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Bryostatin 1 Induces Prolonged Activation of Extracellular Regulated Protein Kinases in and Apoptosis of LNCaP Human Prostate Cancer Cells Overexpressing Protein Kinase Cα

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ABSTRACT

Previously, we reported that 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced apoptosis of LNCaP human prostate cancer cells was accompanied by prolonged translocation of protein kinase C (PKC)α to non-nuclear membranes and that TPA-resistant LNCaP cells had down-regulated PKCα. Here we show that 10 nM bryostatin 1 induced transient membrane translocation and down-regulation of PKCα, prolonged translocation of PKCδ and ϵ to non-nuclear membranes, and did not induce cell death but blocked TPA-induced apoptosis. To test the hypothesis that inhibition of TPA-induced apoptosis by bryostatin 1 was due to down-regulation of PKCα, we inducibly overexpressed PKCα in LNCaP cells. Overexpression of PKCα alone did not induce apoptosis, even in clones that contained much more membrane-bound, active PKCα than was observed in TPA-treated untransfected LNCaP cells. However, the addition of 10 nM bryostatin 1 to PKCα-overexpressing LNCaP cells did not yield down-regulation of PKCα and induced extensive apoptosis. Immunoblot analysis revealed that TPA induced prolonged hyperphosphorylation of Raf-1 and activation of extracellular-regulated/mitogen-activated protein kinases 1 and 2 in untransfected LNCaP cells, as did bryostatin 1 in PKCα-overexpressing cells. On the other hand, bryostatin 1 induced only transient hyperphosphorylation of Raf-1 and activation of extracellular-regulated/mitogen-activated protein kinases 1 and 2 in untransfected LNCaP cells. These results confirm a role of prolonged membrane-associated PKCα in PKC activator-mediated LNCaP apoptosis and suggest involvement of the mitogen-activated protein kinase pathway.

Prostate cancer is the most common cancer and, despite recent progress in early detection, the second leading cause of cancer deaths among men in the United States (Landis et al., 1999). Although androgen ablation induces apoptosis of normal prostate epithelial cells and regression of early-stage prostate cancer, this treatment is not curative for prostate cancer due to the resurgent growth of androgen-independent cells. Even many androgen-sensitive prostate tumors appear to be resistant to induction of apoptosis; androgen withdrawal affects those tumors by decreasing proliferation (Westin et al., 1995). Thus, much effort is being directed at increasing and understanding the mechanisms of apoptosis in prostate cancer.

The tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has been shown to induce extensive death of LNCaP human prostate cells with features of apoptosis, including cell shrinkage, chromatin condensation, and internucleosomal DNA fragmentation (Day et al., 1994). LNCaP cells are stimulated to grow by androgens and become growth arrested but do not die on the removal of androgen (Horoszewics et al., 1983). TPA is well known as an activator, and subsequent down-regulator, of most protein kinase C (PKC) isozymes (Nishizuka, 1995), and more recently, it has been shown to activate chimaerins (Ron and Kazanietz, 1999), which are Rac GTPase-activating proteins, and RasGRP (Ebinu et al., 1998), a Ras GTP exchange protein. TPA is also a potent tumor promoter in mouse skin (Slaga, 1983). Bryostatin 1 is a macrocyclic lactone that also activates and down-regulates PKCisozymes but with a different pattern of modulation of different isozymes relative to TPA in many cell types (Blumberg and Pettit, 1992). It has been shown to have antineoplastic activity in several tumor types and is not a tumor promoter (Blumberg and Pettit, 1992); thus, it is a more attractive therapeutic agent than TPA. Here, we measured the effects of bryostatin 1 on growth and induction of

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ABBREVIATIONS: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ERK, extracellular-regulated/mitogen-activated protein kinase; FBS, fetal bovine serum; PKC, protein kinase C; tet, tetracycline; tTA, tetracycline-repressible transactivator protein; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt.

apoptosis by TPA in parental and PKCα-overexpressing LNCaP cells to increase our knowledge of the mechanism of PKC activator-induced apoptosis and to assess the efficacy of bryostatin 1 as an agent for prostate cancer.

TPA has been shown to induce growth, differentiation, or death of cultured cells, depending on the cell type and culture conditions (Clemens et al., 1992). Growth-stimulatory effects of TPA have most typically been attributed to activation of PKC and the extracellular-regulated/mitogen-activated protein kinase (ERK) pathway (Seger and Krebs, 1995). There is accumulating evidence that in cell types and under conditions where activation of the ERK pathway is prolonged, increased expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, hypophosphorylation of the retinoblastoma protein (Rb), and growth inhibition without apoptosis ensue (Liu et al., 1996; Woods et al., 1997). Prolonged activation of the ERK pathway was not thought previously to trigger apoptosis, but TPA-induced LNCaP apoptosis has been shown to involve increased $p21^{WAF1/CIP1}$ expression and Rb hypophosphorylation and is dependent on Rb (Zhao et al., 1997). We showed that TPA-induced LNCaP apoptosis was accompanied by prolonged translocation of PKCα to non-nuclear membranes, implying prolonged activation, and that TPA-resistant LNCaP cells had down-regulated that isozyme (Powell et al., 1996).

Although bryostatin 1 has also been shown to induce growth of some cell types, it more frequently inhibits growth and/or opposes the effects of TPA (Blumberg and Pettit, 1992). In some cell types, inhibition of TPA effects by bryostatin 1 has been associated with differential down-regulation of certain PKC isozymes by bryostatin 1 compared with TPA (Isakov et al., 1993; Szallasi et al., 1994a,b), but it has been difficult to attribute the effects of bryostatin 1 to activation or down-regulation of particular PKC isozymes. Also, the biological effects of bryostatin 1 may in some cases be PKC-independent (Szallasi et al., 1996). Here, we show that 10 nM bryostatin 1 induced prolonged translocation of PKCδ and ε to non-nuclear membranes in LNCaP cells but transient membrane translocation and down-regulation of PKCα and did not induce cell death. Pretreatment of untransfected LNCaP cells with bryostatin 1 yielded down-regulation of PKCα much sooner than with TPA alone and completely inhibited TPA-induced cell death. Overexpression of PKCα in both cytoplasm and non-nuclear membranes did not by itself induce death of LNCaP cells but changed the effect of bryostatin 1 to induction of apoptosis. We also show a strong correlation between prolonged membrane translocation of PKCα in the presence of TPA or bryostatin 1 and prolonged hyperphosphorylation of Raf-1 and activation of ERKs 1 and 2. These results confirm a role of prolonged PKCα activation in the induction of LNCaP apoptosis and suggest that the induction of cell death may require prolonged activation of the mitogen-activated protein kinase pathway.

Materials and Methods

Cell Culture. LNCaP cells were obtained from American Type Culture Collection (Rockville, MD) and maintained as monolayer cultures in RPMI 1640 (Memorial Sloan-Kettering Cancer Center media preparation facility) supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine (complete RPMI) in a humidified atmosphere at 37° and 5% CO_2. LNGK9 cells, a subclone of LNCaP cells stably expressing a tetracycline (tet)-repressible transactivator protein (tTA; Gossen and Bujard, 1992), were previously generated in our laboratory (Gschwend et al., 1997) and maintained in complete RPMI containing 150 μg/ml hygromycin (Calbiochem, La Jolla, CA) and 1 μg/ml tet.

Stable Transfection of PKCα cDNA. The plasmids pUHD 15–1 and pUHD 10-3 (Gossen and Bujard, 1992) were kindly provided by M. Gossen and H. Bujard (University of Heidelberg, Heidelberg, Germany). pUHD 15–1 contains a chimeric gene coding for the repressor protein of the *Escherichia coli* Tn*10*-specified tet resistance operon fused to the C-terminal activation domain of the herpes simplex virus virion protein 16. Expression of this fusion protein, tTA, is under the control of a human cytomegalovirus (hCMV) promoter and enhancer that constitutively drive expression in most mammalian cells. The pUHD 10-3 plasmid contains seven operator sequences from the *E. coli* tet resistance operon upstream of an hCMV promoter without enhancer. This inducible operator/promoter element can be activated by tTA in the absence of tet but is inactive in many cell types in the absence of tTA or in the presence of tTA plus 1 μg/ml tet, which prevents tTA from binding to the promoter (Gossen and Bujard, 1992). A previously characterized subclone of LNCaP cells stably transfected with the tTA vector pUHD 15-1 and a hygromycin-selectable vector, *pgkhyg* (gift of M. Jasin), designated LNGK9 (Gschwend et al., 1997), was used for the transfection of PKCα cDNA. Transient transfection of LNGK9 with a reporter vector containing a firefly luciferase gene downstream of the same inducible operator/promoter sequence as in plasmid pUHD 10-3 yielded barely detectable luciferase activity in the presence of 1 μg/ml tet and about 10^4-fold higher luciferase activity in the absence of tet (Gschwend et al., 1997). The full-length human PKCα cDNA (Finkenzeller et al., 1990), generously provided by H. Hug, was subcloned into the *Eco*RI site of pUHD 10-3, and clones were selected with PKCα cDNA in the sense orientation relative to the operator/promoter sequence. For stable transfection of LNGK9 cells, the PKCα cDNA in pUHD 10-3 and a neomycin-selectable vector, pcDNA3 (InVitrogen, San Diego, CA), were purified by alkaline lysis followed by cesium chloride density centrifugation (Sambrook et al., 1989). Aliquots containing 10 μg of PKCα/pUHD 10-3 and 5 μg of pcDNA3 were preincubated for 15 min with 30 μl of LipoFECTAMINE (Life Technologies, Gaithersburg, MD) in 200 μl of serum-free, antibiotic-free RPMI medium, diluted to 4 ml with the same medium, and then added to 75-cm^2 culture flasks (Corning Glass Works, Corning, NY) containing LNGK9 cells that had been plated 48 h earlier at a density of 2×10^6 cells per flask. After a 6-h incubation at 37°C, the transfection mixture was replaced with 15 ml of complete RPMI containing 1 μg/ml tet (Sigma Chemical Co.). At 48 h later, the cells were trypsinized, and different dilutions were seeded onto 100-mm plates in complete RPMI supplemented with 500 μg/ml G418 (Life Technologies), 150 μg/ml hygromycin (Calbiochem), and 1 μg/ml tet. After incubation at 37°C for 2 to 3 weeks, individual colonies were isolated using sterile cloning rings, trypsinized, and plated onto 12-well plates. Clones were expanded and maintained in complete RPMI with 500 μg/ml G418, 150 μg/ml hygromycin, and 1 μg/ml tet, except when tet was removed for various times in certain experiments as indicated.

Viable Growth Assays. The effects of bryostatin 1 and TPA on viable growth of untransfected and PKCα-transfected LNCaP cells were measured by a colorimetric assay using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT; Roehm et al., 1991). Untransfected LNCaP cells were maintained and plated for these experiments in the absence of tet. PKCα-overexpressing cells were isolated and maintained in medium containing 1 μg/ml tet and either plated in the continued presence of tet or removed from tet for 1 week before plating in the absence of tet. Cells were plated in 200 μl of complete medium onto flat-bottom 96-well tissue culture plates at 5000 cells/well and 6 wells/condition. TPA (10 nM), bryostatin 1 (10 nM), TPA plus bryostatin 1 (10 nM

each), or ethanol vehicle (final concentration, 0.006%) was added 3 days later. The number of viable cells, including both adherent cells and cells floating in the medium, were counted 1, 2, and 3 days after drug addition as described (Roehm et al., 1991). Briefly, XTT (final, 0.2 mg/ml) and phenazine methosulfate (final, 25 μM) were added to the medium covering the cells in a volume of 50 μl/well. After incubation for 4 h at 37°C, $A_{450} - A_{650}$ of the medium in each well was measured in a Molecular Devices (Menlo Park, CA) UV_{max} kinetic microplate reader. A standard curve was constructed by assaying known numbers of viable LNCaP cells, determined by trypan blue exclusion, and plotting $A_{450} - A_{650}$ versus cell number. XTT, TPA, and phenazine methosulfate were purchased from Sigma Chemical Co.. Bryostatin 1 was purchased from LC Laboratories (Woburn, MA).

Growth of untransfected and PKCα-transfected LNCaP cells in the presence and absence of tet was determined using a hemacytometer by counting cells that excluded trypan blue. Untransfected LNCaP cells were either never exposed to tet or cultured with 1 μg/ml tet in the medium for 1 week before plating in the same medium for counting. PKCα-transfected LNCaP clones were isolated, maintained, and plated in medium containing 1 μg/ml tet, or tet was removed 1 week before plating for this experiment in the absence of tet. Cells were plated in triplicate onto 6-well plates at 10^5 cells/well. At 24, 48, 72, 96, and 120 h later, detached and adherent cells were harvested by centrifugation and trypsinization, respectively; pooled; and counted. Cell numbers from the period of exponential growth were analyzed by linear regression, and cell doubling times were calculated by dividing log2 by the slopes of the regression lines. Statistical analyses of cell numbers and growth rates were made by paired, two-tailed t tests.

Immunoblot Analyses. For the detection of PKC isozymes α, δ, ϵ, η,and ζ, 6×10^6 cells were plated per flask in 150-cm^2 flasks and bryostatin 1 (10 nM), TPA (10 nM), or ethanol vehicle (final concentration, 0.006%) was added 3 days later. Cells were lysed at different times after drug addition, and cytoplasmic and non-nuclear membrane fractions were prepared and semipurified on DE-52 columns as described (Huang et al., 1986), with some modifications (Powell et al., 1996). Aliquots containing 40 and 20 μg of cytoplasmic and non-nuclear membrane protein, respectively, were electrophoresed on SDS-6% polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes. Based on average yields from separation and DE-52 purification of cytoplasmic and membrane fractions, the amount of cytoplasmic protein obtained per cell was about four times the amount of membrane protein per cell. Thus, 20 μg of membrane proteins represents about twice as many cells as 40 μg of cytoplasmic proteins. Immunoblot analyses were performed with affinity-purified, PKC isozyme-specific anti-peptide polyclonal antibodies; horseradish peroxidase-linked donkey anti-rabbit Ig as secondary antibody (1:5000 diluted; Amersham, Arlington Heights, IL); and enhanced chemiluminescence detection (ECL detection reagents, Amersham). PKCα antibodies were obtained from Life Technologies. PKCδ and ϵ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). PKCη antibodies were from Calbiochem. PKCζ antibodies were from Upstate Biotechnology (Lake Placid, NY). Specificities of the PKC antibodies were assessed by the manufacturers and by our immunoblot analyses of PKCα- and δ-overexpressing LNCaP cells, PKCζ-overexpressing rat prostate cells, and purified, recombinant PKCϵ and η (Calbiochem).

For the detection of Raf-1 and dual-phosphorylated ERKs 1 and 2, 1×10^6 cells were plated per flask in 25-cm^2 flasks, and TPA (10 nM), bryostatin 1 (10 nM), or ethanol vehicle (final concentration, 0.006%) was added 3 days later. Total cell lysates were prepared at different times after drug addition, and aliquots containing 50 μg of protein were run on SDS-6% (for Raf-1) or SDS-8% (for ERKs 1 and 2) polyacrylamide gels. Transfers to polyvinylidene difluoride membranes and immunoblot analyses were performed as for PKC isozymes, using affinity-purified anti-peptide polyclonal antibodies against Raf-1 (Santa Cruz Biotechnology) and dual-phosphorylated active site of ERKs 1 and 2 (Promega Corp., Madison, WI).

Detection of DNA Fragmentation. Detection of apoptotic cells by in situ labeling of DNA ends (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) was performed as described (Gavrieli et al., 1992), with some modifications. Detached and adherent cells were harvested separately from 25-cm^2 flasks by centrifugation and trypsinization, followed by centrifugation, respectively, and washed once in ice-cold PBS. Aliquots were mixed with 0.4% trypan blue (Sigma Chemical Co.) for counting, and the remaining cells were fixed in 50 μl of 10% neutral formaldehyde. The fixed cells were spread on glass slides, air-dried, washed, and then treated with 0.1% H_2O_2 for 15 min. After repeated washing, cells were covered with 100 μl of terminal deoxynucleotidyl transferase buffer containing 30 U of terminal deoxynucleotidyl transferase and 5 μM biotin-16-dUTP (both from Boehringer-Mannheim Biochemicals, Indianapolis, IN) and incubated for 1 h at 37°C. Reactions were stopped, and nonspecific binding was blocked as described (Gavrieli et al., 1992). Then the slides were incubated with avidin and biotinylated horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA) for 30 min at room temperature in a humid chamber. Peroxidase was detected with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.), and slides were counterstained with hematoxylin, dehydrated, and mounted. For quantification of DNA fragmentation, 500 cells were examined at 400× magnification.

Assay of PKC Activity in Isolated Membranes. Direct measurement of total PKC activity in its native membrane-associated state was performed according to the method of Chakravarthy et al. (1994). Cells (6×10^6/flask) were plated in 150-cm^2 flasks, and TPA (10 nM), bryostatin 1 (10 nM), or ethanol vehicle (final concentration 0.006%) was added 3 days later. After 4 h of drug or vehicle treatment, non-nuclear membranes were isolated as described and resuspended by vortexing in 0.5 ml of 2× assay buffer [50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 μM $CaCl_2$, 200 μM sodium vanadate, 200 μM sodium pyrophosphate, 2 mM NaF, 200 μM phenylmethylsulfonyl fluoride, and 20 μg/ml concentration of both aprotinin and leupeptin (Sigma Chemical Co.) and 1 nM Calyculin A (Life Technologies)]. Protein concentrations were measured with a Bio-Rad (Hercules, CA) Protein Assay kit, using BSA as standard, and the resuspended membranes were diluted to 800 μg protein/ml in 2× assay buffer. Then, 50-μl aliquots of membrane suspensions, containing 40 μg of protein, were preincubated at 30°C with 10 μl of 750 μM PKC selective peptide substrate (FKKSFKL-NH_2; LC Laboratories) with or without 10 μl of inhibitory PKCα pseudosubstrate peptide (Life Technologies) in a total volume of 100 μl. Reactions were started by the addition of 10 μl of ATP solution (0.5 mM ATP plus 0.5 μCi of [γ-^{32}P]ATP, 3000 Ci/mmol, in 50 mM Tris-HCl, pH 7.5), incubated at 30°C for 10 min, and terminated by the addition of 10 μl of 5% acetic acid. Samples were centrifuged for 5 min at 14,000 rpm and 4°C, and then 30-μl aliquots of the supernatants were spotted onto phosphocellulose discs. The disks were washed 3× for 2 min in 5% acetic acid, and cpm of bound, ^{32}P-labeled peptide was measured in a scintillation counter.

Results

Effects of Bryostatin 1 on LNCaP Cell Growth and TPA-Induced Cell Death. An initial dose-response analysis indicated that moderate doses of bryostatin 1, from 0.6 to 20 nM in the presence of 10% FBS, slightly increased the viable growth rate of LNCaP cells (Fig. 1A), although none of the doses yielded growth rates significantly different ($P < .05$) from those of vehicle-treated cells. Higher doses of bryostatin 1 yielded progressively less growth stimulation, but little or no growth inhibition relative to vehicle-treated LNCaP cells was observed at up to 1 μM. The extensive LNCaP cell death induced by 10 nM TPA (83% loss of viability from

the first to third day after drug addition) was completely blocked by the addition of bryostatin 1 (10 nM) 1 h before TPA (Fig. 1, A and B).

Effects of Bryostatin 1 on Membrane Translocation and Down-Regulation of PKC Isozymes. Previous RNase protection assays revealed the presence of PKCα, δ, ϵ, η, ζ, and μ mRNAs in LNCaP cells, but PKCβ, γ, and θ mRNAs were undetectable (Powell et al., 1996; data not shown). Immunoblot analyses revealed that LNCaP cells growing in 10% FBS contained substantial cytoplasmic pools of PKCα, δ, ϵ, and η, with minimal, high, moderate, and low amounts of those isozymes, respectively, in non-nuclear membranes (Fig. 2A; Powell et al., 1996). Treatment of LNCaP cells with 10 nM bryostatin 1 resulted in translocation of PKCα, δ, and ϵ from cytoplasm to non-nuclear membranes and down-regulation of the cytoplasmic pools, of which PKCϵ was most rapid. PKCα was down-regulated from non-nuclear membranes to almost undetectable levels 12 to 18 h after the addition of bryostatin 1, but membrane levels of PKCδ and ϵ were only partially down-regulated through 48 h (Fig. 2A). Membrane PKCδ content at 48 h, measured by densitometry, was 45% of the peak level induced by bryostatin 1 and 70% of the high level in untreated cells. Membrane PKCϵ content at 48 h was 52% of the peak level after bryostatin 1 and 125% of the moderate level in untreated cells. PKCη levels were not substantially altered by bryostatin 1, except for a transient increase in non-nuclear membranes 1 to 8 h after drug addition. Levels of PKCμ protein, which contains a transmembrane domain and usually is constitutively membrane bound, were not analyzed. Densitometric quantification of the PKCα immunoblot data showed that 10 nM TPA yielded much higher and more prolonged amounts of PKCα in LNCaP membranes than did bryostatin 1 (Fig. 2D, TPA data taken from Powell et al., 1996). A 12-fold increase in PKCα mRNA level was observed in LNCaP cells 6 to 9 h after thr addition of 10 nM TPA (Powell et al., 1996) but not after the addition of bryostatin 1 (data not shown). That is the most likely reason that the cytoplasmic PKCα protein level recovered temporarily after an initial partial down-regulation by TPA but did not recover after rapid down-regulation by bryostatin 1 (Fig. 2, A and D). No PKCα was detectable in LNCaP nuclei 1, 4, 8, or 12 h after the addition of bryostatin 1 or TPA (data not shown).

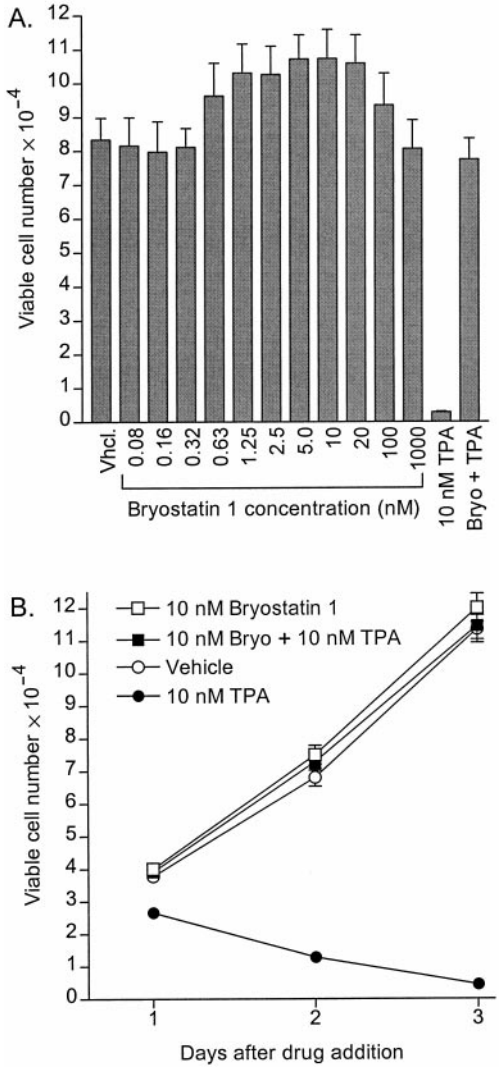

Fig. 1. Effects of bryostatin 1 on LNCaP cell growth and TPA-induced cell death. LNCaP cells were plated onto 96-well plates at 5000 cells/well, 6 wells/condition, in RPMI 1640 containing 10% FBS. Three days later, bryostatin 1, TPA (10 nM), bryostatin 1 plus TPA (each 10 nM), or ethanol vehicle (final concentration, 0.006%) was added. When both bryostatin 1 and TPA were added, bryostatin 1 was added 1 h before TPA. The numbers of viable cells, including both adherent cells and cells floating in the medium, were counted 72 h after drug addition (A) or 24, 48, and 72 h after drug addition (B) by a colorimetric assay, using the tetrazolium salt XTT (Roehm et al., 1991), as described in *Materials and Methods*. Vhcl., 0.006% ethanol vehicle; Bryo + TPA, 10 nM bryostatin 1 followed by 10 nM TPA.

Relative to bryostatin 1 alone, the addition of 10 nM TPA 1 h after bryostatin 1 yielded a partial recovery of cytoplasmic PKCα and higher membrane PKCα content at 5 and 9 h after the addition of bryostatin 1, but PKCα still was down-regulated from both cytoplasm and non-nuclear membranes by 13 h (Fig. 2, B and D). DNA fragmentation has not been detected in LNCaP cells until 18 to 24 h after the addition of TPA (Day et al., 1994). Thus, bryostatin 1 or bryostatin 1 plus TPA yielded down-regulation of PKCα before the onset of detectable DNA fragmentation.

PKCζ, although not directly activated by TPA or bryostatin 1, was examined because of its reported role in apoptosis (Diaz-Meco et al., 1996). In untreated LNCaP cells, PKCζ was abundantly expressed in cytoplasm, barely detectable in non-nuclear membranes, and undetectable in nuclei. Prolonged treatment with 10 nM TPA, 10 nM bryostatin 1, or bryostatin 1 followed by TPA did not alter that distribution, except for a slight increase in non-nuclear membranes 4 to 8 h after the addition of bryostatin 1 plus TPA (Fig. 2C).

Inducible Overexpression of PKCα in LNCaP Cells. LNCaP cells constitutively expressing tTA (clone LNGK9) were further transfected stably with human PKCα cDNA, and several clones were isolated. The removal of tet for more than 1 month did not alter the morphology or induce extensive cell death of any clones. Immunoblot analyses of lysates prepared from clones that had been maintained in the absence of tet for 2 weeks revealed in most clones very high overexpression of PKCα protein relative to lysates prepared from the same clones grown in the continuous presence of tet or untransfected LNCaP cells. Three representative PKCα transfected clones, designated LNα 1, LNα 17, and LNα 20, were chosen for further study. In the presence of tet, the amount of PKCα expressed in the cytoplasm and non-nuclear

membranes of those three clones was similar to or slightly higher than that in untransfected or tTA-expressing LNCaP cells (Fig. 3). The amount of background expression in the clones in the presence of tet was directly proportional to the amount of overexpression in the absence of tet. The removal of tet for 12 days yielded high levels of PKCα in both cytoplasm and non-nuclear membranes of LNα clones. In clone LNα 20, slightly elevated cytoplasmic and membrane PKCα levels were apparent 48 h after the removal of tet, increased substantially over the next 3 days, and reached a peak about 5 days after the removal of tet (Fig. 3). Previously, we showed that membrane PKCα levels reach a peak in untransfected LNCaP cells 3 to 4 h after the addition of 10 nM TPA (Powell et al., 1996). The amount of membrane PKCα in all three LNα clones in the absence of tet or exogenous PKC activators was much greater than the maximum amount found in the membranes of TPA-treated, untransfected LNCaP cells (Fig. 3).

RNase protection analyses of PKCδ, ϵ, η, ζ, and μ and immunoblot analyses of PKCδ and ϵ revealed no changes in expression of those isozymes in PKCα-overexpressing clones LNα 17 and LNα 20 grown in the absence of tet for 2 weeks (data not shown). Also, PKCβ mRNA, which was undetectable by RNase protection in untransfected LNCaP cells, remained undetectable in clones LNα 17 and LNα 20 in the presence of high PKCα overexpression (data not shown). Both α- and β-chimaerins, a second class of proteins activated by phorbol esters (Ron and Kazanietz, 1999), were undetectable by RNase protection in untransfected LNCaP cells (Powell et al., 1996) and were not examined in this study.

An assay for direct measurement of total PKC activity in isolated non-nuclear membranes without the addition of exogenous activators to the reactions (Chakravarthy et al., 1994) was used to assess the amount of active PKC in the membranes of PKCα-overexpressing cells. In that assay, total PKC activity is defined as the amount of phosphorylation of a PKC substrate peptide that can be blocked by inclusion of a PKCα pseudosubstrate peptide. Untransfected LNCaP cells and LNα17 cells incubated with 1 μg/ml tet showed low basal membrane PKC activity (Fig. 4). The treatment of untransfected LNCaP cells with 10 nM TPA or bryostatin 1 for 4 h before isolation of membranes raised the membrane PKC activity by 4.5- and 2.6-fold, respectively. This increase presumably reflected both the increased membrane PKC content caused by those drugs and the presence of TPA or bryostatin 1 in the membranes. The removal of tet from LNα17 cells for 12 days resulted in about 19-fold higher membrane PKC activity than in LNα17 cells grown in the presence of tet and untransfected LNCaP cells (Fig. 4). Thus, a substantial

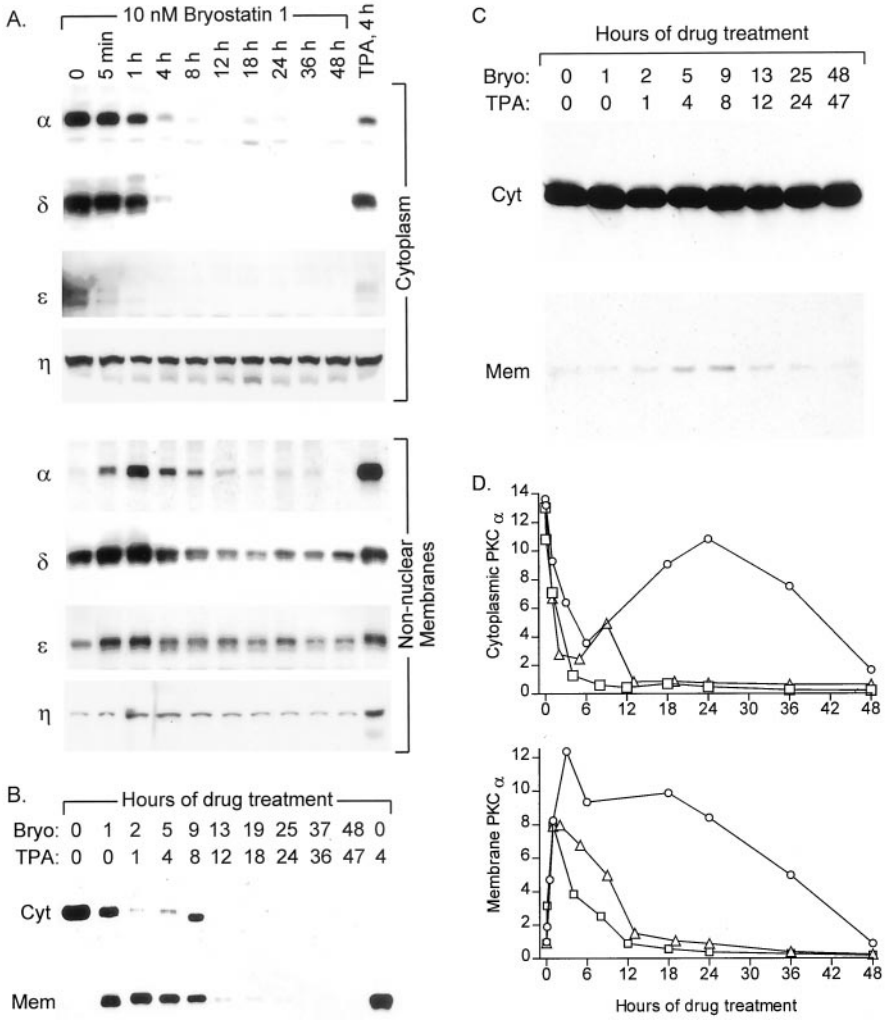

Fig. 2. Effects of bryostatin 1 or bryostatin 1 plus TPA on membrane translocation and down-regulation of PKC isozymes in LNCaP cells. Three days after plating 6×10^6 LNCaP cells in 150-cm^2 flasks, bryostatin 1 and/or TPA was added to the medium for the indicated times before lysing the cells. Cytoplasmic and non-nuclear membrane fractions were separated, and immunoblots with anti-PKCα, δ, ϵ, and η polyclonal antibodies were performed as described in *Materials and Methods*. A, cells were treated for up to 48 h with 10 nM bryostatin 1 or 4 h with 10 nM TPA. B and C, 10 nM bryostatin 1 was added to the medium, followed 1 h later by 10 nM TPA. PKCα (B) and PKCζ (C) were detected in cytoplasm (Cyt) and non-nuclear membranes (Mem) up to 48 h after the addition of bryostatin (47 h after addition of TPA). Far right lane of B, cells were treated with 10 nM TPA alone for 4 h (control). D, plot of densitometric scans of cytoplasmic and non-nuclear membrane PKCα in LNCaP cells after treatment with 10 nM TPA (○; data from Powell et al., 1996), 10 nM bryostatin 1 (□; from A), or bryostatin 1 plus TPA (10 nM each, △; from B). All data are from cells that were adherent to the flask surface at the time of lysis. Each experiment was performed at least twice with similar results.

portion of the overexpressed PKCα in the membranes of LNα17 cells appeared to be in an active state without the addition of TPA or bryostatin 1. The treatment of LNα17 cells with bryostatin 1 for 4 h, in the absence of tet, increased membrane PKC activity an additional 3-fold, to about 58-fold higher than in untreated LNCaP cells.

Growth and Bryostatin 1-Induced Apoptosis of LNCaP Cells Overexpressing PKCα. Growth of untransfected LNCaP cells was not affected by the presence of 1 μg/ml tet in the culture medium (Fig. 5A). PKCα-transfected clones LNα 1, 17, and 20 grew slightly faster in the presence of 1 μg/ml tet than did untransfected LNCaP cells (doubling times, 1.54 ± 0.14, 1.41 ± 0.12, and 1.42 ± 0.12 days for LNα 1, 17, and 20, respectively; 1.74 ± 0.16 days for LNCaP), although the differences were not significant (Fig. 5, A and B, $P \geq .05$). The removal of tet for 12 days slightly reduced the growth rates of the clones that overexpressed higher amounts of PKCα (LNα 17 and 20 doubling times, 1.63 ± 0.17 and 1.64 ± 0.15 days, respectively), but the differences did not reach significance (Fig. 5,A and B).

In the presence of 1 μg/ml tet, clones LNα 1, 17, and 20 responded to TPA and bryostatin 1 as did untransfected LNCaP cells (i.e., 10 nM TPA induced extensive cell death that could be blocked by 10 nM bryostatin 1; data not shown). However, after the induction of PKCα overexpression by the removal of tet, bryostatin 1 induced extensive cell death in clones LNα 1, 17, and 20 (Fig. 6), as did TPA alone (Fig. 6) or bryostatin 1 plus TPA (data not shown). Cell death induced by bryostatin 1 in clone LNα 17 was accompanied by a similar extent of DNA fragmentation, detected by in situ labeling of DNA ends, as in TPA-treated, untransfected LNCaP cells (Table 1). The highest percentages of cells exhibiting DNA fragmentation were among cells that had detached from the flask surface, which were the majority of cells by the second day of drug treatment. No DNA fragmentation was induced by bryostatin 1 in LNα 17 cells grown in the presence of tet or by the removal of tet from LNα 17 cells for 3, 4, 5, or 7 days in the absence of exogenous PKC activator (Table 1). The percentage of cells shown to be nonviable by absorption of trypan blue was slightly lower than the percentage of cells exhibiting DNA fragmentation after 2 days of drug treatment but slightly higher after 3 days of drug.

Effect of Bryostatin 1 on Subcellular Distribution of PKCα in PKCα-Overexpressing Clone LNα 17. The addition of bryostatin 1 to clone LNα 17, grown in the absence of tet for 12 days, yielded a slight increase in cytoplasmic and non-nuclear membrane PKCα content over the next 36 h but almost no down-regulation of PKCα from cytoplasm or non-nuclear membranes up to 48 h later, at which time more than half of the cells had detached from the flask surface (Fig. 7). Only cells that remained adherent 48 h after the addition of bryostatin 1 had a somewhat lower membrane content of PKCα, but this amount was still far more than the amount translocated by 4 h of TPA alone in untransfected LNCaP cells (Fig. 7).

Effects of TPA and Bryostatin 1 on Raf-1 Mobility in Control and PKCα-Overexpressing LNCaP Cells. Immunoblot analyses revealed the presence of all known Raf isozymes, Raf-1 and A- and B-Raf, in LNCaP cells. Raf-1 was chosen for analysis here because its decreased gel mobility has been shown to correlate with hyperphosphorylation and

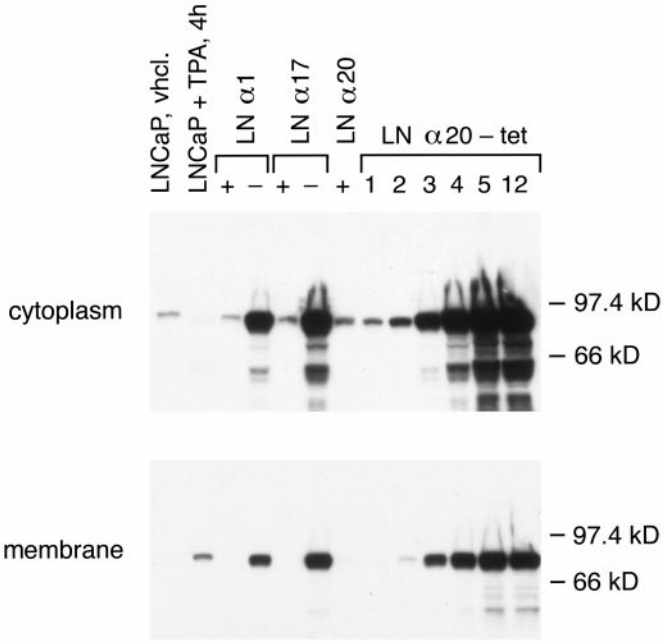

Fig. 3. Inducible overexpression of PKCα in cytoplasm and non-nuclear membranes of transfected LNCaP clones. LNCaP clones LNα 1, 17, and 20, expressing tTA and stably transfected with PKCα cDNA in pUHD 10-3, were grown in the continuous presence of 1 μg/ml tet (+), in the absence of tet for 12 days (−), or after removal of tet for 1 to 12 days (LNα 20 −tet). Untransfected LNCaP cells were grown in the absence of tet and treated for 4 h with ethanol vehicle (Vhcl.; final concentration, 0.006%) or 10 nM TPA before lysis. Cytoplasmic and non-nuclear membrane fractions were prepared and immunoblot analysis with anti-PKCα polyclonal antibodies was performed as described in *Materials and Methods*. Molecular weights of standard proteins are indicated at right.

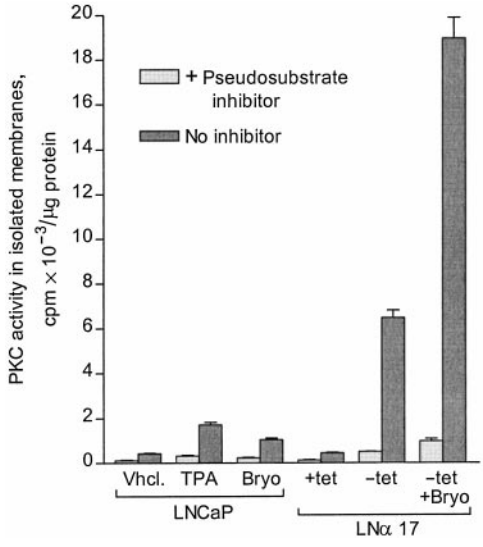

Fig. 4. Total PKC activity in isolated non-nuclear membranes. Untransfected LNCaP cells were grown in the absence of tet. LNα 17 cells were grown in the continuous presence of 1 μg/ml tet (+tet) or removed from tet 1 week before plating in the absence of tet (−tet) for this experiment. Cells (6×10^6/flask) were plated in 150-cm^2 flasks, and 10 nM TPA or bryostatin 1 (Bryo) or ethanol vehicle (Vhcl.; final concentration, 0.006%) was added 3 days later for 4 h before the cells were lysed. Non-nuclear membranes were prepared, and phosphorylation of the PKC-selective peptide substrate FKKSFKL-NH_2 was measured as described (Chakravarthy et al., 1994; see *Materials and Methods*). Values plotted are mean cpm $\times 10^{-3}$ of ^{32}P incorporated into FKKSFKL-NH_2/mg of membrane protein in the absence minus that in the presence of an inhibitory PKCα pseudosubstrate peptide. Reactions were performed in triplicate, and the entire experiment was repeated with similar results.

increased activity (App et al., 1991). Raf-1 appeared in untransfected LNCaP cells grown in RPMI plus 10% FBS and LNα 17 cells grown in the same medium containing 1 μg/ml tet as a major band of about 74 kDa and a minor band of slightly higher molecular mass (bands 1 and 2, Fig. 8A). The treatment of untransfected LNCaP cells with 10 nM TPA resulted in the prolonged appearance of two additional bands of slightly reduced mobility, usually indicative of hyperphosphorylated Raf-1 (bands 3 and 4, Fig. 8A). The lower-mobility bands 3 and 4 were detectable from 1 to 18 h after the addition of TPA, peaking at 8 h. Thereatment of untransfected LNCaP cells or LNα 17 cells plus tet with 10 nM bryostatin 1 also yielded lower-mobility bands 3 and 4 but only transiently (Fig. 8A). Bands 3 and 4 were readily apparent 1 h after the addition of bryostatin 1 but were barely detectable at 4 h and were undetectable thereafter. The induction of PKCα overexpression, by the removal of tet from the medium of LNα 17 cells, did not yield detectable changes in Raf-1 mobility 1 to 4 days later, as measured at 12-h intervals (data not shown). However, the addition of 10 nM bryostatin 1 to LNα 17 cells that had been grown in the absence of tet for 12 days yielded prolonged hyperphosphorylation of Raf-1, similar to TPA treatment of untransfected LNCaP cells (Fig. 8A).

Effects of TPA and Bryostatin 1 on ERK Activation in Control and PKCα-Overexpressing LNCaP Cells.

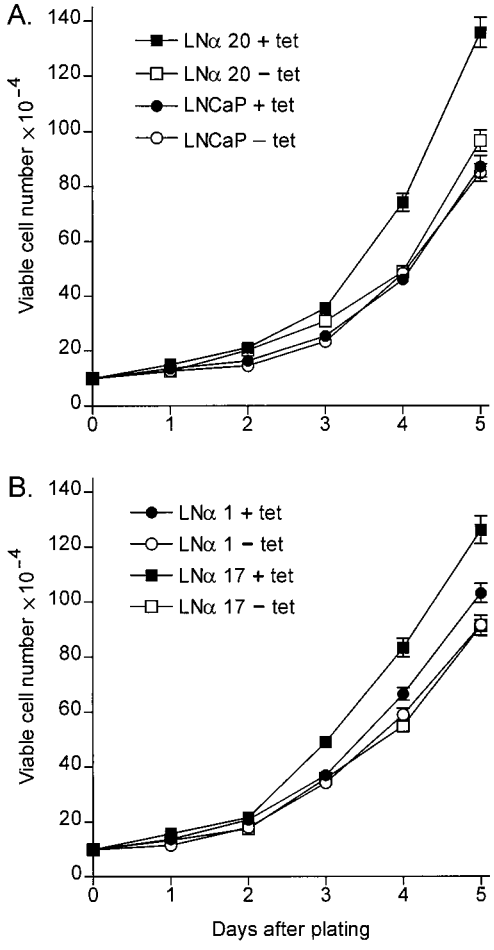

Fig. 5. Growth of PKCα-transfected clones in the presence and absence of tet. Cells were plated in triplicate onto 6-well plates at 1×10^5 cells/well. and adherent plus detached cells that excluded trypan blue were counted daily using a hemacytometer. Untransfected LNCaP cells were either never exposed to tet (−tet) or cultured with 1 μg/ml tet in the medium for 1 week before plating (+tet) in the same medium for counting. Clones LNα 1, 17, and 20 were isolated, maintained, and plated in medium containing 1 μg/ml tet (+tet), or tet was removed 1 week before plating for this experiment in the absence of tet.

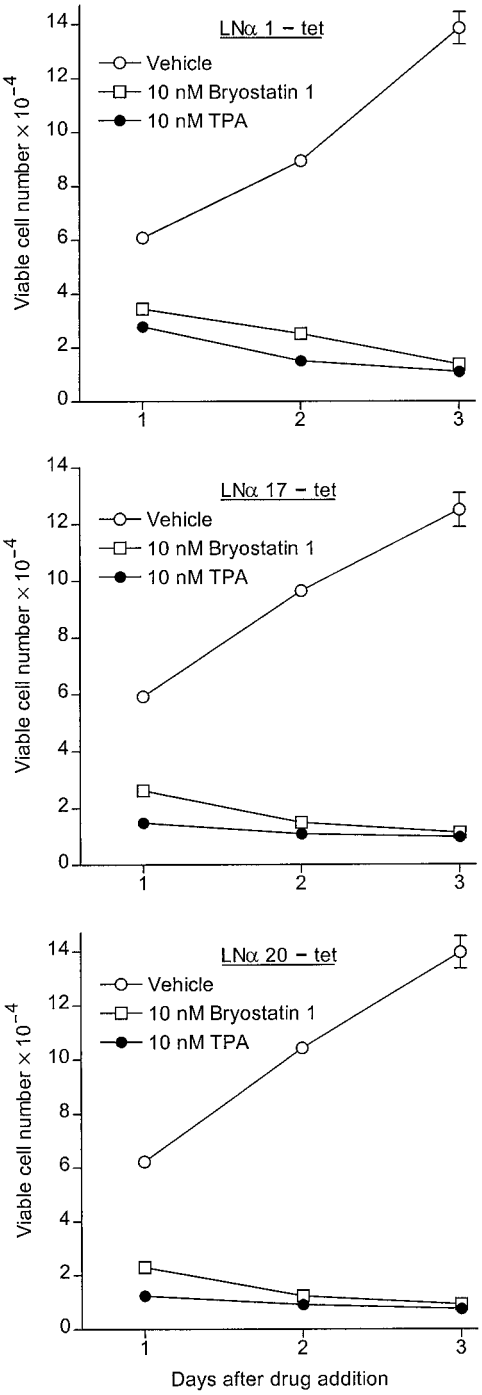

Fig. 6. Effects of bryostatin 1 on growth of PKCα-overexpressing LNCaP clones. LNCaP clones LNα 1, 17, and 20 were maintained in medium containing 1 μg/ml tet and then were grown for 1 week and plated for this experiment in the absence of tet. Cells were plated onto 96-well plates at 5000 cells/ well, 6 wells/condition, and TPA (10 nM), bryostatin 1 (10 nM), or ethanol vehicle (final concentration, 0.006%) was added 3 days later. The numbers of viable cells, including both adherent and detached cells, were counted 1, 2, and 3 days after drug addition by a colorimetric assay, using the tetrazolium salt XTT (Roehm et al., 1991), as described in *Materials and Methods*.

LNCaP cells express ERKs 1 and 2 (data not shown), but the amounts of those proteins in the dual-phosphorylated, active states are barely detectable in untransfected LNCaP cells grown in RPMI plus 10% FBS (Fig. 8B, t = 0). The addition of 10 nM TPA to untransfected LNCaP cells yielded prolonged activation of ERKs 1 and 2, whereas 10 nM bryostatin 1 yielded only transient activation of those proteins, prominent at 1 h but not detectable thereafter, in untransfected LNCaP cells or LNα 17 cells grown in the presence of 1 μg/ml tet (Fig. 8B). The addition of 10 nM bryostatin 1 to LNα 17 cells grown in the absence of tet for 12 days yielded prolonged activation of ERKs 1 and 2 (Fig. 8B). Those data parallel the effects of TPA and bryostatin 1 on Raf-1 mobility shown in Fig. 8A.

An additional control suggesting a role of prolonged ERK activation in PKC activator-induced cell death is provided by androgen-insensitive PC-3 cells, which are almost completely resistant to growth-inhibitory effects of TPA (Young et al., 1994). The treatment of PC-3 cells with 10 nM TPA yielded abundant membrane translocation of PKCα, δ, and ε for at least 48 h (data not shown) but only minor activation of ERKs 1 and 2, peaking 4 h after the addition of TPA (Fig. 8B).

Discussion

Previously, we reported that TPA-induced apoptosis of LNCaP cells involves prolonged membrane translocation of PKCα, δ, and ε and that TPA-resistant LNCaP cells, derived by culturing the cells in progressively higher concentrations of TPA, had down-regulated PKCα and δ (Powell et al., 1996). Here, we show that 10 nM bryostatin 1 blocked induction of LNCaP apoptosis by TPA and induced prolonged membrane translocation of PKCδ and ε but only transient membrane translocation of PKCα before down-regulation. Similar differential regulation of PKCα, δ, and ε by 1 μM bryostatin 1 in primary mouse keratinocytes has been reported (Szallasi et al., 1994a). Inducible overexpression revealed that the prolonged presence of a large amount of PKCα in LNCaP nonnuclear membranes did not by itself alter the morphology of the cells or induce apoptosis but overwhelmed the ability of 10 nM bryostatin 1 to down-regulate that isozyme and rendered the cells sensitive to bryostatin 1-induced apoptosis. Those results suggest strongly that the inhibitory effect of bryostatin 1 in untransfected cells was due to down-regulation of PKCα and that prolonged activation of PKCα is necessary for the induction of LNCaP apoptosis by PKCactivators.

The observation that a negligible proportion of the large amount of PKCζ in LNCaP cytoplasm was translocated by TPA, bryostatin 1, or bryostatin 1 plus TPA is in agreement with most other reports (Huwiler et al., 1994) and suggests that PKCζ is not a direct mediator of PKCactivator-induced LNCaP cell death. However, although it is likely that PKCζ requires membrane association for full activation in vivo (Chou et al., 1998), the evidence is not yet conclusive.

Although DNA fragmentation usually is a mark of apoptosis and trypan blue absorption of necrosis, DNA fragmentation detectable by the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling assay also may occur during necrosis, and most apoptotic cells eventually undergo secondary necrosis (Kroemer et al., 1998). Previous reports have shown clearly that TPA-treated LNCaP cells exhibit hallmarks of apoptosis such as chromatin condensation, internucleosomal DNA fragmentation, and cleavage of nuclear poly(ADP-ribose) polymerase (Day et al., 1994; Zhao et al., 1997). Although it is likely that apoptosis is the predominant mode of TPA- and bryostatin 1-induced death of LNCaP and LNα cells, respectively, the high numbers of trypan blue-positive cells (Table 1) suggests a possible contribution by primary necrosis. A more complete time course with more precise assays will be required to measure the relative contributions of those two modes of cell death.

We have shown that TPA-induced apoptosis of LNCaP cells is preceded by increased expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and hypophosphorylation of the retinoblastoma protein (Rb) and is dependent on Rb

TABLE 1

Effects of tet removal and PKC activators on DNA fragmentation

LNα 17 cells were isolated and maintained in medium containing 1 μg/ml tet, and tet either remained in the medium until harvest (+) or was removed from the medium for the indicated number of days before harvest (− days). Untransfected LNCaP cells were maintained in the absence of tet. Cells (750,000) were plated in 25-cm^2 flasks, with three flasks per condition. TPA (final, 10 nM), bryostatin 1 (Bryo; final, 10 nM), or ethanol vehicle (Vhcl.; final, 0.006%) was added to the medium 3 days after plating, and cells were harvested 2 or 3 days after drug addition or 2 days after vehicle addition. Cells that excluded (Viable) or were permeable to trypan blue (Dead) were counted, and the percentage of viable plus dead cells that were dead was calculated (% Dead). The percentage of cells with fragmented DNA (DNA frag) was determined as described in the text. Detached cells (det) were included in the table only when the fraction of detached cells was greater than 0.1% of the total number of cells in adherent (adh) plus detached fractions.

Cells	Tet (1 μg/ml)	Drug (10 nM)	Cell numbers			DNA Fragmentation
			Viable	Dead	% Dead	
				$\times 10^{-4}$		% cells
LNα 17 adh	+	Vhcl.	379 ± 31	4.08 ± 0.51	1.07 ± 0.08	0.00
LNα 17 adh	−3 days	Vhcl.	347 ± 29	3.48 ± 0.28	0.99 ± 0.06	0.13 ± 0.07
LNα 17 adh	−4 days	Vhcl.	353 ± 33	3.93 ± 0.20	1.10 ± 0.14	0.00
LNα 17 adh	−5 days	Vhcl.	338 ± 38	3.66 ± 0.29	1.07 ± 0.18	0.00
LNα 17 adh	−7 days	Vhcl.	313 ± 27	3.68 ± 0.38	1.16 ± 0.09	0.13 ± 0.13
LNCaP adh		Vhcl.	267 ± 22	3.08 ± 0.31	1.14 ± 0.09	0.00
LNCaP adh		TPA, 2 days	45.8 ± 5.0	4.63 ± 0.42	9.18 ± 1.0	16.6 ± 1.3
LNCaP det		TPA, 2 days	11.2 ± 1.8	45.2 ± 3.2	80.1 ± 7.4	88.4 ± 5.8
LNCaP adh		TPA, 3 days	16.3 ± 2.3	3.15 ± 0.45	16.2 ± 1.1	15.9 ± 1.4
LNCaP det		TPA, 3 days	2.74 ± 0.49	40.9 ± 5.4	93.7 ± 8.8	81.0 ± 5.6
LNα 17 adh	+	Bryo, 2 days	366 ± 30	4.45 ± 0.48	1.20 ± 0.11	0.00
LNα 17 adh	−11 days	Bryo, 2 days	42.4 ± 3.7	5.95 ± 0.43	12.3 ± 0.93	17.3 ± 1.8
LNα 17 det	−11 days	Bryo, 2 days	10.7 ± 1.3	49.1 ± 4.7	82.1 ± 6.6	91.5 ± 5.8
LNα 17 adh	−12 days	Bryo, 3 days	11.5 ± 2.1	2.31 ± 0.34	16.7 ± 1.7	16.3 ± 1.1
LNα 17 det	−12 days	Bryo, 3 days	2.95 ± 0.36	42.6 ± 4.9	93.5 ± 9.0	82.7 ± 5.2

(Zhao et al., 1997). Furthermore, the addition of bryostatin 1 to PKCα-overexpressing LNCaP cells, but not overexpression of PKCα alone, induced p21$^{WAF1/CIP1}$ expression, Rb hypophosphorylation, and cleavage of the caspase substrate nuclear poly(ADP-ribose) polymerase (Zhao et al., 1997). TPA-induced growth arrest and differentiation of leukemic cells also were found to be associated with increased expression of p21$^{WAF1/CIP1}$ and hypophosphorylation of Rb and were shown to require activation of the ERK pathway (Liu et al., 1996). In addition, constitutively active Raf-1 was reported recently to arrest growth of LNCaP cells (Ravi et al., 1999), and active B-Raf or Raf 1 has been shown in other cell types to induce expression of p21$^{WAF1/CIP1}$ and growth arrest (Woods et al., 1997). Numerous reports have implicated PKC in activation of Raf-1 (see later). Here, we show that TPA, but not bryostatin 1, induced in LNCaP cells prolonged hyperphosphorylation of Raf-1, presumed from decreased gel mobility, and activation of ERKs 1 and 2. Hyperphosphorylation of Raf-1 and activation of ERKs in response to bryostatin 1 were only transient, despite prolonged membrane translocation of PKCδ and ϵ. Our finding that overexpression of PKCα prolonged the activation of Raf-1 and ERKs 1 and 2 by bryostatin 1 suggests that, at least in LNCaP cells, PKCα is a more potent activator of Raf-1 than PKCδ and ϵ.

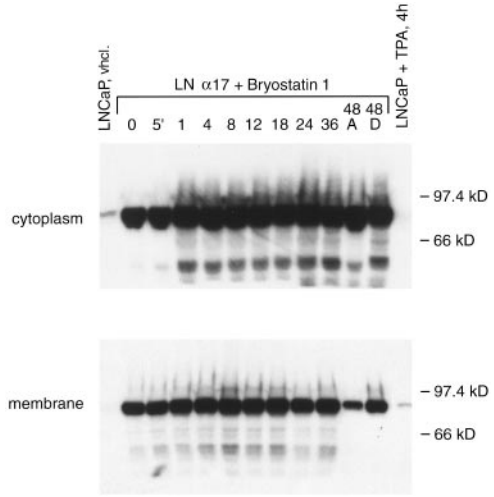

Fig. 7. Effects of bryostatin 1 on cytoplasmic and non-nuclear membrane content of PKCα in PKCα-overexpressing LNCaP clone LNα 17. Three days after plating 6×10^6 cells in 150-cm^2 flasks, cells were treated for up to 48 h with 10 nM bryostatin 1 or for 4 h with 10 nM TPA. Cells were lysed at the indicated times after drug addition, cytoplasmic and non-nuclear membrane fractions were separated, and immunoblots with PKCα-specific, anti-peptide polyclonal antibodies were performed as described in *Materials and Methods*. Numbers above lanes indicate times of drug treatment in h, except 5′ = 5 min. 48hA and 48hD indicate adherent and detached cells, respectively, 48 h after the addition of bryostatin 1. Molecular weights of standard proteins are indicated at right. Two separate experiments were performed with similar results.

The induction of PKCα overexpression in clone LNα 17 in the absence of exogenous PKC activators yielded far more membrane PKC activity than did the treatment of untransfected LNCaP cells with TPA for 4 h (Fig. 4), yet it did not yield hyperphosphorylation of Raf-1 or apoptosis. The absence of an effect of PKCα overexpression alone suggests that either the PKCα was not fully active in the membranes or additional proteins activated by TPA or bryostatin 1 are necessary to properly target PKCα to Raf. A previous report by the developers of the PKC activity assay used here suggested that a substantial portion of membrane PKC found in numerous cell types in the absence of an exogenous activator is in an inactive state (Chakravarthy et al., 1994). That appears to be the case in our PKCα-overexpressing cells, because treatment of LNα17 cells, grown in the absence of tet, with bryostatin 1 for 4 h yielded an additional 3-fold increase in membrane PKC activity (Fig. 4). Although it is possible that the proportion of membrane PKCα that was active in LNα 17 cells grown in the absence of tet was insufficient for hyperphosphorylation of Raf-1, the huge amount of active membrane PKCα in those cells in the absence of bryostatin 1 renders that possibility unlikely. It is more likely that one or more additional proteins are necessary to participate in hyperphosphorylation of Raf or to properly target

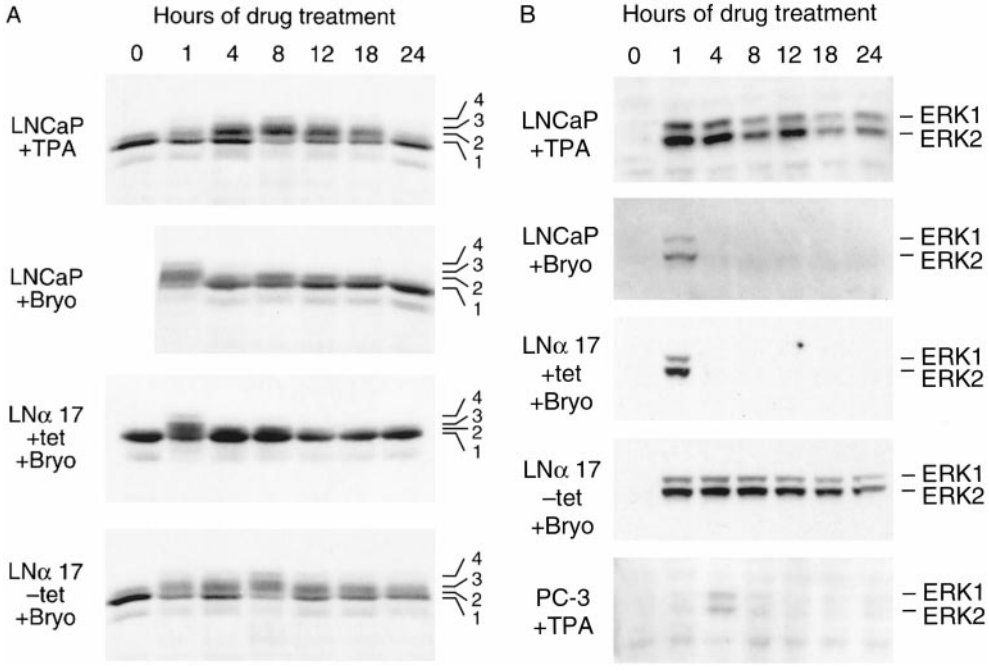

Fig. 8. Effects of TPA and bryostatin 1 on Raf-1 gel mobility and activation of ERKs 1 and 2. Cells were treated for the indicated numbers of hours with 10 nM TPA or 10 nM bryostatin 1 (Bryo), and then total cell lysates were prepared and immunoblot analyses of Raf-1 (A) or dual-phosphorylated ERKs 1 and 2 (B) were performed as described in *Materials and Methods*. LNCaP, untransfected LNCaP cells; LNα 17 + tet, LNα 17 cells grown in the continuous presence of 1 μg/ml tet; LNα 17 + tet, LNα 17 cells grown in the absence of tet for 12 days; PC-3, untransfected PC-3 cells (TPA-resistant control) The entire experiment was performed twice with similar results.

PKCα to Raf. Those proteins might include PKCδ, ε, η, or μ; PKC-binding proteins; or a recently described Ras-GTP exchange protein, rasGRP (Ebinu et al., 1998). The latter protein is intriguing because it has been shown to be activated by TPA and in turn activates Ras, which may be required for membrane localization of Raf (Marais et al., 1998). We have found that rasGRP mRNA is expressed at a low level in LNCaP cells but is undetectable in TPA-resistant PC-3 cells (C.T. Powell, unpublished data).

Exogenous overexpression of wild-type PKCα and ε and deletion mutants of PKCα, ε, and η have been reported to activate Raf-1 in COS and NIH 3T3 cells in the absence of an exogenous PKC activator (Cai et al., 1997; Schonwasser et al., 1998). On the other hand, another group reported that among overexpressed, constitutively active point mutants, PKCδ, but not PKCα and ε, induced activation of Raf-1 in COS cells (Ueda et al., 1996). Although some of the discrepancies among different reports might be explained by postulating that different activating mutations of PKC isozymes might alter specificity for or targeting to Raf-1 in different ways, the finding that overexpression of wild-type PKCα and ε can activate Raf-1 clearly differs from our data and may reflect cell type differences. Alternatively, our measure of Raf-1 gel mobility may not be as sensitive as the assay used by Cai et al. (1997), which included in vitro activation of mitogen-activated protein kinase kinase by immunoprecipitated, overexpressed Raf-1. In any case, overexpression of PKCα in LNCaP cells was not sufficient to induce p21 expression (Zhao et al., 1997) or apoptosis. These results differ from those of Blagosklonny (1998), who found that overexpression of PKCα alone yielded $p21^{WAF1/CIP1}$ expression and growth arrest of SKBR3 breast cancer cells. Although Raf-1 hyperphosphorylation was not examined in that study, a possible explanation, if one assumes that Ras is required for activation of Raf-1, is that SKBR3 cells contain higher constitutive Ras activity than LNCaP cells when grown in the presence of 10% FBS.

Constitutive overexpression of PKCα did slow the growth of LNCaP cells slightly (Fig. 5), and the growth rates were inversely proportional to the amount of overexpressed PKCα. It is not clear whether the slower growth rate of PKCα-overexpressing LNCaP cells is due to kinase activity of PKCα or to a nonspecific effect of the presence of an overwhelming amount of PKCα in the cells. This is complicated further by the finding that in the presence of tet, LNα 17 and LNα 20 cells grew somewhat faster than untransfected LNCaP cells, possibly due to cloning variations. It is also possible that the small amount of background PKCα expression in clones LNα 17 and 20 in the presence of tet was sufficient to stimulate growth slightly but insufficient to induce detectable changes in Raf-1 or ERK activation or $p21^{WAF1/CIP1}$ expression in the absence of an exogenous PKC activator.

It is important to point out that LNCaP cell death is associated with very prolonged activation of PKC. The growth of LNCaP cells in culture is normally stimulated by 10% FBS, which contains growth factors that would be expected to activate PKC transiently through the production of diacylglycerol. Even TPA-mediated PKC activation is more commonly associated with increased growth than with death (Clemens et al., 1992; Nishizuka, 1995). At least in NIH 3T3 cells, TPA mitogenicity is associated with transient membrane translocation of PKCα (Szallasi et al., 1994b). Our data are in agreement with a previous report that induction of T cell hybridoma apoptosis by anti-CD3 antibodies correlates with membrane translocation of PKCα for at least 3 h (Jin et al., 1992). It would be interesting to know whether activation of PKCα was sufficiently prolonged in those cells to induce p21 expression.

Acknowledgments

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