Bryostatin 1 Inhibits Phorbol Ester-Induced Apoptosis in Prostate Cancer Cells by Differentially Modulating Protein Kinase C (PKC) δ Translocation and Preventing PKC δ -Mediated Release of Tumor Necrosis Factor- α

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ABSTRACT

Bryostatin 1, a macrocyclic lactone that has been widely characterized as an ultrapotent protein kinase C (PKC) activator, displays marked pharmacological differences with the typical phorbol ester tumor promoters. Bryostatin 1 impairs phorbol 12-myristate 13-acetate (PMA)-induced tumor promotion in mice and is in clinical trials as an anticancer agent for a number of hematopoietic malignancies and solid tumors. In this study, we characterized the effect of bryostatin 1 on LNCaP prostate cancer cells, a cellular model in which PKC isoforms play important roles in the control of growth and survival. Although phorbol esters promote a strong apoptotic response in LNCaP cells via PKC δ -mediated release of TNF α , bryostatin 1 failed to trigger a death effect even at high concentrations, and it pre-

vented PMA-induced apoptosis in these cells. Mechanistic analysis revealed that bryostatin 1 is unable to induce TNF α release, and it impairs the secretion of this cytokine from LNCaP cells in response to PMA. Unlike PMA, bryostatin 1 failed to promote the translocation of PKC δ to the plasma membrane. Moreover, bryostatin 1 prevented PMA-induced PKC δ peripheral translocation. Studies using a membrane-targeted PKC δ construct revealed that the peripheral localization of the kinase is a requisite for triggering apoptosis in LNCaP cells, arguing that mislocalization of PKC δ may explain the actions of bryostatin 1. The identification of an antiapoptotic effect of bryostatin 1 may have significant relevance in the context of its therapeutic efficacy.

Introduction

Activation of protein kinase C (PKC) isoforms leads to a plethora of cellular responses, including mitogenesis, cell growth arrest, survival, apoptosis, differentiation, and transformation. This family of serine-threonine kinases has been originally identified as the major cellular target for the phorbol esters and related tumor promoters. Biochemical and structural analysis established that two subgroups, namely the classical (cPKC α , β I, β II, and γ) and the novel (nPKCs δ , ϵ , η , and θ) PKCs, bind phorbol esters with nanomolar affinity and respond to the lipid second-messenger diacylglycerol (DAG), a product of membrane phospholipid hydrolysis. Phorbol esters and DAG bind to the C1 domains present in

the N-terminal regulatory region of PKCs, an event that triggers the activation of these kinases (Griner and Kazanietz, 2007). Members of the PKC family have been extensively implicated in the control of the cell cycle, apoptotic cascades, and survival pathways. For example, PKC δ activation leads to cell growth arrest through multiple mechanisms, including up-regulation of the cell cycle inhibitor p21^{cip1}, reduction in cyclin levels, and dephosphorylation of the retinoblastoma protein. PKC δ is required for the apoptotic cell death induced by phorbol esters and chemotherapeutic agents such as etoposide. On the other hand, PKC ϵ generally activates survival and mitogenic pathways in normal and cancer cells (Reyland et al., 1999; Blass et al., 2002; Basu and Sivaprasad, 2007; Griner and Kazanietz, 2007).

Pharmacological manipulation of PKC isoforms gained importance not only for understanding the basic biological processes and players in DAG signaling but also because of its therapeutic relevance. In fact, phorbol esters, DAG analogs, and other PKC activators are in clinical trials for cancer

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ABBREVIATIONS: PKC, protein kinase C; DAG, diacylglycerol; CM, conditioned medium; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; PMA, phorbol 12-myristate 13-acetate; RNAi, RNA interference; siRNA, small interfering RNA; TNF, tumor necrosis factor; DAPI, 4,6-diamidino-2-phenylindole; PEP005, ingenol-3-angelate; myr, myristoylated; TACE, tumor necrosis factor- α -converting enzyme; n, novel; c, classical.

treatment (Barry and Kazanietz, 2001; Schaar et al., 2006). The bryostatins, natural products isolated from the marine organism *Bugula neritina*, are atypical PKC modulators. These compounds are ultrapotent activators of PKC isozymes in vitro that bind to C1 domains in cPKCs and nPKCs (Kazanietz et al., 1994). However, although bryostatins mimic several phorbol ester responses, they display unusual pharmacological properties because they fail to induce phorbol ester-like responses in many cellular models. More interestingly, bryostatins functionally antagonize phorbol ester responses that they themselves are unable to elicit, including tumor promotion (Hennings et al., 1987; Szallasi et al., 1994). Bryostatin 1, a prototype bryostatin analog, exhibits characteristic anticancer effects, because it inhibits tumor growth, invasion, and angiogenesis, and it is currently in phase I and II clinical trials against a number of cancers, both alone and in combination with other antineoplastic agents (Propper et al., 1998; Varterasian et al., 2000; Zonder et al., 2001; Clamp et al., 2003; Roberts et al., 2006).

Although phorbol esters exert major effects on cell survival and proliferation, a number of cell types undergo apoptotic cell death in response to phorbol ester treatment (Powell et al., 1996; Reyland et al., 2000; Gonzalez-Guerrico and Kazanietz, 2005). LNCaP prostate cancer cells emerged as one of the most extensively characterized models that undergo apoptosis in response to PKC activators. Our laboratory established that in LNCaP prostate cancer cells, phorbol 12-myristate 13-acetate (PMA) induces apoptosis primarily through the activation of PKC δ (Tanaka et al., 2003; Gonzalez-Guerrico and Kazanietz, 2005). A thorough analysis of the mechanisms underlying this response led to the identification of a PKC δ -mediated autocrine loop that involves the secretion of death factors, primarily TNF α . Impairing this autocrine loop essentially abolishes phorbol ester-induced apoptosis (Gonzalez-Guerrico and Kazanietz, 2005).

In the present studies, we established that the failure of bryostatin 1 to cause LNCaP cell death is due to its inability to translocate PKC δ to the plasma membrane and promote TNF α release. Moreover, bryostatin 1 functionally antagonizes peripheral PKC δ translocation and TNF α release from prostate cancer cells in response to phorbol ester stimulation.

Materials and Methods

Materials. PMA was purchased from LC Laboratories (Woburn, MA). Bryostatin 1 was purchased from EMD Biosciences (Gibbstown, NJ). 4', 6-Diamidino-2-phenylindole (DAPI) was obtained from Roche Diagnostics (Indianapolis, IN). Recombinant human TNF α was purchased from PeproTech Inc. (Rocky Hill, NJ).

Cell Culture. LNCaP cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum from HyClone Laboratories (Logan, UT), penicillin (100 U/ml), and streptomycin (100 μ g/ml) at 37°C in a humidified 5% CO $_2$ atmosphere. Cells were used from passages 2 to 8.

Western Blot Analysis. Cells were harvested in lysis buffer containing 50 mM Tris-HCl, pH 6.8, 10% glycerol, and 2% β -mercaptoethanol. Equal amounts of protein (20 μ g protein/lane) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA). After blocking with 5% milk/0.1% Tween 20 in Tris-buffered saline, membranes were incubated with primary antibodies against PKC α , myc (Millipore Corporation), PKC δ (Cell Signaling Technology Inc., Danvers, MA), PKC ϵ (Santa Cruz Biotechnology Inc., Santa Cruz, CA), vinculin, or β -actin (Sigma-Aldrich, St. Louis,

MO). After extensive washing, membranes were incubated for 1 h with either anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:5000; Bio-Rad Laboratories, Hercules, CA). Bands were visualized by enhanced chemiluminescence.

RNA Interference. Double-stranded siRNAs for PKC isozymes were purchased from Dharmacon RNA Technologies (Lafayette, CO). The following target sequences were used: PKC α 1, CCAUCCGCUC-CACACUAAA; PKC α 2, GAACAACAAGGAAUGACUU; PKC δ 1, CCAUGAGUUUAUCGCCACC; PKC δ 2, CAGCACAGAGCGUG-GGAAA; PKC ϵ 1, GUGGAGACCUCUAUGUUUCA; and PKC ϵ 2, GACGUGGACUGCACAAGA. As controls, we used either Silencer Negative Control siRNA1 or Silencer Negative Control siRNA2 (Ambion, Austin, TX). siRNAs (120 pmol) were transfected into LNCaP cells (2×10^6) using the Amaxa nucleofactor (Amaxa Biosystems, Gaithersburg, MD), and experiments were carried out 48 h after transfection (Gonzalez-Guerrico and Kazanietz, 2005).

Treatment of LNCaP Cells and Generation of Conditioned Medium. LNCaP cells (~70% confluent) were treated for 1 h with PMA or bryostatin 1 alone or in combination, or vehicle. After 24 h, cells were collected for the determination of apoptosis. Alternatively, conditioned medium (CM) was collected, filtered through a 13-mm syringe filter (0.45 μ m pore size; Fisher Scientific, Waltham, MA), added to naive LNCaP cells (~70% confluence), and apoptosis was determined 24 h later.

Generation of Myristoylated PKC δ Constructs. PKC δ containing a C-terminal myc-tag was polymerase chain reaction-amplified from pShooterPKC δ (generous gift from Dr. Chaya Brodie, Henry Ford Hospital, Detroit, MI) and flanked with 5'-XbaI and 3'-EcoRI restriction sites. The polymerase chain reaction product was cloned into XbaI and EcoRI sites in pCMV6myr (kindly provided by Dr. Alex Toker, Harvard Medical School, Boston, MA). In the resulting construct (PKC δ myr), PKC δ is N-terminally fused to a myristoylation signal sequence and contains a C-terminal myc-tag.

Transfections. Expression vectors encoding PKC δ cyto, PKC δ myr, or empty vector (pcDNA3.1) were transfected (2 μ g) into LNCaP cells using the Amaxa Nucleofactor according to the manufacturer's instructions. Cells were assayed for apoptosis 24 h after transfection.

Apoptosis Assay. Apoptosis was determined as described previously (Tanaka et al., 2003; Xiao et al., 2008).

Immunofluorescence and Confocal Microscopy. For localization studies of GFP-fused PKCs, LNCaP cells were transfected with 2 μ g of pEGFP-N1-PKC α , pEGFP-N1-PKC δ , or pEGFP-N1-PKC ϵ using the Amaxa Nucleofactor and plated on coverslips in 12-well plates. After 48 h, cells were stimulated with PMA and/or bryostatin 1, washed with PBS, and fixed with precooled methanol (-20°C). Samples were stained with DAPI (1 μ g/ml) for 10 min at 4°C, mounted on a glass slide, and visualized with a Zeiss LSM510 META NLO laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NJ). In a different set of experiments, LNCaP cells transfected with plasmids encoding myc-tagged PKCs were fixed as described above and incubated with a rabbit polyclonal anti-myc antibody for 1 h at room temperature in 5% bovine serum albumin (1:500), followed by incubation with a secondary Alexa Fluor 488-coupled antibody (1:2000; Invitrogen, Carlsbad, CA) for 1 h at room temperature. Slides were washed, counterstained with DAPI, and visualized by confocal microscopy.

Enzyme-Linked Immunosorbent Assay. TNF α levels in CM were determined with a Human ELISA Development Kit from PeproTech Inc. in 96-well plates. Measurements were done in triplicate.

Statistical Analysis. Analysis of variance was performed using GraphPad Prism software built-in analysis tools (GraphPad Software, Inc., San Diego, CA). The confidence interval was set to 95%. A $p < 0.05$ was considered statistically significant.

Results

Bryostatin 1 Fails to Induce Cell Death and Inhibits PMA-Induced Apoptosis in LNCaP Cells. Most PKC ago-

nists, including phorbol esters, DAGs, and ingenol-3-angelate (PEP005), bind to the C1 domains in cPKCs and nPKCs leading to kinase activation *in vitro*. In androgen-dependent prostate cancer cells, these PKC activators induce a strong apoptotic response (Garcia-Bermejo et al., 2002; Ogbourne et al., 2004; Gonzalez-Guerrico and Kazanietz, 2005; Xiao et al., 2009). However, previous studies showed that long-term treatment (24–72 h) of LNCaP prostate cancer cells with bryostatin 1, another C1 domain ligand for PKCs, does not affect LNCaP cell viability. Such long-term treatment causes a marked down-regulation of PKC isozymes, primarily PKC α (Gschwend et al., 2000). We were interested in the effects of short-term treatment with bryostatin 1 on LNCaP cells, an effect that should be independent of PKC down-regulation. Indeed, short treatment (1 h) of LNCaP cells with bryostatin 1 (0.1–100 nM) did not appreciably change the expression of PKC α , PKC δ , or PKC ϵ , the 3 phorbol ester- and bryostatin-responsive PKCs expressed in these cells (Fig. 1A). As we reported previously (Tanaka et al., 2003), a 1-h treatment with PMA caused a marked apoptotic response in LNCaP cells. On the other hand, bryostatin 1 failed to cause apoptosis at all concentrations tested (0.1–100 nM) under similar experimental conditions. Moreover, coincubation of bryostatin 1 with PMA inhibited the apoptotic effect of the phorbol ester in a dose-dependent manner (Fig. 1, B and C). In agreement with a previous study (Gschwend et al., 2000), a 24-h treatment with bryostatin 1 also failed to promote LNCaP apoptotic cell death. Representative micrographs of LNCaP cultures and nuclear DAPI stainings in response to the different treatments are shown in Fig. 1C. These results suggest that bryostatin 1 acts as a functional antagonist of PMA-induced apoptosis in LNCaP prostate cancer cells.

Bryostatin 1 Prevents PMA-Stimulated Release of TNF α from LNCaP Cells. Previous studies from our labo-

ratory established that PMA promotes the release of death factors from LNCaP cells, primarily TNF α . RNA interference (RNAi) depletion or inhibition of TACE (the enzyme responsible for TNF α shedding), neutralization of TNF α in the CM, or blockade/depletion of TNF α receptors markedly reduces PMA-induced apoptosis in LNCaP cells, arguing for a critical involvement of this autocrine loop in the death effect of the phorbol ester (Gonzalez-Guerrico and Kazanietz, 2005). To determine whether alterations in autocrine mechanisms are responsible for the inhibition of PMA-induced apoptosis by bryostatin 1, we compared the apoptotic activity of CM collected from cells treated with vehicle (CM-vehicle), bryostatin 1 (CM-Bryo1), PMA (CM-PMA), or bryostatin 1 and PMA (CM-Bryo1/PMA). When added to naive LNCaP cells (recipient cells), CM-PMA induced a prominent apoptotic response, as we reported previously (Gonzalez-Guerrico and Kazanietz, 2005). On the other hand, like CM-vehicle, CM-Bryo1 failed to induce apoptosis. It is noteworthy that the apoptotic effect of CM-Bryo1/PMA was significantly lower than that of CM-PMA (Fig. 2A). These results suggest that bryostatin 1 not only fails to promote the release of death factors from LNCaP cells, it also prevents the secretion of apoptogenic factors that are otherwise released in response to phorbol ester stimulation.

Next, we examined the effect of bryostatin 1 on TNF α release from LNCaP cells. In our previous studies (Gonzalez-Guerrico and Kazanietz, 2005; Xiao et al., 2009) we established that PMA causes a marked release of this cytokine, as determined by ELISA, and that this effect was abolished by either TACE inhibition or TACE RNAi depletion. We have extensively ruled out that the effect was related to any carryover of C1 domain ligand (Gonzalez-Guerrico and Kazanietz, 2005). Using a similar approach, we found that unlike PMA, bryostatin 1 treatment failed to induce any measurable

Fig. 1. Short-term bryostatin 1 treatment prevents PMA-induced apoptosis in LNCaP cells. **A**, LNCaP cells were treated with different concentrations of bryostatin 1 (0.1–100 nM, 1 h) and subjected to Western blot analysis using specific antibodies for PKC isozymes. **B**, LNCaP cells were treated for 1 h with PMA or bryostatin 1 alone or in combination at the concentrations indicated in the figure, and the percentage of apoptotic cells determined 24 h later. The effect of 24 h treatment with bryostatin 1 is also shown. Data represent the mean $\pm$ S.E.M. of three independent experiments. **C**, representative micrographs of LNCaP cell cultures (left) and apoptotic cells, as indicated with arrows (right), 24 h after treatment with PMA and/or bryostatin 1 (100 nM).

release of TNF α from LNCaP cells, and more importantly, it essentially blunted TNF α release by PMA (Fig. 2B).

To determine whether the reduced secretion of TNF α is a critical determinant for the impaired apoptotic response of PMA by bryostatin 1, we pursued a rescue approach adding exogenous TNF α to the CM. We reasoned that by supplementing this cytokine to CM-Bryo1/PMA to levels similar to those normally observed in CM-PMA, we would be able to restore the apoptotic response. A caveat in this same experiment is that the final concentration of TNF α was <20% than that added to the CM [$y = 180.2x + 2.4$, $R^2 = 0.9999$; where y is TNF α in CM (in picograms per milliliter) and x is added TNF α (in nanograms per milliliter)]. Possible reasons for this reduced recovery may be decreased stability and/or misfolding of the synthetic TNF α . It is noteworthy that the addition of exogenous TNF α was able to rescue the proapoptotic effect of CM-Bryo1/PMA in a concentration-dependent manner (Fig. 2C). Based on the linear correlation established between the added and measured TNF α concentrations in the CM, we were able to estimate the actual TNF α concentrations in CM-Bryo1/PMA. Data clearly show that complete rescue could be achieved at concentrations of TNF α that were in a range similar to those present in the CM (compare with Fig. 2B).

PKC δ Is a Key Regulator of PMA-Induced TNF α Release from LNCaP Cells. A previous report showed that the ability of bryostatin 1 to impair PMA-induced apoptosis is related to the ability to down-regulate PKC α from LNCaP cells, although unlike our studies, that was a long-term (24–72 h) treatment (Gschwend et al., 2000). Because our previous studies assigned a key role for PKC δ in PMA-induced apoptosis (Tanaka et al., 2003), we decided to explore this issue in more detail by means of an RNAi approach. The three PMA-responsive PKCs expressed in LNCaP cells were individually knocked down using two different siRNA duplexes in each case, which minimized misinterpretation of results because of “off-target” effects of RNAi. We success-

fully achieved isozyme-specificity depletion for each PKC with either duplex relative to cells transfected with control siRNA (Fig. 3A). Our data revealed that the apoptotic effect of PMA was substantially reduced in PKC δ -knockdown LNCaP cells but not in cells in which either PKC α or PKC ϵ was depleted (Fig. 3B). A slightly higher apoptotic effect of PMA was observed in PKC ϵ -depleted LNCaP cells, consistent with its prosurvival effect in this model (McJilton et al., 2003; Meshki et al., 2010). Most importantly, only PKC δ depletion could significantly impair TNF α release by the phorbol ester, whereas depletion of either PKC α or PKC ϵ failed to block secretion of the death factor (Fig. 3C). These data not only suggest that PKC δ is crucial for PMA-induced TNF α release in LNCaP cells, they also argue in favor of PKC δ as the main PKC implicated in the protection of PMA-induced apoptosis by bryostatin 1.

Bryostatin 1 Selectively Inhibits the Translocation of PKC δ to the Plasma Membrane in LNCaP Cells. At this point, we raised the question of whether bryostatin 1 could induce changes in the subcellular localization of PKC δ . It is well established that translocation of PKC isozymes is a hallmark of enzyme activation. We speculated that the failure of bryostatin 1 to induce apoptosis in LNCaP cells may relate to its failure to translocate PKC δ to the plasma membrane, which may ultimately lead to the inability to release TNF α . To this end, we assessed the translocation using GFP-fused PKCs. LNCaP cells were transfected with pEGFP-N1-PKC α , pEGFP-N1-PKC δ , or pEGFP-N1-PKC ϵ , and 48 h after transfection, cells were treated with PMA or bryostatin 1, alone or in combination. Localization of the GFP-fused PKCs was assessed by confocal microscopy. PMA redistributed PKC α and PKC ϵ to the plasma membrane, whereas PKC δ is dually translocated to the plasma and nuclear membranes (Figs. 4, C, G, and K). Similar patterns of translocation for these PKCs have been reported in various cell types (Wang et al., 1999a, 2004; Garcia-Bermejo et al., 2002). Like PMA, bryostatin 1 caused significant translocation of PKC α and

Fig. 2. Bryostatin 1 prevents the release of TNF α from LNCaP cells. **A**, LNCaP cells were treated with bryostatin 1 (10 nM) and/or PMA (100 nM) or vehicle. CM was collected 24 h later and added to a naive culture of LNCaP cells. The percentage of apoptotic cells was determined 24 h later. Data represent the mean $\pm$ S.E.M. of three independent experiments; *, $p < 0.05$. **B**, TNF α levels were measured by ELISA in CM collected 24 h after the different treatments. Data represent the mean $\pm$ S.E.M. of three independent experiments. **C**, LNCaP cells were treated with vehicle, PMA, or bryostatin 1 in combination with PMA at the concentrations indicated. CM was collected 24 h later and added to naive LNCaP cells. After 24 h, the percentage of apoptotic cells was determined. CM-Bryo1/PMA was supplemented with TNF α at the indicated concentrations. The concentration of TNF α in the CM-Bryo1/PMA adjusted by ELISA is also shown. Data represent the results of three independent experiments (mean $\pm$ S.E.M.).

PKC ϵ to the cell periphery, although the effect was less pronounced for PKC α (Fig. 4, B and J). On the other hand, bryostatin 1 caused a pronounced translocation of PKC δ to the nuclear membrane, but remarkably, translocation to the plasma membrane could not be readily detected (Fig. 4F). PMA retained its ability to translocate PKC α and PKC ϵ to the cell periphery when LNCaP cells were cotreated with bryostatin 1 (Fig. 4, D and L). In contrast, peripheral relocalization of PKC δ by PMA was significantly reduced in cells treated with bryostatin 1, whereas accumulation of PKC δ at the nuclear membrane remained essentially unaffected (Fig. 4H). Therefore, these findings demonstrate a different sub-cellular relocalization of PKC δ depending on the ligand. Most importantly, they reveal that bryostatin 1 has the ability to

prevent the translocation of PKC δ to the plasma membrane without significantly altering the distribution of other PKCs. It is therefore conceivable that translocation of PKC δ to the plasma membrane is a requisite for the induction of apoptosis in LNCaP cells by PKC activators.

Translocation of PKC δ to the Plasma Membrane Is Essential for the Induction of Apoptosis in LNCaP Cells. Localization of PKC isozymes is key for determining the nature of the cellular response to PKC activators. PKCs can differentially translocate to multiple compartments in a cell type-specific manner, leading ultimately to a differential access to substrates or accessory interacting proteins (Schechtman and Mochly-Rosen, 2002; Gomel et al., 2007). Our data point to plasma membrane redistribution of PKC δ as an essential step for PMA-induced apoptosis in LNCaP cells. To test this hypothesis, we generated a construct in which PKC δ was fused at the N terminus to a myristoylated (myr) tag that directs the kinase to the plasma membrane and at the C terminus to an myc-tag for detection. LNCaP cells were transfected with plasmids encoding either wild-type PKC δ , which localizes essentially in the cytoplasm (PKC δ_{cyto}) or the membrane-targeted PKC δ (PKC δ_{myr}), and apoptosis was assessed 24 h later. Transfection efficiencies were ~15%, as determined with vectors encoding GFP (data not shown). Expression by Western blot is shown in Fig. 5A. Upon ectopic expression of PKC δ_{cyto} in LNCaP cells, we could not detect any measurable apoptotic response. In contrast, ectopic expression of PKC δ_{myr} resulted in significant cell death (Fig. 5B). Similar effects were observed upon expression of a constitutively active PKC δ_{myr} mutant (data not shown). To confirm the localization of PKC δ_{myr} at the plasma membrane, confocal microscopy was performed. As shown in Fig. 5C, this modified the form of the kinase localized exclusively in the cell periphery, whereas PKC δ_{cyto} was found essentially in the cytoplasm of LNCaP cells. Thus, targeting PKC δ to the plasma membrane is sufficient to promote apoptosis in LNCaP cells. Taken together, our data strongly argue that the lack of an apoptotic response in

Fig. 3. PKC δ is essential for TNF α release induced by PMA. LNCaP cells were transfected with different siRNA duplexes (1 or 2) for each PKC isozyme or a control siRNA. After 48 h, cells were treated with PMA (100 nM, 1 h). A, Western blot for PKC α , PKC δ , and PKC ϵ 48 h after transfection. Similar results were observed in three independent experiments. B, effect of individual PKC isozyme depletion on PMA-induced apoptosis, as determined 24 h after treatment. Data represent the mean $\pm$ S.E.M. of three independent experiments, *, $p < 0.05$ versus control siRNA. C, TNF α levels in CM from vehicle- or PMA-treated LNCaP cells subject to PKC isozyme RNAi depletion. Data represent the mean $\pm$ S.E.M. of three independent experiments; *, $p < 0.05$ versus control siRNA.

Fig. 4. Bryostatin 1 impairs PMA-induced peripheral translocation of PKC δ in LNCaP cells. LNCaP cells expressing GFP-PKC α , GFP-PKC δ , or GFP-PKC ϵ were stimulated for 20 min with vehicle, 100 nM PMA, or bryostatin 1, alone or in combination. Localization was determined by confocal microscopy. Representative micrographs are shown. Similar results were observed in multiple cells in at least three separate experiments.

response to bryostatin 1 and the abrogation of PMA-induced apoptosis by bryostatin 1 are due to the inability of PKC δ to translocate to the plasma membrane.

Discussion

Studies in animal models established that the marine-derived macrocyclic lactone bryostatin 1 displays pronounced antitumor activity. Moreover, remarkable antiproliferative and apoptotic activities for bryostatin 1 have been reported in numerous cancer cell lines (Stone et al., 1988; Wang et al., 1998, 1999b). Bryostatin 1 is in phase II clinical trials for the treatment of various solid cancers, such as melanoma, colorectal, ovarian and cervical cancers, and hematopoietic cancers (Propper et al., 1998; Varterasian et al., 2000; Zonder et al., 2001; Clamp et al., 2003; Roberts et al., 2006). The effectiveness of bryostatin 1 for the treatment of prostate cancer still remains to be determined. Our limited understanding on how bryostatin 1 modulates prostate cancer cellular fate and exerts actions in prostate models via its main targets, the PKC isozymes, has greatly limited the development of pre-clinical studies with this drug for prostate cancer.

Unlike phorbol esters and DAG analogs, the typical PKC activators, bryostatin 1 is unable to kill LNCaP prostate cancer cells. Moreover, bryostatin 1 treatment prevents PMA from inducing apoptosis in LNCaP cells. Our previous studies established that PKC δ is the main mediator of phorbol ester-induced apoptosis in LNCaP cells (Tanaka et al., 2003;

Gonzalez-Guerrico and Kazanietz, 2005; Xiao et al., 2009). However, despite the ability of bryostatin 1 to strongly activate PKC δ in vitro (Kazanietz et al., 1994), this compound failed to translocate PKC δ to the plasma membrane in LNCaP cells, arguing that the cellular context is key for determining the outcome of the bryostatin 1 response. It is noteworthy that bryostatin 1 treatment was unable to stimulate TNF α release from LNCaP cells. PMA, on the other hand, depends on the autocrine secretion of this cytokine for killing prostate cancer cells (Gonzalez-Guerrico and Kazanietz, 2005). The impaired TNF α secretion by PMA when cells are simultaneously treated with bryostatin 1 seems to fully account for the functional antagonism. Indeed, adding back this cytokine at final concentrations similar to those normally observed in CM-PMA fully restores the apoptotic activity. It is interesting that bryostatin 1 is able to promote apoptosis and TNF α release from leukemia cells (Cartee et al., 2003; Wang et al., 2003), suggesting a strict cell type-dependence for the differential killing ability of this agent. TNF α release from prostate cancer cells by PMA is mediated by PKC δ (Gonzalez-Guerrico and Kazanietz, 2005). This paradigm has been validated recently in vascular smooth muscle cells (Reddy et al., 2009). Although the mechanistic basis of this process is not yet understood, PKC has been implicated in TACE activation and TNF α ectodomain shedding in several models (Shao et al., 2003; Wheeler et al., 2003). TACE may be a direct PKC substrate, a paradigm that would certainly require PKC δ relocation to the plasma membrane. Indeed, several putative PKC phosphorylation sites could be predicted in TACE using the NetPhosK 1.0 Server with Evolutionary Stable Sites filtering (<http://www.cbs.dtu.dk/services/NetPhosK/>). Alternatively, TACE may be phosphorylated by PKC downstream effector kinases, as reported previously (Díaz-Rodríguez et al., 2002). In any given scenario, our data established that relocation of PKC δ to the plasma membrane of LNCaP cells is required for promoting an apoptotic response. Remarkably, expression of a membrane (myr)-targeted PKC δ in LNCaP cells is sufficient to promote apoptosis. Moreover, limiting the access of PKC δ to the plasma membrane prevents the apoptotic effect of PMA.

The ability of bryostatin 1 to differentially translocate PKC isozymes is a well documented phenomenon. Early studies using GFP-tagged PKCs revealed distinctive patterns of translocation for PKC isozymes in living cells in response to different PKC activators and other stimuli. In this regard, it is striking that ligands that are all capable of binding with high affinity to the C1 domains confer such distinct patterns of PKC relocation, a paradox that is particularly observed for PKC δ (Wang et al., 1999a, 2000). The pattern of translocation of PKC δ in LNCaP cells resembles that observed originally in Chinese hamster ovary cells, in which bryostatin 1 translocates PKC δ predominantly to the nuclear membrane (Wang et al., 1999a). Our studies revealed that in LNCaP cells, bryostatin 1 prevents PKC δ translocation to the plasma membrane by PMA but still allows for nuclear membrane relocation of PKC δ and without significantly affecting translocation of PKC α or PKC ϵ to the plasma membrane. It is noteworthy that an early study showed that bryostatin 1 differentially protects PKC δ from down-regulation by PMA (Lorenzo et al., 1997), suggesting major differences in the regulation of each PKC isozyme by bryostatins. Our results strongly support the hypothesis that bryostatin 1 exerts its

Fig. 5. Targeted localization of PKC δ to the plasma membrane induces apoptosis in LNCaP cells. LNCaP cells were transfected with myc-tagged vectors encoding PKC δ cyto, PKC δ myr, or empty vector as a control. A, expression was determined by Western blot using an anti-myc-tag antibody. B, percentage of apoptotic cells 24 h after transfection. Data represent the mean $\pm$ S.E.M. of three independent experiments. C, localization of PKC δ cyto and PKC δ myr in LNCaP cells 24 h after transfection.

antiapoptotic effects in LNCaP cells through a functional antagonism driven by mislocalization of PKC δ , ultimately restraining the access of this kinase to plasma membrane substrates. The basis for the differential subcellular translocation for PMA and bryostatins 1 remains to be determined. The complexities of the mechanisms governing the interactions between the C1 domain in PKCs, C1 domain ligands, and the lipid bilayers are indeed poorly understood. Studies by the Blumberg laboratory established that a key determinant for conferring ligand selectivity for PKC translocation is lipophilicity (Wang et al., 2000). It is tempting to speculate that PMA and bryostatins 1 exert their differential relocalization patterns based on distinct hydrophobic interactions with the lipid bilayer. C1 domain ligands fit in a hydrophilic cleft that renders the C1 domain surface more hydrophobic, thus allowing for the insertion of this domain in the lipid bilayer. Additional interactions between the side chains of the ligand and the membrane may be therefore key for dictating intracellular selectivity through specific association with membrane components. Another exciting possibility that needs to be explored is that ligands modulate the association of PKCs to interacting proteins in a differential manner.

In conclusion, bryostatins 1 is not an efficient killing agent for LNCaP prostate cancer cells because of its inability to release TNF α , a necessary step for the apoptotic effect induced by PKC activators. Moreover, bryostatins 1 possesses antiapoptotic activity in LNCaP cells. Because several chemotherapeutic agents depend on PKC δ to promote prostate cancer cell death (Sumitomo et al., 2002), our studies also suggest that bryostatins 1 may have limited effectiveness in combination therapies with certain anticancer agents for prostate cancer treatment.

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References

- Barry OP and Kazanietz MG (2001) Protein kinase C isozymes, novel phorbol ester receptors and cancer chemotherapy. *Curr Pharm Des* 7:1725–1744.
- Basu A and Sivaprasad U (2007) Protein kinase Cepsilon makes the life and death decision. *Cell Signal* 19:1633–1642.
- Blass M, Kronfeld I, Kazimirsky G, Blumberg PM, and Brodie C (2002) Tyrosine phosphorylation of protein kinase Cdelta is essential for its apoptotic effect in response to etoposide. *Mol Cell Biol* 22:182–195.
- Cartee L, Maggio SC, Smith R, Sankala HM, Dent P, and Grant S (2003) Protein kinase C-dependent activation of the tumor necrosis factor receptor-mediated extrinsic cell death pathway underlies enhanced apoptosis in human myeloid leukemia cells exposed to bryostatins 1 and flavopiridol. *Mol Cancer Ther* 2:83–93.
- Clamp AR, Blackhall FH, Vasey P, Soukop M, Coleman R, Halbert G, Robson L, Jayson GC, and Cancer Research UK Phase I/II Committee (2003) A phase II trial of bryostatins-1 administered by weekly 24-hour infusion in recurrent epithelial ovarian carcinoma. *Br J Cancer* 89:1152–1154.
- Diaz-Rodríguez E, Montero JC, Esparís-Ogando A, Yuste L, and Pandiella A (2002) Extracellular signal-regulated kinase phosphorylates tumor necrosis factor alpha-converting enzyme at threonine 735: a potential role in regulated shedding. *Mol Biol Cell* 13:2031–2044.
- García-Bermejo ML, Leskow FC, Fujii T, Wang Q, Blumberg PM, Ohba M, Kuroki T, Han KC, Lee J, Marquez VE, et al. (2002) Diacylglycerol (DAG)-lactones, a new class of protein kinase C (PKC) agonists, induce apoptosis in LNCaP prostate cancer cells by selective activation of PKCalpha. *J Biol Chem* 277:645–655.
- Gomel R, Xiang C, Finniss S, Lee HK, Lu W, Okhrimenko H, and Brodie C (2007) The localization of protein kinase Cdelta in different subcellular sites affects its proapoptotic and antiapoptotic functions and the activation of distinct downstream signaling pathways. *Mol Cancer Res* 5:627–639.
- Gonzalez-Guerrico AM and Kazanietz MG (2005) Phorbol ester-induced apoptosis in prostate cancer cells via autocrine activation of the extrinsic apoptotic cascade: a key role for protein kinase C delta. *J Biol Chem* 280:38982–38991.
- Griner EM and Kazanietz MG (2007) Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer* 7:281–294.
- Gschwend JE, Fair WR, and Powell CT (2000) Bryostatins 1 induces prolonged activation of extracellular regulated protein kinases in and apoptosis of LNCaP

- human prostate cancer cells overexpressing protein kinase Calpha. *Mol Pharmacol* 57:1224–1234.
- Hennings H, Blumberg PM, Pettit GR, Herald CL, Shores R, and Yuspa SH (1987) Bryostatins 1, an activator of protein kinase C, inhibits tumor promotion by phorbol esters in SENCAR mouse skin. *Carcinogenesis* 8:1343–1346.
- Kazanietz MG, Lewin NE, Gao F, Pettit GR, and Blumberg PM (1994) Binding of [26–3H]bryostatins 1 and analogs to calcium-dependent and calcium-independent protein kinase C isozymes. *Mol Pharmacol* 46:374–379.
- Lorenzo PS, Bögi K, Acs P, Pettit GR, and Blumberg PM (1997) The catalytic domain of protein kinase Cdelta confers protection from down-regulation induced by bryostatins 1. *J Biol Chem* 272:33338–33343.
- McJilton MA, Van Sikes C, Wescott GG, Wu D, Foreman TL, Gregory CW, Weidner DA, Harris Ford O, Morgan Lasater A, Mohler JL, et al. (2003) Protein kinase Cepsilon interacts with Bax and promotes survival of human prostate cancer cells. *Oncogene* 22:7958–7968.
- Meshki J, Caino CC, von Burstin VA, Griner EM, and Kazanietz MG (2010) Regulation of prostate cancer cell survival by protein kinase C epsilon involves Bad phosphorylation and modulation of the TNFalpha/JNK pathway. *J Biol Chem* doi:10.1074/jbc.M110.128371.
- Ogbourne SM, Suhrbier A, Jones B, Cozzi SJ, Boyle GM, Morris M, McAlpine D, Johns J, Scott TM, Sutherland KP, et al. (2004) Antitumor activity of 3-ingenyl angelate: plasma membrane and mitochondrial disruption and necrotic cell death. *Cancer Res* 64:2833–2839.
- Powell CT, Britts NJ, Stec D, Hug H, Heston WD, and Fair WR (1996) Persistent membrane translocation of protein kinase C alpha during 12–O-tetradecanoylphorbol-13-acetate-induced apoptosis of LNCaP human prostate cancer cells. *Cell Growth Differ* 7:419–428.
- Propper DJ, Macaulay V, O'Byrne KJ, Braybrooke JP, Wilner SM, Ganesan TS, Talbot DC, and Harris AL (1998) A phase II study of bryostatins 1 in metastatic malignant melanoma. *Br J Cancer* 78:1337–1341.
- Reddy AB, Ramana KV, Srivastava S, Bhatnagar A, and Srivastava SK (2009) Aldose reductase regulates high glucose-induced ectodomain shedding of tumor necrosis factor (TNF)-alpha via protein kinase C-delta and TNF-alpha converting enzyme in vascular smooth muscle cells. *Endocrinology* 150:63–74.
- Reyland ME, Anderson SM, Matassa AA, Barzen KA, and Quissell DO (1999) Protein kinase C delta is essential for etoposide-induced apoptosis in salivary gland acinar cells. *J Biol Chem* 274:19115–19123.
- Reyland ME, Barzen KA, Anderson SM, Quissell DO, and Matassa AA (2000) Activation of PKC is sufficient to induce an apoptotic program in salivary gland acinar cells. *Cell Death Differ* 7:1200–1209.
- Roberts JD, Smith MR, Feldman EJ, Cragg L, Millenson MM, Roboz GJ, Honeycutt C, Thune R, Padavic-Shaller K, Carter WH, et al. (2006) Phase I study of bryostatins 1 and fludarabine in patients with chronic lymphocytic leukemia and indolent (non-Hodgkin's) lymphoma. *Clin Cancer Res* 12:5809–5816.
- Schaar D, Goodell L, Aisner J, Cui XX, Han ZT, Chang R, Martin J, Grospe S, Dudek L, Riley J, et al. (2006) A phase I clinical trial of 12-O-tetradecanoylphorbol-13-acetate for patients with relapsed/refractory malignancies. *Cancer Chemother Pharmacol* 57:789–795.
- Schechtman D and Mochly-Rosen D (2002) Isozyme-specific inhibitors and activators of protein kinase C. *Methods Enzymol* 345:470–489.
- Shao MX, Ueki IF, and Nadel JA (2003) Tumor necrosis factor alpha-converting enzyme mediates MUC5AC mucin expression in cultured human airway epithelial cells. *Proc Natl Acad Sci USA* 100:11618–11623.
- Stone RM, Sarihan E, Pettit GR, and Kufe DW (1988) Bryostatins 1 activates protein kinase C and induces monocytic differentiation of HL-60 cells. *Blood* 72:208–213.
- Sumitomo M, Ohba M, Asakuma J, Asano T, Kuroki T, Asano T, and Hayakawa M (2002) Protein kinase Cdelta amplifies ceramide formation via mitochondrial signaling in prostate cancer cells. *J Clin Invest* 109:827–836.
- Szallasi Z, Smith CB, Pettit GR, and Blumberg PM (1994) Differential regulation of protein kinase C isozymes by bryostatins 1 and phorbol 12-myristate 13-acetate in NIH 3T3 fibroblasts. *J Biol Chem* 269:2118–2124.
- Tanaka Y, Gavrielides MV, Mitsuuchi Y, Fujii T, and Kazanietz MG (2003) Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. *J Biol Chem* 278:33753–33762.
- Varterasian ML, Mohammad RM, Shurafa MS, Hulburd K, Pemberton PA, Rodriguez DH, Spadoni V, Eilender DS, Murgo A, Wall N, et al. (2000) Phase II trial of bryostatins 1 in patients with relapsed low-grade non-Hodgkin's lymphoma and chronic lymphocytic leukemia. *Clin Cancer Res* 6:825–828.
- Wang QJ, Bhattacharyya D, Garfield S, Nacro K, Marquez VE, and Blumberg PM (1999a) Differential localization of protein kinase C delta by phorbol esters and related compounds using a fusion protein with green fluorescent protein. *J Biol Chem* 274:37233–37239.
- Wang QJ, Fang TW, Fenick D, Garfield S, Bienfait B, Marquez VE, and Blumberg PM (2000) The lipophilicity of phorbol esters as a critical factor in determining the pattern of translocation of protein kinase C delta fused to green fluorescent protein. *J Biol Chem* 275:12136–12146.
- Wang QJ, Lu G, Schlackohl WA, Goerke A, Larsson C, Mischak H, Blumberg PM, and Mushinski JF (2004) The V5 domain of protein kinase C plays a critical role in determining the isoform-specific localization, translocation, and biological function of protein kinase C-delta and -epsilon. *Mol Cancer Res* 2:129–140.
- Wang S, Guo CY, Castillo A, Dent P, and Grant S (1998) Effect of bryostatins 1 on paclitaxel-induced apoptosis and cytotoxicity in human leukemia cells (U937). *Biochem Pharmacol* 56:635–644.
- Wang S, Wang Z, Boise LH, Dent P, and Grant S (1999b) Bryostatins 1 enhances paclitaxel-induced mitochondrial dysfunction and apoptosis in human leukemia cells (U937) ectopically expressing Bcl-xL. *Leukemia* 13:1564–1573.
- Wang S, Wang Z, Dent P, and Grant S (2003) Induction of tumor necrosis factor by bryostatins 1 is involved in synergistic interactions with paclitaxel in human myeloid leukemia cells. *Blood* 101:3648–3657.
- Wheeler DL, Ness KJ, Oberley TD, and Verma AK (2003) Protein kinase Cepsilon is linked to 12-O-tetradecanoylphorbol-13-acetate-induced tumor necrosis factor-alpha

- ectodomain shedding and the development of metastatic squamous cell carcinoma in protein kinase Cepsilon transgenic mice. *Cancer Res* **63**:6547–6555.
- Xiao L, Caino MC, von Burstín VA, Oliva JL, and Kazanietz MG (2008) Phorbol ester-induced apoptosis and senescence in cancer cell models. *Methods Enzymol* **446**:123–139.
- Xiao L, Eto M, and Kazanietz MG (2009) ROCK mediates phorbol ester-induced apoptosis in prostate cancer cells via p21Cip1 up-regulation and JNK. *J Biol Chem* **284**:29365–29375.

Zonder JA, Shields AF, Zalupski M, Chaplen R, Heilbrun LK, Arlauskas P, and Philip PA (2001) A phase II trial of bryostatín 1 in the treatment of metastatic colorectal cancer. *Clin Cancer Res* **7**:38–42.

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