Enhancement of Cisplatin Sensitivity of Cisplatin-Resistant Human Cervical Carcinoma Cells by Bryostatin 1

Sanghamitra Mohanty, Jie Huang, and Alakananda Basu

Abstract

Purpose: Bryostatin 1, a unique protein kinase C (PKC) activator, is already in the clinical trials. An understanding of complex regulation of PKC by bryostatin 1 is essential for effective use of bryostatin 1 in the clinic. We have previously shown that the ability of bryostatin 1 to enhance cisplatin sensitivity correlated with its ability to down-regulate PKCδ in HeLa cells. We have investigated how bryostatin 1 influences PKCδ regulation in cisplatin-resistant HeLa (HeLa/CP) cells, and if bryostatin 1 could be used to reverse cisplatin resistance.

Experimental Design: Phorbol 12,13-dibutyrate (PDBu), bryostatin 1, and small interfering RNA were used to manipulate PKC level/activation status. Cell death was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Annexin V dye-binding assay, and analysis of hypodiploid peak in a flow cytometer.

Results: Bryostatin 1 elicited a biphasic concentration response on PKCδ down-regulation and cisplatin-induced cell death in HeLa/CP cells; the maximum effect was achieved with 1 nmol/L bryostatin 1. Down-regulation of PKCα increased with increasing concentrations of bryostatin 1. PDBu induced down-regulation of PKCα in HeLa and HeLa/CP cells but it had little effect on PKCδ down-regulation in HeLa/CP cells. However, both PDBu and bryostatin 1 enhanced the sensitivity of HeLa/CP cells to cisplatin. Knockdown of PKCδ by small interfering RNA inhibited cisplatin-induced apoptosis but knockdown of PKCα enhanced cisplatin-induced cell death.

Conclusions: These results suggest that although PKCδ acts as a proapoptotic protein, full-length PKCδ may inhibit cisplatin-induced cell death. Thus, persistent activation/down-regulation of PKCδ by bryostatin 1 was associated with cisplatin sensitization. Furthermore, PKCα acts as an antiapoptotic protein and down-regulation of PKCα by PDBu was associated with cellular sensitization to cisplatin.

Cis-Diamminedichloroplatinum(II) or cisplatin is one of the most effective anticancer drugs used in the treatment of solid tumors, including ovarian, testicular, cervical, and small cell lung cancers (1, 2). The acquisition of resistance by tumor cells to cisplatin is one of the major problems in cisplatin therapy. Although DNA is the primary target of cisplatin, other cellular factors can influence its cytotoxicity. We, and others, have shown that the protein kinase C (PKC) signaling pathway plays an important role in regulating cisplatin sensitivity (3–9).

PKC represents a family of at least 10 isozymes that have been categorized into three groups: conventional or cPKCs (α, βI, βII, and γ), novel or nPKCs (δ, ε, η, and θ), and atypical or aPKCs (ζ and λ/ι; refs. 10–13). Whereas cPKCs are dependent on Ca^{2+} and diacylglycerol for activity, nPKCs are Ca^{2+}-independent. aPKCs are insensitive to both Ca^{2+} and diacylglycerol. Tumor-promoting phorbol esters are potent activators of PKCs and can substitute for diacylglycerol (10). Prolonged cellular exposure to phorbol esters can lead to depletion or down-regulation of conventional and novel PKCs (10, 13, 14).

PKCδ has been intimately associated with DNA damage–induced apoptosis (15, 16). There are, however, controversies regarding how PKCδ influences apoptosis. PKCδ is a substrate for caspase-3 and proteolytic activation of PKCδ has been linked to DNA damage–induced apoptosis (15, 16). Several studies suggested that activation of PKCδ was associated with cell death (17–20). However, PKCδ has been shown to promote cell survival and chemotherapeutic drug resistance in human non–small cell lung cancer cells (21). We have shown that prolonged cellular exposure to PKC activators that led to down-regulation of PKCδ enhanced cell death induced by the DNA-damaging agent cisplatin (5, 9). Paradoxically, rottlerin, a pharmacologic inhibitor of PKCδ, prevented cisplatin-induced apoptosis (8, 9).

Bryostatin 1, a macrocyclic lactone derived from the marine bryozoan *Bugula neritina*, belongs to a unique class of PKC activators (22, 23). It binds to and activates PKC, but it can also act as a partial agonist and often antagonizes its own effect or the effects of phorbol esters (23, 24). Unlike phorbol esters, bryostatin 1 lacks tumor-promoting activity and is an important

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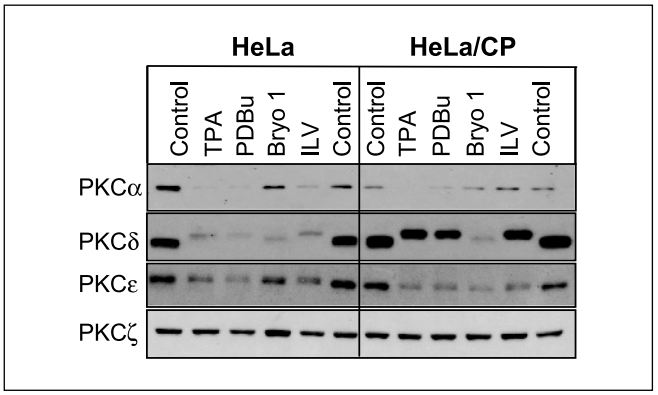

Fig. 1. Effects of PKC activators on the down-regulation of PKC isozymes in HeLa and HeLa/CP cells. Cells were treated with or without 100 nmol/L 12-*O*-tetradecanoylphorbol 13-acetate, 1 μmol/L PDBu, 1 nmol/L bryostatin 1, or 10 μmol/L indolactam V for 24 hours. Western blot analyses were done with total cell lysates using antibodies specific to PKC isozymes. Results are representative of at least three independent experiments.

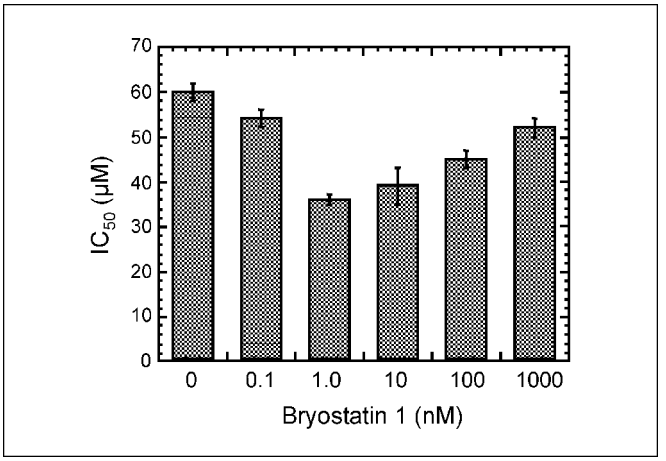

Fig. 3. Effects of different concentrations of bryostatin 1 on the sensitivity of HeLa/CP cells to cisplatin. Cells were pretreated with different concentrations of bryostatin 1 and then exposed to varying concentrations of cisplatin. Cell survival was determined by the MTT assay as described in Materials and Methods. IC_{50} values were determined from the cell survival curves.

candidate for anticancer therapy (24–26). Although bryostatin 1 does not affect proliferation of HeLa cells by itself, it enhances cellular sensitivity to cisplatin significantly at subnanomolar concentrations (6, 9). It, however, elicits a biphasic effect on cisplatin sensitization (6). We have shown that the biphasic concentration response of bryostatin 1 on cisplatin-induced cell death can be explained by PKCδ down-regulation and caspase activation (9).

We have recently shown that the regulation of PKCδ by phorbol 12,13-dibutyrate (PDBu) was affected in HeLa cells that acquired resistance to cisplatin (27). The level of PKCδ was elevated in cisplatin-resistant HeLa (HeLa/CP) cells and the ability of PDBu to induce down-regulation of PKCδ was compromised in HeLa/CP cells compared with drug-sensitive parental cells (27). Because the regulation of PKCδ by bryostatin 1 and PDBu is distinct and bryostatin 1 is already in clinical trials, we have examined how bryostatin 1 influences PKCδ regulation in cisplatin-resistant HeLa cells, and if bryostatin 1 could be used to circumvent cisplatin resistance.

Materials and Methods

Materials. 12-*O*-Tetradecanoylphorbol 13-acetate and PDBu were purchased from LC Service Corporation (Woburn, MA). Cisplatin and MTT were from Sigma (St. Louis, MO). Small interfering RNAs (siRNA) and polyclonal antibodies to PKCδ and PKCε were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody to PKCζ, and monoclonal antibody to PKCα were from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal antibody to caspase-3 and monoclonal antibody to poly(ADP-ribose) polymerase were from PharMingen (San Diego, CA). Annexin V conjugated to Alexa Fluor 488 and propidium iodide were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase–conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson ImmunoResearch Lab., Inc. (West Grove, PA). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL).

Cell culture and transfection. Human cervical carcinoma HeLa cells and its cisplatin-resistant variants (HeLa/CP) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 2 mmol/L glutamine, and kept in a humidified incubator at 37°C with 95% air and 5% CO_2. HeLa cells were transfected with full-length PKCδ cloned into pcDNA5 using LipofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and selected using geneticin.

Assessment of cell viability by MTT Assay. Exponentially growing cells were plated in microtiter plates and incubated at 37°C in 5% CO_2. The following day, cells were pretreated with or without PKC activators as indicated in the text and then with different concentrations of cisplatin. The number of viable cells was determined using the dye MTT as previously described (5).

Assessment of apoptosis by flow cytometric analysis. Cells were pretreated with PKC activators for the indicated periods of time and then treated with cisplatin. At the end of the incubation, cells were harvested and washed with PBS. Nuclei were isolated, stained with propidium iodide, and DNA content was analyzed using a flow cytometer (Coulter Epics, Miami, FL; ref. 28).

Annexin V/propidium iodide binding assay. Cells were treated with or without PKC activators and then with cisplatin for 3 hours.

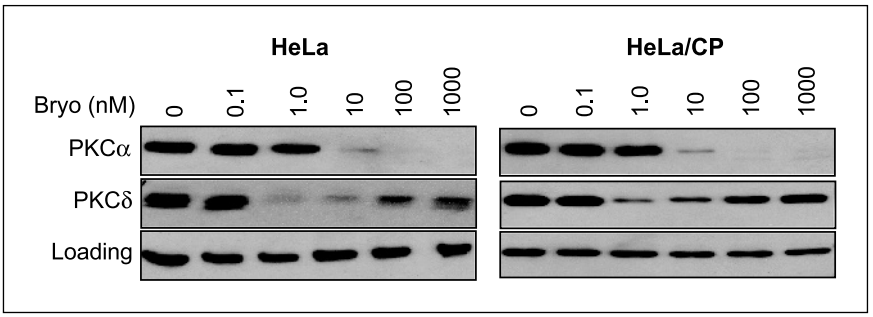

Fig. 2. Effects of different concentrations of bryostatin 1 on down-regulation of PKC isozymes in HeLa and HeLa/CP cells. Cells were treated with different concentrations of bryostatin 1 for 24 hours and Western blot analyses were done with total cell lysates. Results are representative of at least three independent experiments.

Cisplatin-containing media was removed and cells were incubated in drug-free media. At the end of the incubation, both detached cells and attached cells were collected and washed with PBS. Cells were then stained with Annexin V-Alexa 488 conjugate and propidium iodide according to the manufacturer's protocol and analyzed using a flow cytometer (Coulter Epics, Miami, FL).

Immunoblot analysis. Cell extracts containing equal amounts of proteins were electrophoresed by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane. Immunoblot analyses were done as described before (9).

Knockdown of PKC isozymes. Control siRNA or siRNA targeted against PKCα or PKCδ were introduced into HeLa cells using LipofectAMINE 2000 (Invitrogen) and manufacturer's protocol. Briefly, cells were seeded 1 day before transfection. LipofectAMINE 2000 and siRNA diluted in Opti-MEM were mixed gently at a ratio of 300 ng LipofectAMINE 2,000:133 ng siRNA and incubated at room temperature for 15 to 20 minutes. Culture medium was replaced with Opti-MEM and 100 μL of siRNA/LipofectAMINE 2000 complexes were added to cells. After 4 to 6 hours, fresh culture medium was added to cells; 48 hours following siRNA transfection, cells were treated with cisplatin.

Results

Differential effects of protein kinase C activators on protein kinase Cδ down-regulation. We have recently shown that the ability of PDBu to down-regulate PKCδ was compromised in HeLa cells that acquired resistance to cisplatin (HeLa/CP; ref. 27). In the present study, we compared the effects of several PKC activators that differ structurally and functionally on the down-regulation of PKCδ in HeLa and HeLa/CP cells. Figure 1 shows that prolonged cellular exposure to PKC activators, including 12-*O*-tetradecanoylphorbol 13-acetate, PDBu, bryostatin 1, and indolactam V led to down-regulation of PKCδ in HeLa cells. In contrast, with the exception of bryostatin 1, PKC activators failed to induce substantial down-regulation of PKCδ in HeLa/CP cells. The ability of these PKC activators to induce down-regulation of PKCα and PKCε was comparable in HeLa and HeLa/CP cells, suggesting that there was no general alteration in the degradative pathway. None of the PKC activators had any effect on the down-regulation of phorbol ester – insensitive atypical PKCζ. Thus, the level of PKCζ also served as control for equal loading.

Because bryostatin 1 exhibits biphasic down-regulation of PKCδ in HeLa cells (9), we examined the effect of increasing concentrations of bryostatin 1 on PKCδ down-regulation in HeLa/CP cells. Figure 2 shows that bryostatin 1 induced biphasic down-regulation of PKCδ in both HeLa and HeLa/CP cells. The maximum down-regulation was achieved by 1 nmol/L bryostatin 1 and higher concentrations of bryostatin 1 were less effective than 1 nmol/L bryostatin 1 in inducing down-regulation of PKCδ. In contrast, down-regulation of PKCα by bryostatin 1 increased with increasing concentrations of bryostatin 1, such that 1 nmol/L bryostatin 1 had little effect on PKCα down-regulation, whereas 1 μmol/L bryostatin 1 caused complete down-regulation of PKCα in both HeLa and HeLa/CP cells.

Comparison of the effects of phorbol 12,13-dibutyrate and bryostatin 1 on the sensitization of HeLa and HeLa/CP cells to cisplatin. We have previously shown that bryostatin 1 elicits a biphasic concentration response on cisplatin-induced cell death in HeLa cells (6). To examine if PKCδ down-regulation by bryostatin 1 was associated with cellular sensitization to cisplatin in HeLa/CP cells, we determined the concentration

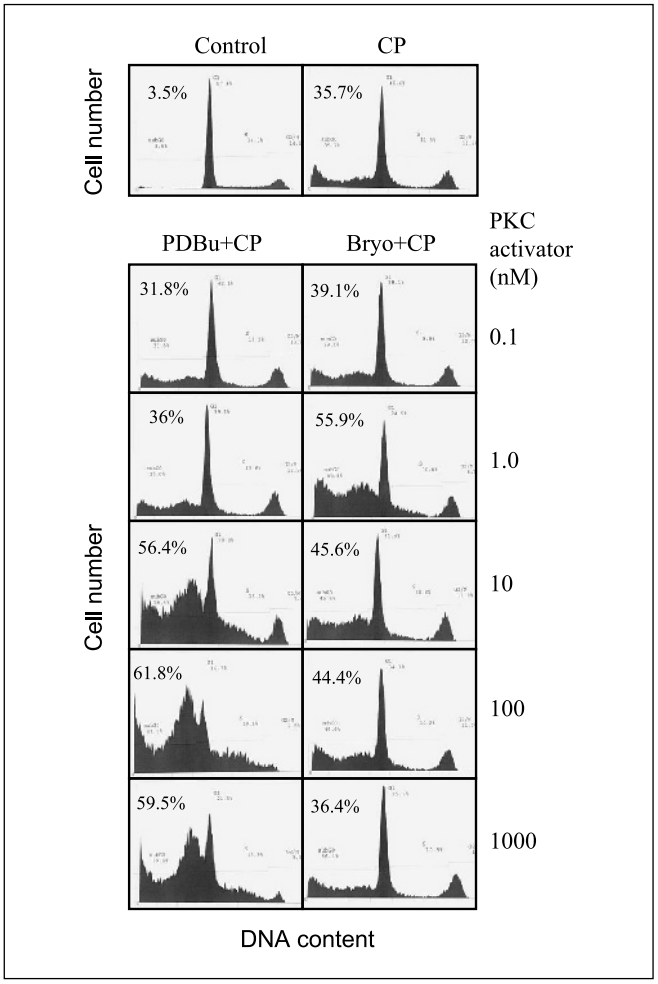

Fig. 4. Concentration-response of PDBu and bryostatin 1 on the sensitization of HeLa/CP cells to cisplatin. Cells were pretreated with or without different concentrations of PDBu or bryostatin 1 and then with 30 μmol/L cisplatin. Nuclei were stained with propidium iodide and DNA was analyzed using a flow cytometer.

response of bryostatin on cellular sensitivity to cisplatin. The IC_{50} of cisplatin at different concentrations of bryostatin 1 was determined from the MTT assay. As shown in Fig. 3, the IC_{50} of cisplatin decreased with increasing concentrations of bryostatin 1 up to 1 nmol/L, and then the ability of bryostatin 1 to enhance cisplatin sensitivity decreased gradually such that 1 μmol/L bryostatin 1 was much less effective than 1 nmol/L bryostatin 1 in enhancing cellular sensitivity to cisplatin. We also assessed cell death by monitoring the appearance of a hypodiploid peak in a flow cytometer. As evident in Fig. 4, bryostatin 1 induced a similar biphasic response in enhancing cisplatin-induced cell death. Whereas treatment of HeLa/CP cells with 30 μmol/L cisplatin resulted in the appearance of 36% cells in the sub-G_1 phase, pretreatment of HeLa/CP cells with 1 nmol/L bryostatin 1 prior to cisplatin treatment increased cell death to 56%. However, 1 μmol/L bryostatin 1 had little effect on cisplatin-induced cell death. Thus, cisplatin sensitization by different concentrations of bryostatin 1 correlated with PKCδ down-regulation.

Because PDBu failed to cause substantial down-regulation of PKCδ in HeLa/CP cells, we compared the ability of PDBu and bryostatin 1 to influence cisplatin sensitivity in HeLa and

HeLa/CP cells. Figure 5 shows that PDBu decreased the IC_{50} of cisplatin from 6.4 to 1.9 μmol/L, whereas bryostatin 1 decreased the IC_{50} of cisplatin from 6.4 to 1.7 μmol/L in HeLa cells. The IC_{50} of CP for HeLa/CP cells was >30 μmol/L. PDBu and bryostatin 1 decreased the IC_{50} of HeLa/CP cells to 16.8 and 14 μmol/L, respectively. This difference was not statistically significant. The concentration response of PDBu shows that the maximum sensitization of HeLa/CP cells to cisplatin was achieved with 100 nmol/L PDBu (Fig. 4). Thus, PDBu was less potent than bryostatin 1. Furthermore, PDBu did not elicit a biphasic concentration response on cisplatin sensitization.

We also monitored apoptotic cell death by the Annexin V dye-binding assay. Cells were costained with the cell-impermeant dye propidium iodide to distinguish apoptotic cells from necrotic cells. When cells undergo apoptosis, phosphatidylserine is flipped from the inner to the outer leaflet of plasma membrane. During the early stage of apoptosis, Annexin V binds to phosphatidylserine on the cell surface. However, during the late stage of apoptosis, Annexin V can enter through the membrane and therefore late-stage apoptosis cannot be distinguished from necrosis. Viable cells are shown at the bottom left quadrant (negative for both Annexin V and propidium iodide). Cells stained with Annexin V conjugate alone (*bottom right quadrant*) represent apoptotic cells, whereas cells costained with Annexin V conjugate and propidium iodide (*top right quadrant*) represent late apoptotic and necrotic cells. We have indicated the total percentage of cell death in Fig. 6. Less than 10% cells underwent apoptosis in untreated HeLa and HeLa/CP cells. Because PKC activators enhance cisplatin-induced cell death, we treated HeLa cells with 5 μmol/L cisplatin that by itself had only a modest effect on cell death, and increased apoptotic cell death from 7.8% to 11.4%. PDBu and bryostatin 1 increased cisplatin-induced cell death in HeLa cells to 62% and 68%, respectively. In HeLa/CP cells, 15 μmol/L cisplatin had little effect on cell death and 45 μmol/L cisplatin increased cell death from 4.9% to 26.6%. Although the effect of bryostatin 1 seems to be less when cells were treated with 15 μmol/L cisplatin, both PDBu and bryostatin 1 were almost equally effective in enhancing cisplatin-induced apoptosis when HeLa/CP cells were treated with 45 μmol/L CP. These results suggest that PKCδ down-regulation was not essential for cisplatin sensitization by PDBu.

Effect of protein kinase Cδ overexpression on cisplatin sensitization by bryostatin 1. Because down-regulation of PKCδ was associated with cisplatin sensitization by bryostatin 1, we examined the effect of PKCδ overexpression on the sensitization of HeLa cells to cisplatin by bryostatin 1. Figure 7A shows that 1 nmol/L of bryostatin 1 caused substantial down-regulation of PKCδ in HeLa cells but bryostatin 1 had little effect on PKCδ down-regulation in PKCδ-overexpressing HeLa cells (HeLa/PKCδ) cells. Ectopic expression of PKCδ alone decreased cisplatin-induced cell death from 19% to 11%. Pretreatment of HeLa cells with bryostatin 1 enhanced cisplatin-induced cell death in HeLa and HeLa/PKCδ cells to 30% and 19%, respectively. Thus, overexpression of PKCδ inhibited cisplatin-induced cell death.

Effect of protein kinase Cδ and protein kinase Cα knockdown on cisplatin sensitization. Because activation of PKC is a prerequisite for its down-regulation, it is difficult to distinguish between persistent activation from down-regulation because residual PKC remains in an active state. To further examine if depletion of PKCδ was necessary for cisplatin sensitization, we used siRNA targeted against PKCδ mRNA to knock down PKCδ. As shown in Fig. 8, we were unable to detect PKCδ in HeLa cells transfected with PKCδ siRNA. However, cisplatin-induced activation of caspase-3 as determined by the increase in processed caspase-3 and cleavage of poly(ADP-ribose) polymerase was reduced in HeLa cells transfected with PKCδ siRNA compared with cells transfected with control siRNA. In contrast, knockdown of PKCα enhanced activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase.

We also examined if knockdown of PKCα and PKCδ by siRNA influences cisplatin-induced cell death when HeLa cells are exposed to cisplatin continuously. We treated cells with 2 μmol/L cisplatin because continuous exposure to 10 μmol/L cisplatin caused extensive cell death. Figure 9 shows that 23% of cells underwent apoptosis when HeLa cells were treated with cisplatin. Knockdown of PKCδ decreased cells in the sub-G_1 phase to 18%, whereas knockdown of PKCα enhanced the percentage of cells in the sub-G_1 phase to 38%. These results show that although depletion of conventional PKCα was associated with cisplatin-induced cell death, depletion of novel PKCδ in fact inhibited cisplatin-induced cell death.

Because the ability of PDBu to down-regulate PKCδ was compromised in cisplatin-resistant HeLa cells yet both

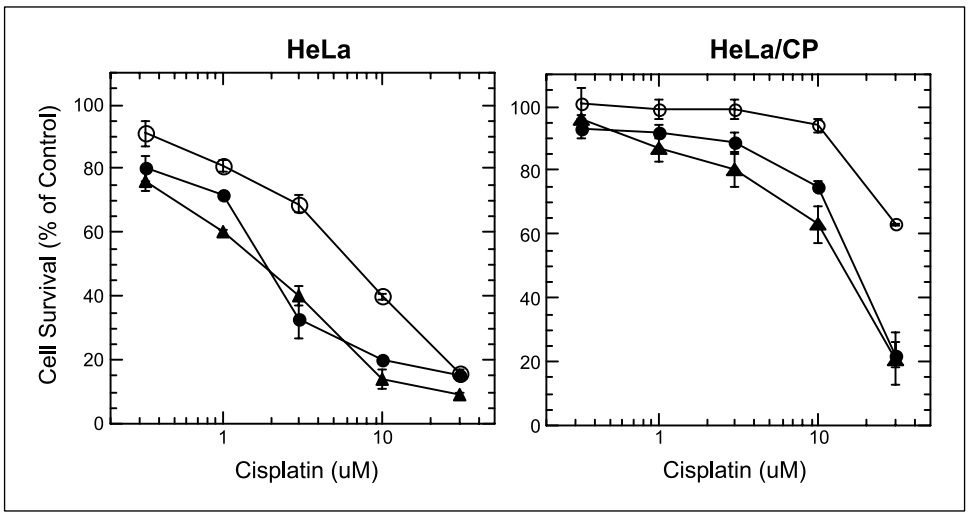

Fig. 5. Comparison of the effects of PDBu and bryostatin 1 on the sensitivity of HeLa and HeLa/CP cells to cisplatin. Cells were treated without (○) or with 1 μmol/L PDBu (●) or 1 nmol/L bryostatin 1 (▲) and then treated with different concentrations of cisplatin. The cell survival was determined by the MTT assay as described in Materials and Methods. Results are representative of at least three independent experiments.

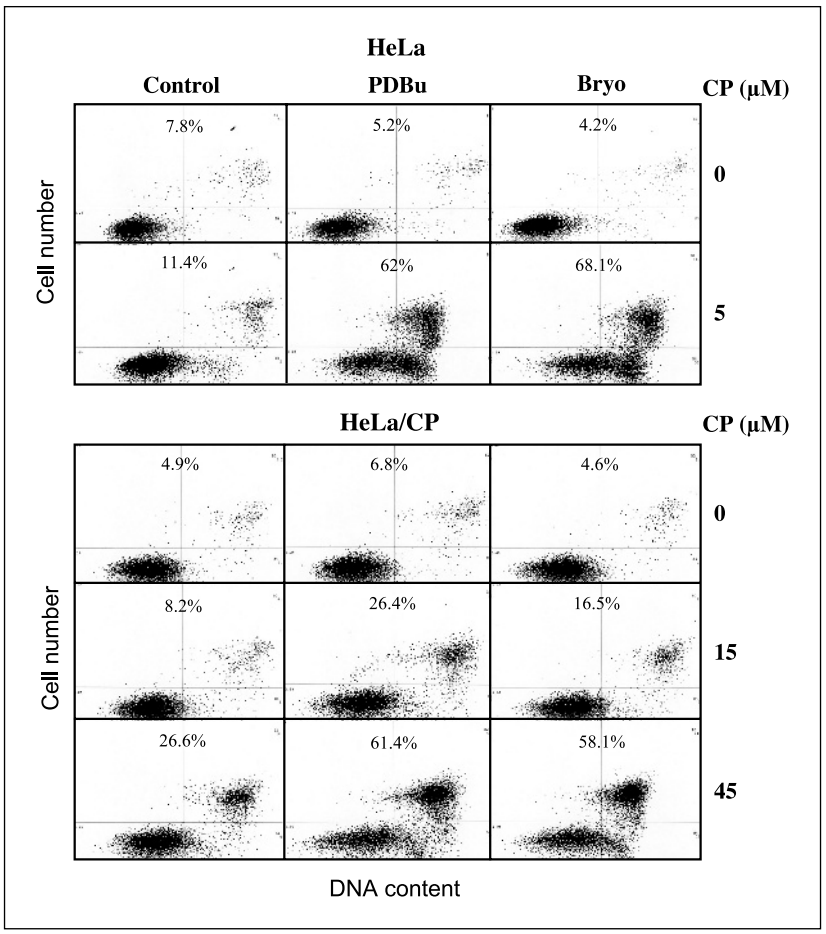

Fig. 6. Comparison of the effects of PDBu and bryostatin 1 on cisplatin-induced apoptosis in HeLa and HeLa/CP cells. Cells were pretreated with 1 μmol/L PDBu or 1 nmol/L bryostatin 1 for 16 hours followed by treatment with cisplatin for 3 hours. Cells were then incubated in drug-free media for 20 hours. The results are representative of two independent experiments.

PDBu and bryostatin 1 sensitized HeLa/CP cells to cisplatin, we examined the consequence of PKCα knockdown on cisplatin-induced cell death in HeLa/CP cells. Figure 10 shows that depletion of PKCα using siRNA, enhanced cisplatin-induced apoptosis as determined by the activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase but depletion of PKCδ inhibited cisplatin-induced apoptosis. These results suggest that the ability of PDBu to sensitize HeLa/CP cells to cisplatin could be explained by down-regulation of PKCα.

Discussion

The results of our present study show that PKC activators, such as PDBu and bryostatin 1 not only sensitize HeLa cells to cisplatin, they also enhance the sensitivity of cisplatin-resistant HeLa cells to cisplatin. However, the mechanism by which they regulate cisplatin sensitivity is distinct. Our results suggest that PKCδ can function as both pro- and antiapoptotic protein, and persistent activation or down-regulation of PKCδ by bryostatin 1 correlated with cellular sensitization to cisplatin. However, depletion of PKCδ was not sufficient to explain cisplatin sensitization by PDBu. We showed that PKCα acts as an antiapoptotic protein and depletion of PKCα was associated with cisplatin sensitization by PDBu.

We have previously shown that bryostatin 1 exhibits biphasic down-regulation of PKCδ (9) and down-regulation of PKCδ by bryostatin 1 correlated with sensitization of HeLa cells to cisplatin (6, 9). Paradoxically, PKCδ inhibitor rottlerin blocked cisplatin-induced cell death (8, 9). These observations raised an important question whether activation or down-regulation of PKCδ was associated with cell death. There are several potential mechanisms that regulate cisplatin-induced cell death, including cisplatin uptake, DNA damage and DNA repair. Although PKC activators caused a modest increase in cisplatin uptake, they had no effect on cisplatin efflux (5, 6, 29). Furthermore, bryostatin 1 reversed the increase in cisplatin uptake by PDBu but had no effect on the rate of cisplatin efflux (29). We have, however, shown that PKC acts upstream of caspases to regulate cisplatin-induced caspase activation and the biphasic concentration response of bryostatin 1 on cisplatin-induced cell death could be explained by its effect on cisplatin-induced caspase activation (9). Furthermore, PKCδ inhibitor rottlerin, which was shown to act at a step subsequent to DNA damage but prior to caspase activation, inhibited cisplatin-induced caspase activation (8, 30).

We have shown that down-regulation of PKCδ by bryostatin 1 also correlated with sensitization of cisplatin-resistant HeLa cells to cisplatin. However, although PDBu failed to induce down-regulation of PKCδ in HeLa/CP cells, both activators were equally effective in sensitizing HeLa/CP cells to cisplatin. These results were based on three independent assays—MTT assay, the appearance of a hypodiploid peak in a flow cytometer, and Annexin V dye binding assay. These results suggest that down-regulation of PKCδ was

not sufficient to explain cisplatin sensitization by PKC activators.

There are differences in how phorbol esters and bryostatin 1 influence cisplatin sensitivity. First, bryostatin 1 is highly potent and it sensitizes cells at subnanomolar concentrations; the maximum sensitization was achieved with 1 nmol/L bryostatin 1. In contrast, PDBu was less potent than bryostatin 1 in enhancing cisplatin-induced cell death and the effects of 10 to 1,000 nmol/L PDBu on cisplatin-induced cell death were comparable. Second, unlike PDBu, bryostatin 1 is a partial agonist and it prevents its own effect or the effects of phorbol esters at higher concentrations (6).

The regulation of PKCs by PDBu and bryostatin 1 is also distinct. HeLa cells express several PKC isozymes, including PKCα, -δ, -ε and -ζ. Unlike PDBu, bryostatin 1 induced biphasic down-regulation of PKCδ and maximum down-regulation of PKCδ was achieved at 1 nmol/L. However, bryostatin 1 did not induce biphasic down-regulation of PKCα. In addition, whereas 1 nmol/L bryostatin 1 was most effective in inducing PKCδ down-regulation, it had little effect on PKCα down-regulation. Thus, in HeLa cells, 1 nmol/L bryostatin 1 that caused maximum sensitization to cisplatin predominantly induced down-regulation of PKCδ, whereas 1 μmol/L PDBu caused down-regulation of both PKCα and PKCδ. In HeLa/CP cells, PDBu primarily caused down-regulation of PKCα because it failed to induce substantial down-regulation of PKCδ.

Because down-regulation of PKCs is a consequence of their activation, reversal of PKCδ down-regulation at higher concentrations of bryostatin 1 suggests that PKCδ that accumulates at higher concentrations of bryostatin 1 is inactive. In addition, because activation of PKC precedes its down-regulation, the

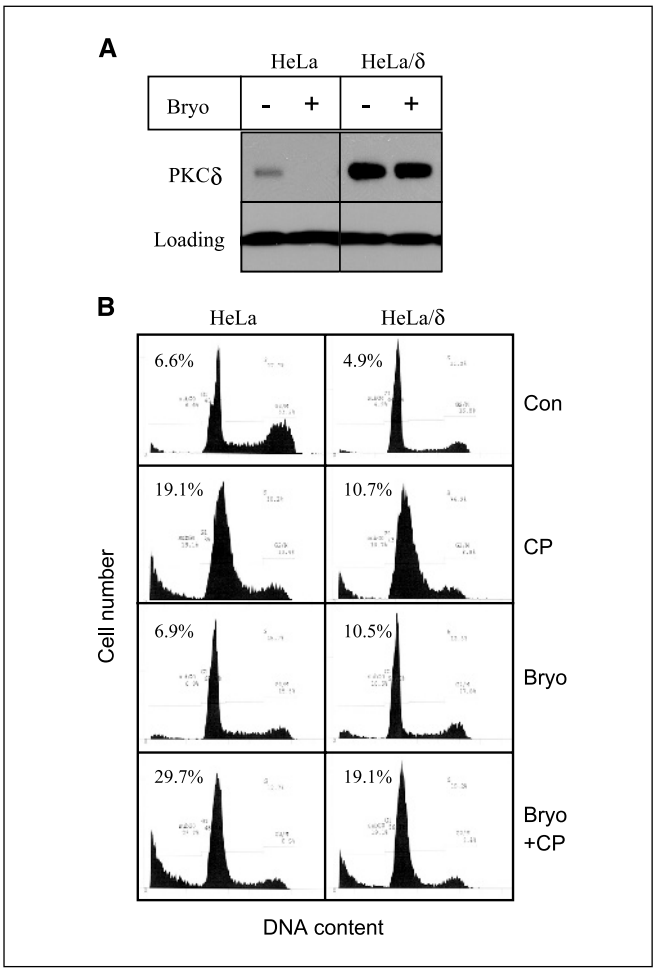

Fig. 7. Effect of PKCδ overexpression on cisplatin-induced cell death. HeLa cells transfected with or without full-length PKCδ were treated with or without 1 nmol/L bryostatin 1 and then with 3.3 μmol/L cisplatin for 24 hours. *A,* Western blot analysis of HeLa and PKCδ overexpressing HeLa cells (HeLa/δ) pretreated with or without 1 nmol/L bryostatin 1 for 24 hours. *B,* nuclei were stained with propidium iodide and DNA was analyzed using a flow cytometer.

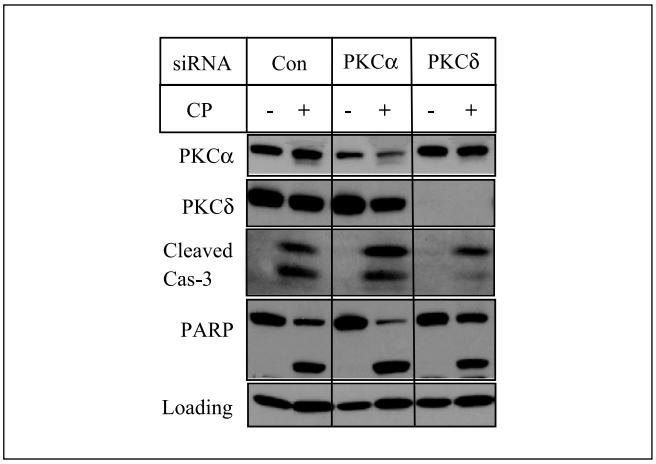

Fig. 8. Effect of knock-down of PKC isozymes on caspase-3 activation in HeLa cells. HeLa cells were transfected with control siRNA or siRNA targeted against PKCα or PKCδ. Cells were then treated with or without 10 μmol/L cisplatin for 2 hours and then incubated in drug-free media for 24 hours. Western blot analyses were done with indicated antibodies. Only the processed forms of 17- and 12-kDa active caspase-3 are shown. Porin was used to control for loading. The results are representative of two independent experiments.

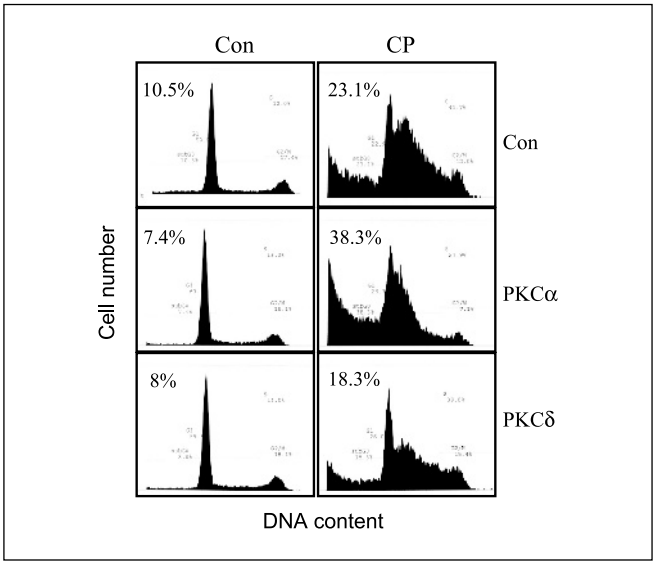

Fig. 9. Effect of knock-down of PKCα and PKCδ on cisplatin-induced apoptosis in HeLa cells. HeLa cells were transfected with control siRNA or siRNA targeted against PKCα or PKCδ. Cells were then treated with or without 2 μmol/L cisplatin for 24 hours. Nuclei were stained with propidium iodide and DNA was analyzed using a flow cytometer.

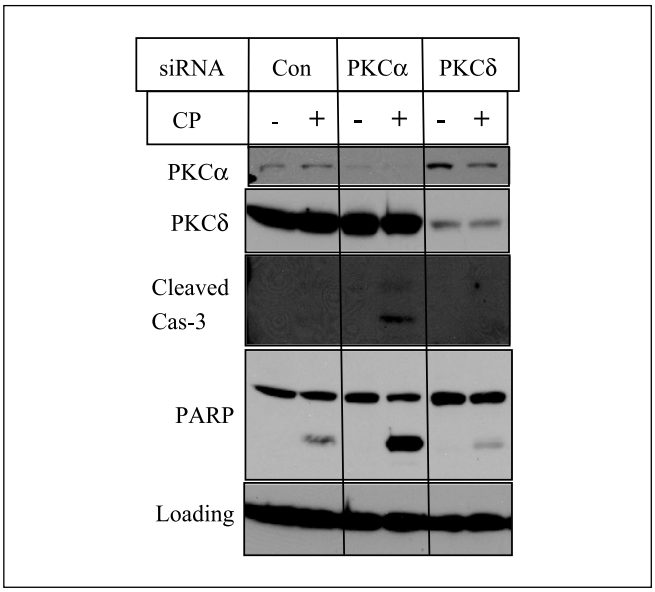

Fig. 10. Effect of knock-down of PKCα and PKCδ on cisplatin-induced apoptosis in HeLa/CP cells. HeLa/CP cells transfected with control siRNA or siRNA targeted against PKCα or PKCδ were treated with or without 30 μmol/L cisplatin for 15 hours. Western blot analyses were done with the indicated antibodies.

residual PKC following down-regulation remains in an active state. The turnover rate of PKCδ is ~8 hours (31) and is greater than most PKCs. Thus, the consequence of PKCδ down-regulation could be distinct from the effect of PKCδ inhibition or depletion.

We therefore examined how knockdown of PKCδ influences cisplatin sensitivity. Our results show that depletion of PKCδ by siRNA targeted against PKCδ in fact reduced cellular sensitivity to cisplatin, suggesting that PKCδ was required for cisplatin-induced cell death. This is consistent with the proapoptotic function of PKCδ. The generation of PKCδ catalytic fragment has been associated with cell death by various apoptotic stimuli (15). Our results suggest that PKCδ may also function as antiapoptotic protein. First, overexpression of PKCδ was associated with cisplatin resistance (27). Second, down-regulation of PKCδ correlated with cisplatin sensitization by bryostatin 1. Finally, ectopic expression of PKCδ in HeLa cells inhibited cisplatin-induced cell death. We propose that whereas proteolytic activation of PKCδ during apoptosis may be associated with cell death, PKCδ holoenzyme inhibits cisplatin-induced cell death. Depletion of PKCδ by siRNA not only removes the full-length antiapoptotic PKCδ but it also prevents generation of cleaved fragments of PKCδ that act as proapoptotic proteins. This may be why the effect of siRNA depletion of PKCδ on cell death was modest. These results also explain why down-regulation of PKCδ correlates with cisplatin sensitization yet PKCδ inhibitor rottlerin prevents cell death by cisplatin. The biphasic response of PKCδ down-regulation and cisplatin sensitization by bryostatin 1 could be explained by the fact that low concentrations of bryostatin 1 caused activation of PKCδ followed by depletion of inactive PKCδ holoenzyme. Accumulation of inactive PKCδ at higher concentrations of bryostatin 1 may prevent cell death by cisplatin. This is consistent with the results that overexpression of PKCδ inhibited cisplatin-induced cell death. The inability of exogenously expressed PKCδ to obliterate responsiveness to bryostatin could be explained by the down-regulation of endogenous PKCδ by bryostatin 1. Knockdown of PKCα, however, enhanced cellular sensitivity to cisplatin. We also found that Gö 6976, which inhibits conventional PKCα, caused substantial increase in the sensitivity of HeLa/CP cells to cisplatin (data not shown), suggesting that PKCα acts as an antiapoptotic protein, and that inhibition of PKCα is associated with cisplatin sensitization. Thus, although PDBu fails to down-regulate PKCδ in HeLa/CP cells, depletion of PKCα by prolonged cellular exposure to PDBu may relieve its antiapoptotic function. Therefore, PKCα may provide a better target in producing cisplatin-induced cell death in HeLa/CP cells.

Bryostatin 1 is already in phase I and II clinical trials, either as a single agent or in combination with chemotherapeutic drugs. Although some studies showed encouraging results, others were not successful (32–35). The regulation of PKC by bryostatin 1 is complex and an understanding of how bryostatin 1 regulates cell death is critical to use it effectively in the clinic. Although phorbol esters cannot be used in the clinic, they provide an important pharmacologic tool to discern the function of PKC. Our results show that PKC activators could be used to enhance cellular sensitivity to cisplatin and shed light on the complex regulation of PKC by bryostatin 1.

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