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Regulation of Caspase Activation and *cis*-Diamminedichloroplatinum(II)-Induced Cell Death by Protein Kinase C†

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ABSTRACT: Activation of caspases is critical for the induction of apoptosis. We have shown previously that cell death mediated by the anticancer agent *cis*-diamminedichloroplatinum(II) (cDDP) is influenced by the protein kinase C (PKC) signal transduction pathway. In the present study, we have examined whether regulation of cDDP sensitivity by PKC involves caspase activation. cDDP caused a time- and concentration-dependent increase in the generation of the catalytic fragment (CF) of novel (n) PKCδ, nPKCϵ, and atypical (a) PKCζ but had little effect on conventional (c) PKCα. Cleavage of PKC isozymes was associated with the activation of caspase-3 and -7 but not of caspase-2. PKC activators enhanced cDDP-induced cleavage of these isozymes and activation of caspase-3. Rottlerin, an inhibitor of nPKCδ, blocked caspase-3 activation and proteolytic cleavage of nPKCδ by cDDP. Bryostatin 1, which elicits a biphasic concentration-response in potentiating cell death by cDDP, exhibited a similar biphasic effect on cDDP-induced activation of caspase-3 and caspase-7 and the cleavage of poly(ADP-ribose) polymerase; while 1 nM bryostatin 1 induced maximum activation of these caspases, 1 μM bryostatin 1 had little effect. z-DEVD-fmk, an inhibitor of caspase-3-like proteases, prevented cDDP-induced cell death. Bryostatin 1 also induced a similar biphasic down-regulation of nPKCδ but not of cPKCα or nPKCϵ. These results suggest that nPKCδ not only acts downstream of caspases but also regulates the activation of caspases and that the biphasic concentration response of bryostatin 1 on cDDP-induced cell death could be explained by its distinct effect on nPKCδ down-regulation and caspase activation.

Caspases, a family of interleukin-1β converting enzyme (ICE)[1]-like cysteine proteases that specifically cleave proteins after Asp residues, play an essential role in the induction of apoptosis (*1*). All caspases exist as inactive proenzymes, which are proteolytically processed to the active heterodimeric form. Caspases can undergo autocatalysis or cleave other caspase zymogens, thus initiating a cascade of events (*1*). To date, 13 caspases have been identified although the regulation of these caspases is largely unknown. While caspase-8, -9, and -10 participate in the initiation phase of apoptosis, caspase-3, -6, and -7 are involved in the execution phase of apoptosis. Activation of these executioner caspases results in the cleavage of critical cellular proteins, including poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, lamin B, and nPKCδ during apoptosis.

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[1] Abbreviations: bryo, bryostatin 1; cDDP, *cis*-diamminedichloroplatinum(II); ICE, interleukin-1β-converting enzyme; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; aPKC, atypical PKC; cPKC, conventional PKC; nPKC, novel PKC; PARP, poly(ADP-ribose) polymerase; z-DEVD-fmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; Ac-DEVD-pNA, acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide.

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We and others have shown that cell death mediated by the chemotherapeutic drug *cis*-diamminedichloroplatinum-(II) (cDDP) is influenced by protein kinase C (PKC) (*2*−*8*), a family of phospholipid-dependent serine/threonine kinases that play a central role in the growth factor signal transduction pathway and regulate a wide variety of cellular functions, including cell proliferation, differentiation, and cell death (*9*−*12*). PKC represents a family of 12 isozymes that have been categorized into 3 groups: group A or cPKCs (α, βI, βII, and γ); group B or nPKCs (δ, ϵ, η, and θ); and group C or aPKCs (ζ and λ/ι) (*10, 11*). In addition, PKCμ resembles nPKCs structurally but aPKCs functionally (*13*). While cPKCs require Ca^{2+} and diacylglycerol/phorbol esters for their activities, nPKCs and aPKCs are Ca^{2+}-independent. aPKCs are also insensitive to diacylglycerol and phorbol esters. PKC isozymes can also be activated by proteolytic separation of the regulatory domain from the catalytic domain. Prolonged cellular exposure to PKC activators can cause degradation or down-regulation of PKC isozymes.

The mechanism by which PKC regulates cDDP sensitivity is unclear. nPKCδ has been shown to be a substrate for caspase-3, and the catalytic fragment of nPKCδ has been directly linked to apoptosis (*14, 15*). We have shown that cells that are resistant to cDDP overexpressed nPKCδ or -ϵ (*7, 8*). In addition, stable transfection of nPKCϵ in rat fibroblasts protected cells against cDDP-induced apoptosis (*6*). Furthermore, prolonged cellular exposure to PKC activators was necessary for any significant sensitization of HeLa cells to cDDP (*4*). These results suggest that nPKC isozymes negatively regulate cDDP-induced cell death. Since caspases have been implicated in the execution of cell death by many apoptotic stimuli, we have examined whether PKC regulates activation of caspases and thus cell death mediated by cDDP.

Bryostatin 1 (bryo), a macrocyclic lactone derived from the marine bryozoan *Bugula neritina*, belongs to a unique class of PKC activators (*16*). It binds to and activates PKC, but it also acts as a partial agonist and often antagonizes its own effect or the effects of phorbol esters (*16, 17*). Unlike phorbol esters, bryo lacks tumor promoting activity and is an important candidate for anticancer therapy (*17*−*19*). Although bryo does not affect proliferation of HeLa cells by itself, it enhances cellular sensitivity to cDDP significantly at subnanomolar concentrations (*5*). It, however, elicits a biphasic effect on cDDP sensitization (*5*). In the present study, we have used this unique compound to demonstrate that PKC functions upstream of caspases to regulate cDDP-induced cell death.

MATERIALS AND METHODS

Materials

PDBu was purchased from LC Service Corp. (Woburn, MA), and MTT was from Sigma (St. Louis, MO). z-DEVD-fmk was obtained from Kamiya Biomedical Co. (Seattle, WA), and Ac-DEVD-pNA was from Calbiochem-Novabiochem Co. (La Jolla, CA). Monoclonal antibody to PKCα was from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal antibody to PKCζ was from GIBCO−BRL (Grand Island, NY), and PKCδ and -ϵ were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies to caspase-2, -3, and -7 were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibody to PARP and polyclonal antibody to caspase-3 were from Pharmingen (San Diego, CA). Horseradish peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmunoResearch Lab. Inc. (West Grove, PA). Poly(vinylidene difluoride) membrane was from Millipore (Bedford, MA), and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL). cDDP and bryo were a generous gift from Bristol-Myers Squibb Co. (Wallingford, CT).

Methods

Cell Culture. Cells were maintained in Dulbecco's modified minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine, and kept in a humidified incubator at 37 °C with 95% air and 5% CO_2.

Assessment of Cell Viability. Exponentially growing cells were plated in microtiter plates and incubated at 37 °C in 5% CO_2. The following day, cells were pretreated without or with PKC modulators and then with different concentrations of cDDP as indicated in the text. The number of viable cells was determined using the dye MTT as described before (*4*).

Immunoblot Analysis. Following treatment with cDDP and PKC modulators, both floating and attached cells were collected, washed twice with cold PBS, and lysed in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EGTA, 1 mM EDTA, 1.0% Triton X-100, 0.5% Nonidet-40, 0.2 mM sodium vanadate, 5 mM benzamidine, and 20 μg/mL each of leupeptin, aprotinin, and soybean trypsin inhibitor. Equal amounts of protein (25−50 μg) from total cellular extracts were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and transferred electrophoretically to a poly(vinylidene difluoride) membrane. Immunoblot analyses were performed with 1:1000 dilution of various antibodies (except for PARP antibody which was used at 1:5000 dilution) as described before (*8*). The blots were visualized using the enhanced chemiluminescence detection reagents and the manufacturer's protocol. Intensities of immunoreactive proteins were quantified by laser densitometry. In each experiment, the same blot was probed with several antibodies, including actin and caspase-2 to control for equal loading.

Caspase Assay. Caspase activity of cell extracts treated with or without cDDP was determined at 37 °C using 200 μM substrate Ac-DEVD-pNA in 50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM DTT, 0.1 mM EDTA, and 0.1% Triton X-100. The release of pNA was measured at 405 nm using a SPECTRAMAX 340 microplate reader (Molecular Devices, Sunnyvale, CA) and SOFTmax PRO software.

RESULTS

Effects of cDDP on PKC Isozyme Content. We and others have shown previously that the PKC signal transduction pathway influences cellular sensitivity to cDDP (*2*−*8*). Since proteolytic activation of nPKCδ has been associated with apoptosis (*14, 15*), we have examined whether cDDP induces cleavage of this PKC isozyme in HeLa cells. Figure 1A shows that cDDP caused a time-dependent decrease in nPKCδ with a concomitant increase in the catalytic fragment of nPKCδ. PKCδ CF appeared after treatment with 20 μM

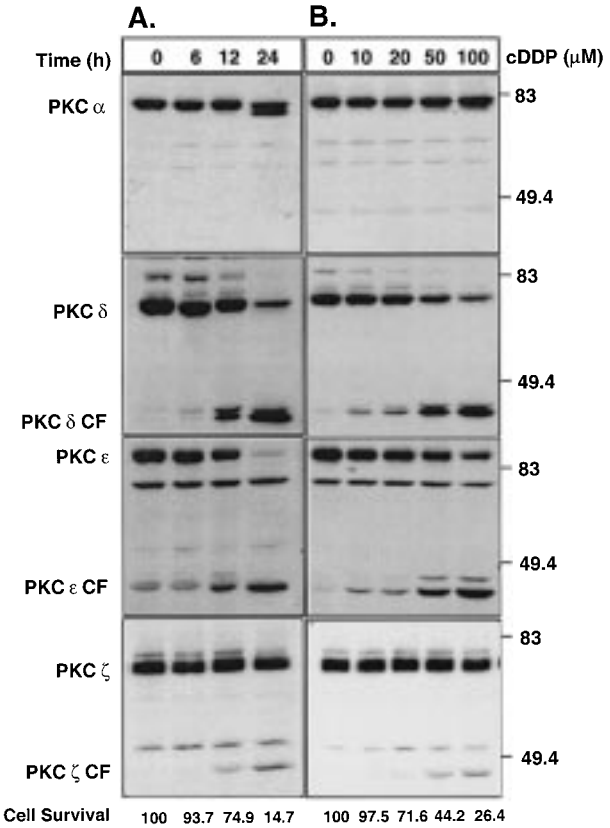

FIGURE 1: Effect of cDDP on the expression of PKC isozymes. HeLa cells were treated with or without cDDP, and Western blot analyses were performed with total cellular extracts using PKC isozyme-specific antibodies as described under Materials and Methods. (A) Cells were treated with 20 μM cDDP for the indicated periods of time. (B) Cells were treated with the indicated concentrations of cDDP for 12 h. The results are representative of 3 experiments. The cell survival was determined by the MTT assay as described under Materials and Methods.

cDDP for at least 6 h and increased significantly by 24 h. cDDP also caused proteolytic cleavage of nPKCϵ and aPKCζ. A 24 h exposure to 20 μM cDDP caused approximately 80% cleavage of nPKCδ and -ϵ. We were unable to detect any catalytic fragment of cPKCα using an antibody that recognizes the catalytic fragment of cPKCα. A 24 h exposure to cDDP, however, resulted in the generation of a faster migrating cPKCα. Figure 1B shows the effect of different concentrations of cDDP on the proteolytic cleavage of PKC isozymes. Treatment of HeLa cells with cDDP for 12 h caused a concentration-dependent increase in the catalytic fragment of nPKCδ and -ϵ with little change in cPKCα expression. The generation of aPKCζ CF was much less compared to nPKCδ or -ϵ. These results suggest that cDDP treatment caused activation of proteases that cleave nPKCδ, -ϵ, and -ζ isozymes.

Effect of PKC Activators on cDDP-Induced Proteolytic Cleavage of PKC Isozymes. We have found that prolonged cellular exposure (>6 h) to PDBu was necessary for any significant sensitization of HeLa cells to cDDP (*4*). To examine whether there was any association between the proteolytic activation of PKC isozymes and cellular sensitization to cDDP by PDBu, we monitored changes in PKC isozyme content in cells that were treated with or without PDBu for 12 h and then with cDDP for an additional 12 h (Figure 2). Under these conditions, PDBu caused an 80% down-regulation of cPKCα. cDDP alone had little effect on the abundance of cPKCα, but it enhanced the degradation of cPKCα by PDBu. PDBu also induced a 75% down-regulation of nPKCδ, and the mobility of nPKCδ from PDBu-treated cells was slower than that from untreated cells, suggesting autophosphorylation of the enzyme. The intensity of nPKCδ CF generated by cDDP was less when cells were pretreated with PDBu, apparently due to down-regulation of this isozyme by PDBu. The abundance of nPKCϵ and aPKCζ CF was increased by PDBu treatment where PDBu caused only 30% or no down-regulation of nPKCϵ and aPKCζ, respectively. Thus, PDBu enhanced cDDP-induced proteolytic cleavage of PKC isozymes.

We also examined the effect of another PKC activator, bryostatin 1 (bryo), that is structurally and functionally distinct from phorbol esters (*16*). We have shown that cDDP sensitization by bryo was concentration-dependent and biphasic; the maximum effect of bryo was seen with 1 nM, but at higher concentrations ($\geq$10 nM), the ability of bryo to sensitize cells to cDDP decreased gradually such that 1 μM bryo had little effect (*5*). To determine whether the biphasic response of bryo on cDDP sensitivity was related to its differential effect on PKC isozymes, we compared the effects of 1 nM and 1 μM bryo on PKC isozyme content. As shown in Figure 2, bryo induced a selective biphasic down-regulation of nPKCδ but not of cPKCα or nPKCϵ; while 1 nM bryo caused an 80% down-regulation of nPKCδ, 1 μM bryo had little effect on nPKCδ down-regulation. Consequently, the abundance of nPKCδ CF generated by cDDP treatment was much greater when cells were treated with 1 μM bryo compared to 1 nM bryo and was almost similar to nPKCδ CF generated by cDDP alone. Thus, a decrease in full-length nPKCδ correlated with cDDP sensitization by bryo. Like PDBu, 1 nM bryo also increased the catalytic fragment of nPKCϵ and aPKCζ, but 1 μM bryo was less effective.

Effects of PKC Modulators on Caspase Activation. Since nPKC isozymes are substrates for caspase-3 and cDDP-induced cleavage of PKC isozymes was increased substantially by PDBu and 1 nM bryo that sensitized cells to cDDP, we examined the effects of PKC activators on caspase activation by monitoring the decrease in levels of the inactive proform which gets proteolytically cleaved upon activation. Figure 3 shows that a 12 h treatment with 20 μM cDDP caused a modest decrease in the inactive 32-kDa procaspase-3 and 1 nM bryo further enhanced cleavage of procaspase-3 by cDDP. In contrast, 1 μM bryo had no additional effect on cDDP-induced caspase-3 activation. A similar result was obtained with caspase-7, which is closely related to caspase-3. The biphasic effect of bryo on caspase-3 activation was also evident when we monitored caspase-3 activation by its ability to cleave its substrate, PARP; 1 nM bryo enhanced cDDP-induced PARP cleavage as evidenced by the decrease in 116-kDa PARP and an increase in the 85-kDa form, but the effect of 1 μM bryo was similar to cells treated with cDDP alone. In several independent experiments, we have found that cDDP neither alone nor in combination with PKC activators had any effect on the activation of caspase-2. In fact, the level of caspase-2 could be used as an internal control to account for any variability associated with the amount of protein loaded in each experiment during electrophoresis. The effect of PDBu on the activation of caspase-3 and caspase-7 and cleavage of

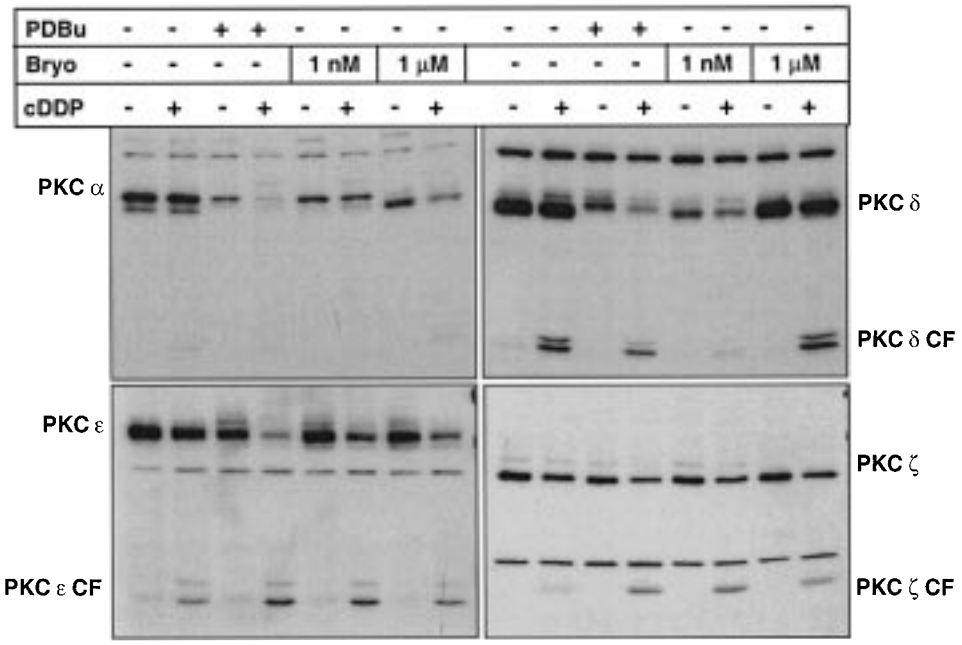

FIGURE 2: Effect of PKC activators on cDDP-induced cleavage of PKC isozymes. Cells were pretreated with or without 1 μM PDBu, 1 nM bryostatin 1, or 1 μM bryostatin 1 for 12 h and then treated with or without 20 μM cDDP for an additional 12 h. Western blot analyses were performed with total cellular extracts using PKC isozyme-specific antibodies. Results are representative of 3−7 experiments.

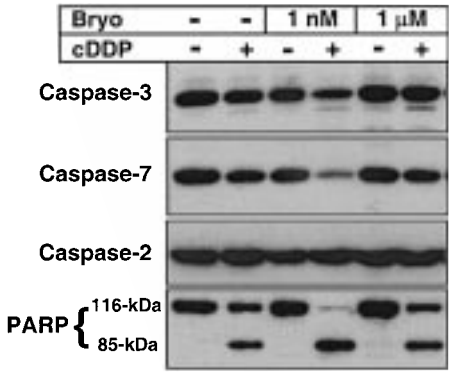

FIGURE 3: Effect of cDDP and bryo on the activation of caspase-3, caspase-7, and caspase-2 and the cleavage of PARP. Cells were treated with 1 nM or 1 μM bryo for 12 h and then treated with 20 μM cDDP for 12 h. Western blot analyses were performed with antibodies against caspase-2, caspase-3, caspase-7, and PARP. Results are representative of 2−4 experiments.

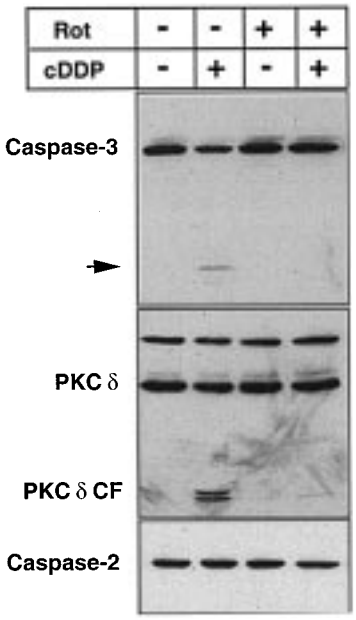

FIGURE 4: Effect of rottlerin on caspase-3 activation and PKCδ cleavage. Cells were pretreated with 10 μM rottlerin for 1 h prior to treatment with 20 μM cDDP for 12 h. Western blot analyses were performed with antibody against PKCδ, caspase-3, and caspase-2. Results are representative of 5 experiments. The arrow indicates the processed form of caspase-3.

PARP was similar to 1 nM bryo (data not shown). To further examine if PKC regulates activation of caspase-3, we examined the effect of rottlerin, an inhibitor of nPKCδ (*20*, *21*), on the reversal of cDDP-induced caspase-3 activation. Figure 4 shows that rottlerin blocked cDDP-induced cleavage of nPKCδ as well as proteolytic activation of caspase-3. These results suggest that nPKCδ not only acts as a substrate for caspase-3 but also regulates its own cleavage by caspases.

Effect of z-DEVD-fmk and Rottlerin on Caspase-3 Activation and cDDP-Induced Cell Death. To investigate if activation of caspase-3 was responsible for cDDP-induced cell death, we examined the effect of z-DEVD-fmk, a cell-permeable inhibitor of caspase-3, on its ability to reverse cDDP-mediated cell death. Since DEVD subsrates may be cleaved by several caspases (*22*), we have used immunoblotting to monitor activation of specific caspases by cDDP and PKC activators. The 32-kDa procaspase-3 is cleaved to 17-kDa and 11-kDa forms upon activation; therefore, we have used an antibody that recognizes both the proform and the larger subunit. Due to low binding of small molecular weight proteins on nitrocellulose, it was, however, difficult to detect the 17-kDa fragment when cells were treated with 20 μM cDDP for only 12 h. As shown in Figure 5, a 24 h exposure to 20 μM cDDP caused a marked decrease in 32-kDa procaspase-3 with an increase in the 17-kDa active form. We could detect a doublet below the 20-kDa region which may represent differentially processed forms of active caspase-3 (*23*). Pretreatment of HeLa cells with 1 nM bryo further enhanced conversion of the 32-kDa proform to the doublet near 17 kDa. z-DEVD-fmk, a cell-permeable inhibi-

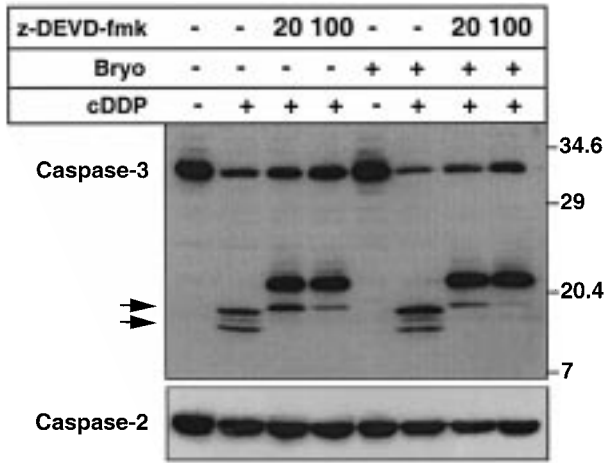

FIGURE 5: Effects of z-DEVD-fmk on cDDP-induced activation of caspase-3 and caspase-2. Cells were treated with 20 or 100 μM z-DEVD-fmk for 3 h and then with 1 nM bryo for 14 h. The treatment with 20 μM z-DEVD-fmk was repeated, and cells were then treated with 20 μM cDDP for 24 h. Western blot analyses were performed with antibodies to caspase-3 and caspase-2. Results are representative of 4 experiments. The arrows indicate the processed forms of caspase-3.

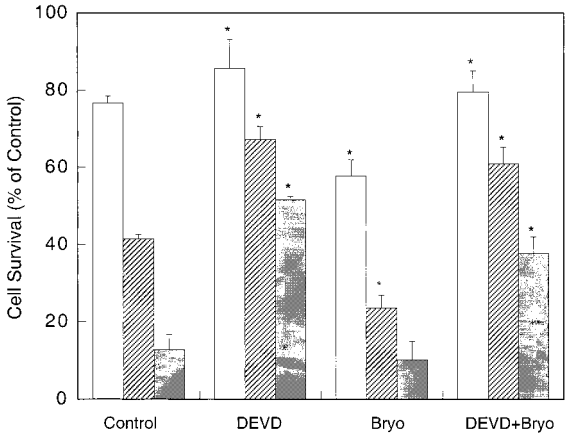

FIGURE 6: Effect of z-DEVD-fmk on cDDP-induced cell death. Cells were treated with 1 nM bryo for 12 h. Following treatment with 100 μM z-DEVD-fmk, cells were incubated with 3, 10, or 30 μM cDDP, and cell survival was determined after 24 h by the MTT assay as described under Materials and Methods. Values are mean $\pm$ standard errors. Statistical significance was determined using the Student's *t* test. Ninety percent confidence limits ($p < 0.05$) were considered significant and are denoted with an asterisk.

tor of caspase-3, blocked the generation of the active forms near the 17-kDa region in a concentration-dependent manner, but a higher molecular weight protein above 20 kDa appeared. We have also performed a parallel experiment to examine the effect of caspase-3 inhibition on cDDP-induced cell death at three different concentrations of cDDP. Figure 6 shows that cDDP caused a concentration-dependent decrease in cell survival; 1 nM bryo enhanced cDDP-induced cell death considerably. Pretreatment of cells with 100 μM z-DEVD-fmk reduced cell death induced by either cDDP or combination of cDDP and bryo at all three concentrations of cDDP. These results suggest that activation of caspase-3 was important for cDDP-induced cell death.

Since rottlerin blocked proteolytic processing of caspase-3 and cleavage of nPKCδ, we examined whether it protects cells against cDDP cytotoxicity. As shown in Figure 7A, treatment of cells with rottlerin inhibited cDDP-induced cell

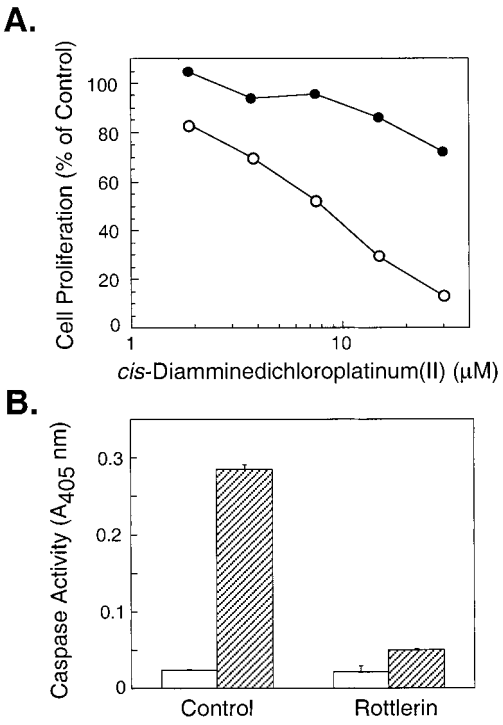

FIGURE 7: Effect of rottlerin on cDDP-induced cell death and caspase activation. (A) Cells were treated without (○) or with (●) 10 μM rottlerin for 1 h and then with different concentrations of cDDP for 24 h. The cell survival was determined after 24 h by the MTT assay as described under Materials and Methods. (B) Cells were treated without or with 10 μM rottlerin and then without (open bar) or with (hatched bar) 20 μM cDDP for 24 h. The caspase activity was determined as described under Materials and Methods.

death. Figure 7B shows that rottlerin also blocked cDDP-induced caspase-3 activation measured using the substrate Ac-DEVD-pNA. These results suggest that inhibition of cDDP-induced caspase-3 activation was responsible for protection against cDDP cytotoxicity by rottlerin.

DISCUSSION

Activation of caspases is central to cell death mediated by a variety of agents although the pathway that leads to caspase activation depends on the apoptotic stimuli. It is generally believed that nPKCδ lies downstream of caspase-3 and proteolytic activation of nPKCδ is responsible for apoptotic execution (*14, 15*). Results of our present study demonstrate that PKC acts upstream of caspase-3 to regulate cisplatin-induced cell death. It has been reported that apoptotic stimuli, such as ionizing radiation or 1-β-D-arabinofuranosylcytosine, cause selective cleavage of nPKCδ but not of cPKCα, cPKCβ, nPKCϵ, or aPKCζ (*14, 15*). We have shown that cDDP induced a time- and concentration-dependent increase in catalytic fragments not only of nPKCδ but also of nPKCϵ and to a lesser extent of aPKCζ. Since both nPKCϵ and aPKCζ contain the sequence DXXD, a potential cleavage site for caspase-3-like proteases, activation of these proteases by cDDP is likely to be responsible for the generation of the catalytic fragments. Although cDDP did not increase the generation of the catalytic fragment of cPKCα, a faster mobility form of PKCα appeared when cells were exposed to cDDP for $\geq$24 h. We speculate that marked activation of caspases may result in cleavage of a small fragment of PKCα because treatment of cells with caspase

inhibitors and/or overexpression of the antiapoptotic protein Bcl-2 prevented the appearance of the faster mobility PKCα (data not shown).

There are several potential mechanisms that may regulate cDDP-induced cell death, including cDDP uptake, DNA damage, and DNA repair (*24*). The observation that z-DEVD-fmk, a cell-permeable inhibitor of caspase-3-like proteases, protected cells against cDDP cytotoxicity demonstrates the importance of these proteases in cDDP-induced cell death. Although z-DEVD-fmk blocked the generation of the 17-kDa active fragment of caspase-3, we consistently detected a higher molecular mass band at above the 20-kDa region. The cleavage of caspase-3 at Asp-175 generates an 11-kDa small subunit and a 21-kDa large subunit, which is further processed at Asp-9 and Asp-28 to remove the *N*-peptide to generate 20-kDa and 17-kDa forms. Thus, the 21-kDa band in the presence of z-DEVD-fmk is presumably the large subunit of caspase-3 that contains the N-terminal peptide (*23*). It has been reported that unlike caspase-1 where the presence of *N*-peptide inhibits substrate hydrolysis, diferentially processed forms of caspase-3 are catalytically active (*23*). Treatment of cells with 100 μM z-DEVD-fmk, however, blocked cDDP-induced increase in caspase activity toward the substrate, Ac-DEVD-pNA. In addtition, z-DEVD-fmk blocked the cleavage of caspase-3 substrates, nPKCδ and PARP (data not shown). HeLa cells also expressed caspase-7, which is closely related to caspase-3, and PKC modulators affected cDDP-induced activation of caspase-7 in a manner similar to caspase-3. The antibody to caspase-7 did not recognize the active catalytic fragment of caspase-7, and, therefore, we were unable to monitor activation of caspase-7 by the generation of the active catalytic fragment. cDDP had no effect on the activation of caspase-2, which was processed by another apoptotic stimulus, tumor necrosis factor-α.

Our study suggests that the activation of caspase-3 by cDDP was regulated by the PKC signal transduction pathway although the critical step has not been identified. First, PKC activators enhanced cDDP-induced conversion of caspase-3 zymogen to the active catalytic form. Second, they enhanced cleavage of caspase-3 substrates, such as nPKCδ and PARP, by cDDP. Third, rottlerin, an inhibitor of nPKCδ, blocked cDDP-induced activation of caspase-3. It is conceivable that PKC can influence cDDP uptake, DNA damage, or DNA repair, as well as later steps in an undefined signaling pathway that results in caspase-3 activation.

We have seen a striking similarity between the concentration response of nPKCδ down-regulation and cDDP sensitization by bryo. This biphasic down-regulation was specific to nPKCδ and was not observed with cPKCα, nPKCε, or aPKCζ. It has been reported that the catalytic domain of nPKCδ confers protection from down-regulation induced by bryo (*25*). At high concentrations, bryo may occupy a low-affinity site and this low-affinity interaction of bryo with PKCδ may be responsible for protection from down-regulation. It has also been reported that the kinase activity is required for down-regulation of PKC isozymes and rottlerin was shown to prevent nPKCδ down-regulation (*21*). We have found that the ability of bryo to activate caspase-3 correlates with its ability to down-regulate nPKCδ. For example, 1 μM bryo failed to down-regulate nPKCδ and did not enhance cDDP-induced caspase activation. We have found that caspase inhibitors did not prevent down-regulation of nPKCδ, suggesting that PKC activators act upstream of caspases.

It is puzzling that while inhibition of proteolytic activation of nPKCδ by rottlerin blocked cDDP-induced cell death, down-regulation of nPKC isozymes enhanced cDDP-induced cell death. The observations that prolonged cellular exposure to PKC activators was necessary for cDDP sensitization (*4*) and overexpression of nPKC isozymes was associated with cDDP resistance (*6*−*8*) indicate that although the catalytic fragment of nPKCδ may be important for cDDP-induced cell death, the holoenzyme protects cells against cDDP cytotoxicity. A similar scenario was observed with the antiapoptotic proteins Bcl-2 and Bcl-X_L (*26*, *27*). While Bcl-2 inhibits caspase activation, it itself is a substrate for caspase-3 and cleavage of Bcl-2 converts it from an antiapoptotic to a proapoptotic protein. There may be a feedback loop betwen nPKCδ and caspase-3, and nPKCδ may regulate its own cleavage in response to apoptotic stimuli. Further studies are needed to determine the precise mechanism by which PKC regulates caspase activation. Since a failure to undergo apoptosis is associated with drug resistance, activation of caspases by PKC provides an important therapeutic strategy for the treatment of cancer.

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