468 cells were obtained from the American Type Culture Collection (Rockville, MD). All cells were grown in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with glutamine (0.6 g/liter), gentamicin (25 mg/liter), and 5% FCS (Biofluids, Rockville, MD). Cultures were grown at 37°C in 5% CO₂ and routinely tested negative for Mycoplasma contamination.

Reagents. Bryostatin 1 was the kind gift of Dr. G. Robert Pettit, Arizona State University (Tempe, AZ). Stock solutions were kept at a concentration of 1 mM in DMSO at -20°C and diluted as required into DMSO or medium. TPA (Sigma, St. Louis, MO) was maintained in DMSO or medium. Reagents used to perform the assay in the presence of 6 mM EGTA were kept on ice for 15 min before TPA, and cells were grown and counted as above. Cell morphology was recorded by photographing cultures after 5 days of incubation with the test agents.

Fractionation and Partial Purification of Protein Kinase C. Subconfluent cells were held overnight in serum-free medium, and then vehicle, bryostatin 1, TPA, or both agents were added for varying time periods. Monolayers were washed; scraped into cold PBS; resuspended in sterile buffer containing 20 mM sodium fluoride, 0.1 mM sodium vanadate, 1% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of leupeptin, and held on ice for 15 min. Cells were then sheared through a 22-gauge needle, sonicated, and centrifuged at 205,000 x g for 15 min at 4°C. The resulting supernatant contained the membrane fraction of the cell preparation.

The membrane and cytosolic fractions were then passed over DEAE-cellulose columns (1-ml bed volume) equilibrated with 25 mM Tris (pH 7.4), 0.5 mM EGTA, 10 μg/ml of leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The column was washed with 5 bed volumes of buffer, and samples were eluted in equilibration buffer with 0.4 M NaCl. The eluates were then brought to 0.1 M NaCl and loaded onto phenyl-Sepharose columns (0.3-ml bed volume; Pharmacia LKB Biotechnolog, Inc.), equilibrated with 7 mM Tris (pH 7.4), 0.1 M EDTA, 0.1 M diithiothreitol, and 2.75 M NaCl. Fractions were recovered by elution in NaCl-free buffer and assayed for protein concentration by measuring the absorbance at 260 nm against a standard curve. All procedures were carried out at 4°C.

Immunoblot Analysis of Protein Kinase C. Immediately after fractionation equal amounts of protein (20 to 85 μg) from purified cell fractions were solubilized in sodium dodecyl sulfate-Laemmli sample buffer, subjected to polyacrylamide gel electrophoresis on 10% polyacrylamide gels, and transferred to nitrocellulose at a constant 60 V for 9 h. The filters were stained with Fast green to confirm efficient transfer and even loading and blocked overnight at room temperature with 3% bovine serum albumin and 0.02% sodium azide in PBS. The blocked filters were incubated for 2 h with antibody specific to PKCα, PKC-βII, or PKC-δ, or PBS for 60 min at room temperature, and washed 5 times with 15 ml of 0.1% (v/v) Tween 20 in PBS for 10 min. The filters were then incubated with 2.5 μCi of 125I-Protein A in 3% (w/v) bovine serum albumin and 0.1% (v/v) sodium azide in PBS, washed as before, and autoradiographed with Kodak XAR-5 film at -70°C.

Assay of Total Protein Kinase C Activity. Fifty-μl aliquots containing equal amounts of protein (10 to 43 μg) were added to a reaction buffer of 25 mM Tris (pH 7.4), 10 μM ATP, 40 μg/ml of phosphatidylserine, 20 mM MgCl₂, 2 mM diithiothreitol, 1 mg/ml of histone, 1 μCi of [32P] ATP, and 2 mM CaCl₂ in a final volume of 150 μl. Reactions were carried out at room temperature for 15 min and stopped by adding 150 μl of 20% trichloroacetic acid. Histone protein was then precipitated onto glass microfiber filters (Whatman), and the incorporation of radiolabeled phosphate into histone IIa was measured in a liquid scintillation counter. Baseline incorporation of 32P was determined by subtracting this from the activity seen in the presence of calcium.

RESULTS

Effect of TPA and Bryostatin 1 on Cell Proliferation and Morphology. The effect of 0.01 to 100 nM bryostatin 1 or TPA on growth of six human breast cancer cell lines over 5 days of culture was determined. As shown in Fig. 1A, TPA significantly inhibited the growth of four of the cell lines tested, including the well-characterized MCF-7 line, in a dose-dependent fashion.

![Fig. 1. Effects of TPA and bryostatin 1 on the growth of human breast cancer cell lines in vitro](cancerres.aacrjournals.org)
In contrast bryostatin 1 slightly inhibited the growth of only one cell line, MCF-7, only at the highest concentration tested, 100 nM. A longer time course with MCF-7 cells confirmed that 100 nM TPA for 12 days inhibited MCF-7 growth by 80% of control, unlike bryostatin 1 which had little effect (Fig. 1B). Interestingly, bryostatin 1 produced minimal growth stimulation of Hs578t cells at the highest dose levels tested (Fig. 1A).

Treatment of cells with bryostatin 1 together with TPA blocks the phorbol ester response in a number of systems. Therefore, we performed competition studies in which MCF-7 cells were treated with bryostatin 1 for 30 min before the addition of TPA. Fig. 2A shows that incubation of MCF-7 cells with bryostatin 1 antagonized the growth-inhibitory effects of 100 nM TPA in a dose-dependent fashion. Equimolar doses of bryostatin 1 completely abrogated the growth-inhibitory effects of TPA. Similar antagonistic effects were seen when exposure of cells to bryostatin 1 was begun 30 min before, at the same time as, or 30 min after treatment with TPA (not shown). Identical dose-dependent abrogation of TPA’s growth-inhibitory effects was also seen with bryostatin 1 in two other cell lines examined, MDA-MB-231 and Hs578t (not shown).

The effects of TPA and bryostatin 1 on the morphology of MCF-7 cells were also examined. The morphological changes seen paralleled the effects on cell growth. As shown in Fig. 2B, treatment of MCF-7 cells with 100 nM TPA alone resulted in characteristic morphological changes after 4 days. Cells became large, rounded, and vacuolar, and giant cells were observed. In contrast 100 nM bryostatin did not alter cell appearance. Furthermore treatment with bryostatin 1 blocked the TPA-mediated morphological changes, again in a dose-dependent fashion. At equimolar doses bryostatin 1 completely abrogated TPA-mediated changes in MCF-7 morphology, and no difference from control cells could be observed. Thus TPA but not bryostatin 1 inhibits the growth and alters the morphology of human breast cancer cell lines, including MCF-7. However, bryostatin 1 abrogates these effects of TPA.

PKC Isotype Expression and Activity in Human Breast Cancer Cells. We next tested whether the divergent effects of bryostatin 1 and TPA in MCF-7 cells could be due to the differential translocation and activation of PKC isotypes. Affinity-purified...
antibodies for PKC-α, PKC-βII, and PKC-γ were used to determine expression of these isotypes in MCF-7 cells by immunoblotting. MCF-7 cells expressed both PKC-α and PKC-γ but not PKC-βII (not shown). Membrane-associated PKC-α was the predominant isofrom, although cytosolic PKC-α was also evident (Fig. 3A). In contrast PKC-γ isoform was detected predominantly in the cytosolic fraction (Fig. 3A).

The effect of treatment with TPA or bryostatin 1 on the localization of PKC-α and PKC-γ in MCF-7 cells was examined. Cells were treated with bryostatin 1 or TPA atug 100 nM for 10 or 30 min, and cytosolic and membrane fractions were analyzed. The results are shown in Fig. 3. No changes were seen in PKC-γ localization with either agent. However, treatment with TPA resulted in the translocation of cytosolic PKC-α to the membrane fraction within 10 min, and the effect persisted for at least 30 min (Fig. 3A). Bryostatin 1 had a different effect. While PKC-α was lost from the cytosolic fraction, suggesting that translocation occurred, there was also clear evidence of loss of PKC-α from the membrane fraction within 10 min, and complete loss by 30 min. More detailed analysis of the time course of bryostatin's effects showed that the loss of PKC-α from the membrane fraction began by 1 min (Fig. 3B). No evidence for an early increase in membrane-associated PKC-α levels was detected, suggesting rapid compensatory degradation of PKC-α.

In view of these divergent effects on the PKC-α isomer, MCF-7 cells were cocultured with equimolar amounts of TPA and bryostatin 1 for 30 min to see if bryostatin 1 would antagonize the TPA effect. Immunoblot analysis showed that the effect of bryostatin 1 was dominant as demonstrated by the loss of detectable membrane-associated PKC-α after 30 min (Fig. 4A). Thus bryostatin 1 can abrogate TPA's effects on growth and morphology of MCF-7 cells, and this antagonism is associated with rapid down-regulation of the PKC-α isofrom in the membrane-bound fraction.

**Fig. 3.** Immunoblot analysis of PKC-α and PKC-γ isoforms in MCF-7 breast cancer cells treated with TPA or bryostatin 1. In A, protein was loaded for electrophoresis at 22.5 μg/well. Odd-numbered lanes, cytosolic fractions of cells; even-numbered lanes, membrane-associated fractions. Lanes 1 and 2, DMSO vehicle; Lanes 3 and 4, 100 nM TPA for 10 min; Lanes 5 and 6, 100 nM bryostatin 1 for 10 min; Lanes 7 and 8, 100 nM TPA for 30 min; Lanes 9 and 10, 100 nM bryostatin 1 for 30 min. In B, protein was applied for electrophoresis at 37.5 μg/well. Odd-numbered lanes, cytosolic fractions of cells; even-numbered lanes, membrane-associated fractions. Lanes 1 and 2, DMSO vehicle; Lanes 3 and 4, 100 nM bryostatin 1 for 1 min; Lanes 5 and 6, 100 nM bryostatin 1 for 5 min; Lanes 7 and 8, 100 nM bryostatin 1 for 10 min; Lanes 9 and 10, 100 nM TPA for 10 min.

To assess further the functional significance of the PKC protein changes seen by immunoblot, we also measured total PKC activity after exposure of MCF-7 cells to 100 nM TPA, bryostatin 1, or the combination. Fig. 4B demonstrates that PKC activity was translocated from the cytosolic to membrane-bound fraction in TPA-treated cells within 30 min. In cells exposed to bryostatin 1 there was evidence of loss of activity from both cytosolic and membrane-bound fractions. Coincubation assays again showed that the bryostatin 1 effect was dominant. Cells which were exposed to TPA and bryostatin 1 simultaneously demonstrated a rapid decrease in enzyme activity in the membrane-bound fraction to levels observed following bryostatin 1 alone. These changes mirror the alterations in PKC-α isoform expression seen with immunoblot analysis (Fig. 4A).

We wished to extend these findings to other human breast cancer cell lines. Similar experiments were performed on MDA-MB-468 and T47D cells. Growth of the MDA-MB-468 cell line was also inhibited by TPA but not by bryostatin 1. As in MCF-7 cells, PKC-α was again the predominant isofrom detected by immunoblot analysis. Treatment with 100 nM bryostatin 1 resulted in a rapid loss of PKC-α within 10 min, while cells treated with 100 nM TPA showed increased amounts of membrane-associated PKC-α at both 10 and 30 min (Fig. 5A). PKC kinase activity was simultaneously translocated from the cytosolic to membrane-associated fractions by treatment with TPA, while the PKC activity was decreased in both cytosolic and membrane fractions following incubation with bryostatin 1 (Fig. 5B).

In order to determine if TPA growth-inhibitory effects were specifically associated with increased PKC-α levels in the mem-
when MCF-7 cells are treated with both agents simultaneously. Cell growth, morphology, and PKC-α isoform levels and activity were seen in the membrane fraction of T47D cells exposed to TPA. Bryostatin 1, however, still caused loss of activity was seen in the membrane fraction of T47D cells. Furthermore, bryostatin 1 can block the effects of TPA on differential actions of the two agents on membrane-associated PKC-α. In two cell lines, MCF-7 and MDA-MB-468, these changes are associated with differential patterns of expression in a variety of cell systems, suggesting that different isoforms may play different roles in the transmembrane signalling process (24).

In the T47D cell line, where TPA is not growth inhibitory, TPA does not appear to cause an increase in membrane-associated PKC-α. These data demonstrate that selective activation of PKC-α can be associated with a dramatic effect on growth and differentiation of human breast cancer cells in vitro.

Our results show that TPA treatment of MCF-7 and MDA-MB-468 cells results in the rapid translocation of PKC activity and the α-isofrom from the cytosolic to the membrane fraction. Bryostatin 1 induces the rapid loss of cytosolic and membrane-associated PKC-α isoform and total kinase activity and blocks the TPA-mediated effects on PKC. Bryostatin 1 is known to be an activator of PKC in a variety of cell systems. It is somewhat puzzling that treatment with this agent resulted only in the down-regulation of PKC activity and PKC-α isoform in the human breast cancer cell lines studied. The data presented here suggest that bryostatin 1 treatment results in a more rapid degradation of the membrane PKC-α. Although we were unable to demonstrate any transient translocational activation of PKC even after only 1 min of bryostatin 1 exposure, the net effect of the bryostatin 1-mediated down-regulation could be to result in a net release of cells from a growth-inhibitory effect mediated by a more prolonged activation of membrane PKC-α. The observation that TPA treatment produces neither growth inhibition nor increases in membrane-associated PKC-α in the T47D cell line suggests that a transient increase in membrane-associated PKC-α is a critical event in TPA-mediated growth inhibition. Such a scenario could account for the observation that bryostatin 1 blocks the growth-inhibitory effects of TPA in certain breast cancer cells. The rapid activation and subsequent down-regulation of PKC activity by bryostatin 1 would suggest that bryostatin 1 treatment results in a more rapid activation and subse-

bran fraction, we examined the effects of TPA and bryostatin on T47D cells, a human breast cancer cell line where TPA caused no inhibition of growth. The results of immunoblot assays of PKC-α levels and PKC activity assays are outlined in Fig. 6. In contrast with MCF-7 and MDA-MB-468 cells, no transient increase in the levels of PKC-α isoforms or PKC activity was seen in the membrane fraction of T47D cells exposed to TPA. Bryostatin 1, however, still caused loss of PKC-α isoform and PKC activity from both cytosolic and membrane-associated fractions.

Thus, TPA and bryostatin 1 have differential effects on cell growth, membrane-associated PKC-α isoform levels, and total PKC activity in at least 3 different human breast cancer cell lines.

DISCUSSION

The protein kinase C family plays a key role in the transduction of external growth and differentiation signals. A number of studies show that the members of the PKC family exhibit differential patterns of expression in a variety of cell systems, suggesting that different isoforms may play different roles in the transmembrane signalling process (24). We have shown here that two potent PKC activators, TPA and bryostatin 1, have divergent effects on the growth and cell morphology of several human breast cancer cell lines. In two cell lines, MCF-7 and MDA-MB-468, these changes are associated with differential actions of the two agents on membrane-associated PKC-α. Furthermore, bryostatin 1 can block the effects of TPA on cell growth, morphology, and PKC-α isoform levels and activity when MCF-7 cells are treated with both agents simultaneously.
then render the cell refractory to the effects of subsequent TPA treatment.

Other groups have looked at the effects of TPA and bryostatin 1 on PKC isomers in systems where these agents have antagonistic effects. For example, phorbol dibutyrate stimulates differentiation of HL60 cells, while bryostatin 1 blocks this morphological effect and stimulates continued cell proliferation (25). Immunoblot analysis shows that HL60 cells express equal amounts of immunoreactive \( \alpha \) and \( \beta_1 \) PKC (23). Treatment of these cells with bryostatin 1 leads to complete translocation of both isoforms from cytosol to cell membrane, while phorbol dibutyrate treatment leads to complete translocation of PKC-\( \alpha \) but only partial translocation of PKC-\( \beta_1 \) to the plasma membrane (23). These data suggest that in this system also the antagonistic actions of TPA and bryostatin 1 may be due to differing effects on specific isoforms of PKC.

While our results are compatible with the hypothesis that the divergent effects of bryostatin 1 and TPA in human breast cancer cells may be explained by different effects on expression and activity of the PKC-\( \alpha \) isoform, it is also possible that the agents interact variably with other cellular target(s). For example, we have not addressed the possibility that the selective activity of these agents on other members of the PKC family not studied here could also explain their different biological effects. Also, studies by Warren et al. (26) have demonstrated the presence in HL60 and C3H/10T1/2 of two 70,000 proteins which are phosphorylated by bryostatin 1 at low (e.g., 6 nM) concentrations but by phorbol esters only at very high doses (e.g., 600 nM). Thus the possible existence of additional non-PKC targets for bryostatin 1 with which phorbol esters interact only at very high doses cannot be excluded.

Finally, although bryostatin 1 does not inhibit growth of human breast cancer cell lines in vitro, its ability to induce the rapid loss of PKC activity from the membrane fraction of treated human breast cancer cells may have significant biological implications. There is a large body of evidence that modulation of PKC may affect the metastatic capacity of tumor cells. For example, Gopalakrishna and Barsky (27) found that the metastatic capacity of B16 melanoma cell sublines was strongly correlated with their basal level of membrane-bound PKC activity. Activation of PKC by brief treatment with TPA rendered the cell refractory to the effects of subsequent TPA concentrations but by phorbol esters only at very high doses (e.g., 600 nM). This thus the possible existence of additional non-PKC targets for bryostatin 1 with which phorbol esters interact only at very high doses cannot be excluded.

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