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Phorbol Myristate Acetate Inhibits Okadaic Acid-Induced Apoptosis and Downregulation of X-Linked Inhibitor of Apoptosis in U937 Cells

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Okadaic acid is a specific inhibitor of serine/threonine protein phosphatase 1 (PP-1) and 2A (PP-2A). The phosphorylation and dephosphorylation at the serine/threonine residues on proteins play important roles in regulating gene expression, cell cycle progression, and apoptosis. In this study, phosphatase inhibitor okadaic acid induces apoptosis in U937 cells via a mechanism that appears to involve caspase 3 activation, but not modulation of Bcl-2, Bax, and Bcl-X_L expression levels. Treatment with 20 or 40 nM okadaic acid for 24 h produced DNA fragmentation in U937 cells. This was associated with caspase 3 activation and PLC-γ1 degradation. Okadaic acid-induced caspase 3 activation and PLC-γ1 degradation and apoptosis were dose-dependent with a maximal effect at a concentration of 40 nM. Moreover, PMA (phorbol myristate acetate), PKC (protein kinase C) activator, protected U937 cells from okadaic acid-induced apoptosis, abrogated okadaic acid-induced caspase 3 activation, and specifically inhibited downregulation of XIAP (X-linked inhibitor of apoptosis) by okadaic acid. PMA cotreated U937 cells exhibited less cytochrome *c* release and sustained expression levels of the IAP (inhibitor of apoptosis) proteins during okadaic acid-induced apoptosis. In addition, these findings indicate that PMA inhibits okadaic acid-induced apoptosis by a mechanism that interferes with cytochrome *c* release and activity of caspase 3 that is involved in the execution of apoptosis.

Key Words: **apoptosis; okadaic acid; PMA; caspase 3; IAP.**

Apoptosis is a distinct form of cell death regulated by internal genetic programs. Apoptosis plays an important role in the maintenance of tissue homeostasis by the selective elimination of excessive cells (1). Apoptosis is a highly organized cell death process characterized by rapid condensation of chromatin, loss of plasma membrane phospholipid asymmetry, activation of protease and endonucleases, segmentation of the cells into membrane-enclosed apoptotic bodies (2).

Okadaic acid (Ok) is a potent inhibitor of protein phosphatase type 1 (PP-1) and type 2A (PP-2A), which dephosphorylate serine and threonine residues (3, 4). The use of okadaic acid has led to the understanding that phosphorylation/dephosphorylation status is related to cell proliferation, differentiation and apoptosis (5–8). Previous observations have reported that okadaic acid treatment of different cells can induce apoptosis, and this induction is attributable to inhibition of phosphatases (9–11). However, okadaic acid has been reported to inhibit apoptosis in some instances (12, 13). In an effort to explore the mechanism of okadaic acid-induced apoptosis and the contribution of PKC to okadaic acid-induced apoptosis, I have examined the possible involvement of caspases and IAP family proteins, which form another important family of cell death regulators (14–17). To do this, I determined the protective effects of the PMA because PMA have been reported to activate PKC activities (18). I hypothesized that PMA would protect U937 cells from okadaic acid-induced apoptosis via a decrease in caspase 3 activity. In the present paper, I show that caspase 3 is actively mediating okadaic acid–induced apoptosis. PMA protect cells against okadaic acid induced apoptosis. PMA abrogated okadaic acid-induced caspase 3 activation and specifically inhibited downregulation of XIAP by okadaic acid. Expression of XIAP protein in this cell line provides partial protection against okadaic acid-induced apoptosis, and this protection is clearly associated with inhibition of okadaic acid-induced caspase 3 activation. Taken together, these results demonstrate that caspase 3 is actively involved in okadaic acid-induced U937 cells.

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MATERIALS AND METHODS

Cells. Human leukemia U937 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle medium, containing 10% fetal calf serum (FCS), 20 mM Hepes buffer and 100 μg/ml gentamicin.

Drugs and materials. Anti-Bcl-2, anti-Bax, anti-Bcl-X_L, and anti-HSP70 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against the following proteins were purchased from the indicated suppliers: PARP from Boehringer Mannheim (Indianapolis, IN), cytochrome *c* from PharMingen (San Diego, CA), actin from sigma (St. Louis, MO), and XIAP, cIAP1, and cIAP2 from R & D systems (Minneapolis, MN). Okadaic acid was purchased from Biomol (Plymouth Meeting, PA).

Western blotting. Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM Mops, 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

Cell count and flow cytometry analysis. Cell counts were performed using a hemocytometer. For flow cytometry analysis, approximately 1×10^6 U937 cells were suspended in 100 μl of PBS, and 200 μl of 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS, and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent-activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content based on red fluorescence.

DNA fragmentation assay. After treatment with okadaic acid, U937 cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10,000*g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 μg/ml of ethidium bromide.

Caspase 3 activity assay. To evaluate caspase 3 activity, cell lysates were prepared after their respective treatment with okadaic acid. Assays were performed in 96-well microtiter plates by incubating 20 μg of cell lysates in 100 μl of reaction buffer (1% NP-40, 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 10% glycerol) containing the caspase 3 substrate (DEVD-pNA) at 5 μM. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

Analysis of cytochrome c release. Cells (2×10^6) were harvested, washed once with ice-cold phosphate buffered saline and gently lysed for 2 min in 80 μl ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 20 mM Tris–HCl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin). Lysates were centrifuged at 12,000*g* at 4°C for 10 min. to obtain the supernatants (cytosolic extracts free of mitochondria) and the pellets (fraction that contains mitochondria). The resulting cytosolic fractions were used for Western blot analysis with an anti-cytochrome *c* antibody.

RNA isolation and reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNA was isolated according to Chomczynski and Sacchi (19). Single-strand cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). The cDNA for XIAP, cIAP2 and actin were amplified by PCR with specific primers. The sequences of the sense and antisense primers for XIAP were 5′-CTTGAGGAGTGTCTGGTAAG-3′ and 5′-GTGACTAGATGTCCACAAGG-3′, respectively. The sequences of the sense and anti-sense primer for cIAP2 were 5′-ACCTAC-CTGTGGAGATGCCT-3′ and 5′-CCAGCACGAGCAAGACTCCT-3′, respectively. Conditions for PCR were 1× (94°C, 3 min); 30× (94°C, 45 s; 58°C, 45 s; and 72°C, 1 min); and 1× (72°C, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

RESULTS

Okadaic Acid Induces Apoptosis in U937 Cells

To assess whether or not apoptosis induced by okadaic acid, cells treated for 24 h with various concentrations of okadaic acid were examined with propidium iodine staining. To quantify the apoptosis percentage, we analyzed the amount of sub-G1 DNA by flow cytometry of fixed nuclei (Fig. 1A). U937 cells were exposed to various concentrations of okadaic acid for 24 h. As shown in Fig. 1B, okadaic acid treatment in U937 cells resulted in a markedly increased accumulation of sub-G1 phase in a dose-dependent manner. To further quantify the difference in DNA fragmentation, we analyzed DNA fragmentation on agarose gel electrophoresis. Following agarose gel electrophoresis of U937 cells treated with various concentrations of okadaic acid for 24 h, a typical ladder pattern of internucleosomal fragmentation was observed (Fig. 1C).

Okadaic Acid Induced Caspase 3 Activation and PLC-γ1 Degradation

We also examined whether okadaic acid induces cell death by modulating the expression of Bcl-2 family members, which ultimately determine cell's response to apoptotic stimuli. Treatment of U937 cells with concentrations of okadaic acid that are sufficient to induced apoptosis did not significantly alter the expression of the Bcl-2, Bcl-X_L, or Bax proteins after 24 h (Fig. 2A). These results indicated that the expression levels of Bcl-2 family proteins had no effect on apoptosis induced by okadaic acid in U937 cells.

To determine whether okadaic acid-induced apoptosis was associated with the activation of the caspase 3, we determined the caspase 3 levels and activity in U937 cells that had been exposed to various concentrations of okadaic acid. As shown in Fig. 2B, okadaic acid treatment caused a decrease in levels of pro-caspase 3 in U937 cells exposed to 10 to 40 nM okadaic acid for 24 h. Caspase 3 represents one of the key protease known to be responsible for cleavage of poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase C δ (PKC-δ), and other substrate (20–22). Among the downstream targets of activated caspase 3 *in vivo*, PLC-γ1 has recently been shown to be cleaved into a 60-kDa fragment (23). Subsequent Western blotting demonstrated proteolytic cleavage of

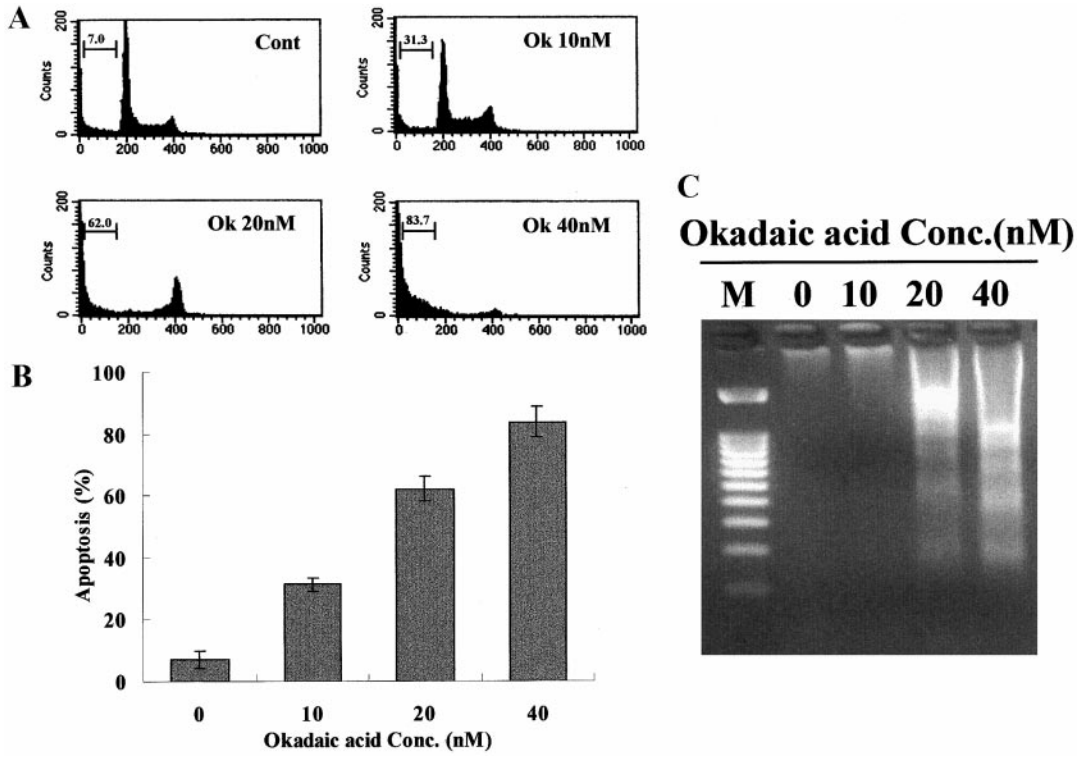

FIG. 1. Okadaic acid-induced apoptosis in U937 cells. (A) FACS analysis of apoptotic cells. Cells were treated for 24 h with the indicated concentrations of okadaic acid and then evaluated for DNA content after propidium iodide staining. (B) The fraction of apoptotic cells as indicated. Data are mean values obtained from three independent experiments and bars represent standard deviations. (C) Fragmentation of genomic DNA in cells were treated for 24 h with indicated concentrations of okadaic acid. Fragmented DNA was extracted and analyzed on 2% agarose gel.

PLC-γ1 in U937 cells after 24 h of okadaic acid. To further investigate and quantitate the proteolytic activity of caspase 3, we performed an *in vitro* assay based on the proteolytic cleavage of DEVD-pN by caspase 3 into the chromophore *p*-nitroanilide (pNA). U937 cells showed a 15-fold increased in DEVD-pNA cleavage after 24 h exposure to 40 nM okadaic acid.

To determine whether activity of caspase 3 was associated with the levels of caspase inhibitors in okadaic acid-induced apoptosis, we determined the expression levels of IAP family proteins in U937 cells that had been exposed to various concentrations of okadaic acid. As shown in Fig. 2C, treatment with okadaic acid did caused a decrease in levels of XIAP and cIAP1, but not cIAP2 in U937 cells exposed to 10 to 40 nM okadaic acid for 24 h. These results indicated that the elevated caspase 3 activity in okadaic acid treated U937 cells are correlated with downregulation of XIAP and cIAP1, but not cIAP2.

PMA Protective Effect on the Okadaic Acid-Induced Apoptosis

To determine whether PMA, protein kinase C (PKC) activator, could prevent okadaic acid-induced apoptosis, U937 cells were treated for 24 h with 20 nM PMA and 40 nM okadaic acid. As shown in Fig. 3A, population of apoptosis obviously decreased in the cultures treated PMA plus okadaic acid compared with the okadaic acid alone treated cells.

To further investigate relationship between PMA protective effect on the okadaic acid-induced apoptosis and caspase 3 activity, U937 cells were treated with okadaic acid in the presence or absence of PMA, and procaspase 3 expression level and caspase 3 activity were measured. Okadaic acid induced a significant reduction of procaspase 3 expression, whereas, PMA cotreatment revealed detectable level of procaspase 3. PMA plus okadaic acid significantly decrease the okadaic acid-induced response as assessed by caspase 3 activities (Fig. 3B).

There is accumulating evidence that the mitochondria play an essential role in many forms of apoptosis by releasing apoptogenic factors, such as cytochrome *c* and apoptosis-inducing factor (AIF) (24). To examine the release of cytochrome *c* in okadaic acid-treated U937 cells, we conducted Western blotting analysis with cytosolic fractions. Importantly, PMA partly blocked okadaic acid-induced release of cytochrome *c* from the mitochondria into the cytoplasm (Fig. 3C). To further characterize the okadaic acid-induced and

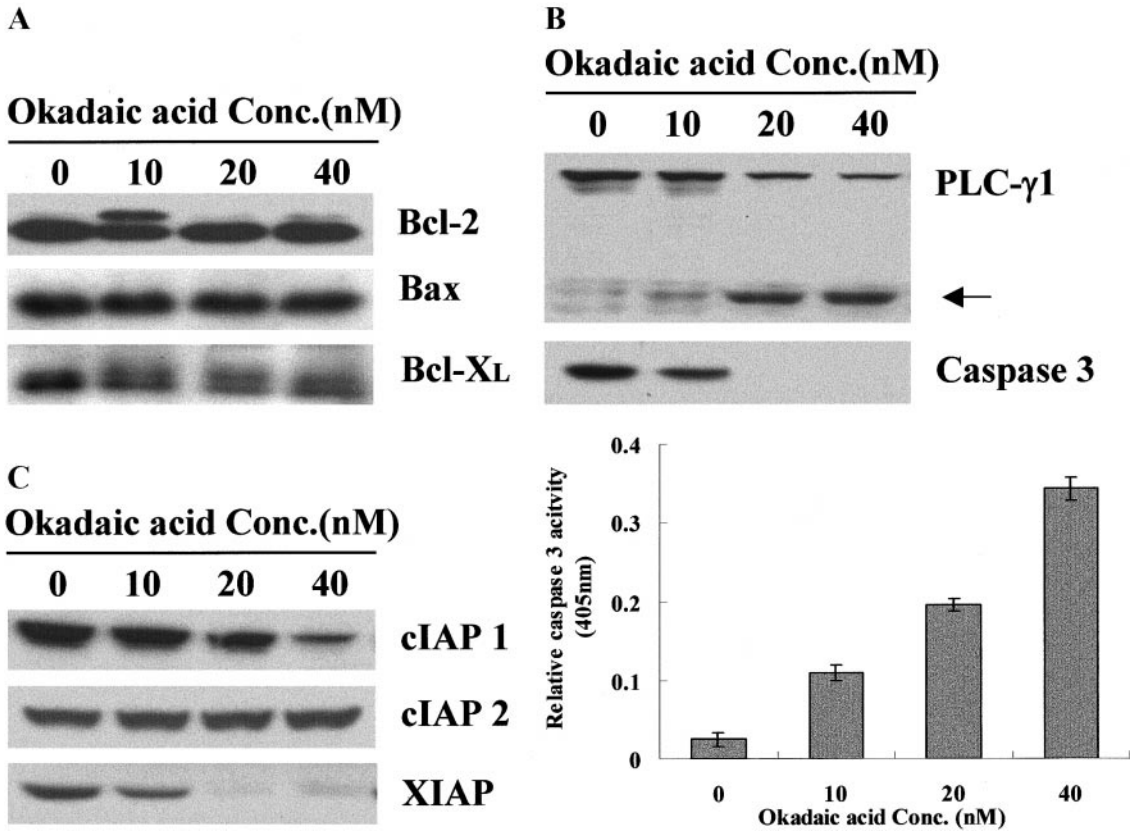

FIG. 2. The expression levels of apoptosis related proteins and effect of okadaic acid on caspase 3 activity in U937 cells. (A) U937 cells were treated with indicated concentrations of okadaic acid. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-Bcl-2, anti-Bcl-X_L and anti-Bax). A representative study is shown; two additional experiments yielded similar results. (B) Cells were treated with the indicated concentrations of okadaic acid. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for caspase 3 and PLC-γ1. The proteolytic cleavage of PLC-γ1 was indicated by arrow. U937 cells were treated with the indicated concentrations of okadaic acid for 24 h and harvested in lysis buffer. Enzymatic activities of caspase 3 were determined by incubation of 20 μg of total protein with 200 μM chromogenic substrate (DEVD-pN) in a 100 μl assay buffer for 2 h at 37°C. The release of chromophore *p*-nitroanilide (pNA) was monitored spectrophotometrically (405 nm). Data are mean values from three independent experiments and bars represent standard deviations. (C) U937 cells were treated with indicated concentrations of okadaic acid. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-cIAP1, anti-cIAP2 and anti-XIP). A representative study is shown; two additional experiments yielded similar results.

PMA-protected nuclear fragmentation in U937 cells, the cells were cultured for 24 h with PMA in the presence of okadaic acid and the DNA was extracted. In okadaic acid treated cells, a DNA fragmentation pattern forming a ladder was observed. However, the degree of ladder formation slightly decreased in PMA and okadaic acid-treated U937 cells (Fig. 3D).

PMA Recovers Okadaic Acid-Mediated XIAP Downregulation by Transcriptional Regulation

IAP-related proteins have been demonstrated to modulate activation of procaspase-3 and caspase cascade (14–17). Modulation of IAP-related protein levels has been associated with apoptosis. Therefore, we examined the effect of PMA and okadaic acid exposure on expression level of IAP-related proteins in U937 cells. Expression levels of the IAP proteins were measured by Western blot after okadaic acid treated with or without PMA. Figure 4A shows that PMA cotreated with okadaic acid recover expression levels of XIAP and cIAP1 in U937 cells. However, the expression levels of cIAP2 and survivin did not altered by treatment with PMA, okadaic acid, and PMA plus okadaic acid. To further elucidate the mechanism responsible for the changes in amounts of XIAP protein, we determined levels of XIAP mRNA by RT-PCR. Treatment with okadaic acid resulted in marked decrease XIAP mRNA levels, however, PMA plus okadaic acid revealed similar levels of untreated control cells (Fig. 4B). There was a correlation between the expression level of XIAP protein and the expression level of XIAP mRNA. Therefore, our results indicated that PMA-mediated XIAP expression was regulated in part at the transcriptional level.

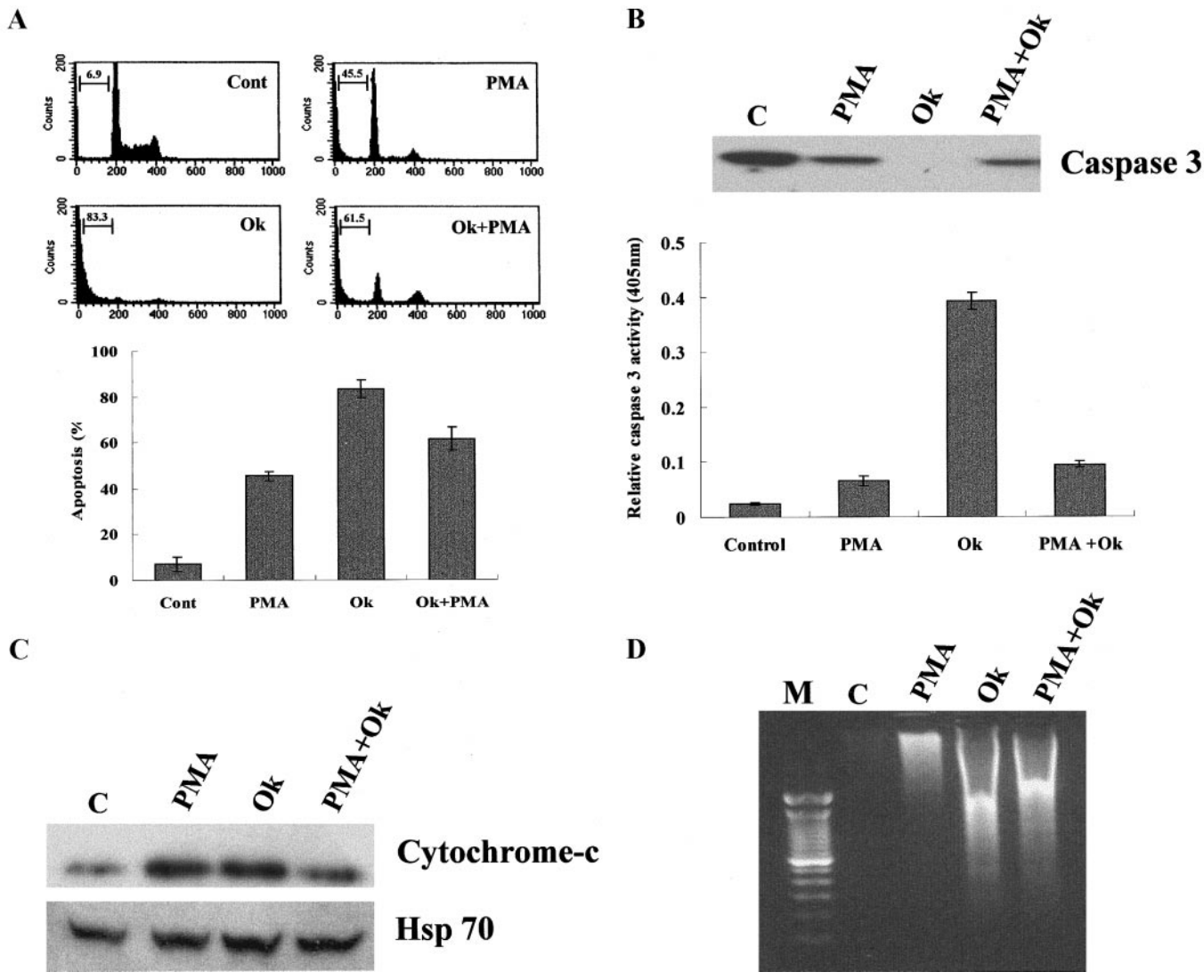

FIG. 3. Effect of PMA on okadaic acid-mediated apoptosis. (A) FACS analysis of apoptotic cells. Cells were treated for 24 h with the 20 nM PMA, 40 nM okadaic acid and 20 nM PMA plus 40 nM okadaic acid, and then evaluated for DNA content after propidium iodide staining. The fraction of apoptotic cells as indicated. Data are mean values obtained from three independent experiments and bars represent standard deviations. (B) Cells were treated with the 20 nM PMA, 40 nM okadaic acid and 20 nM PMA plus 40 nM okadaic acid. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for caspase 3. Caspase 3 activity was determined as described in the legend to Fig. 2. Data are mean values from three independent experiments and bars represent standard deviations. (C) Release of cytochrome *c* in U937 cells. Cells were treated with the 20 nM PMA, 40 nM okadaic acid and 20 nM PMA plus 40 nM okadaic acid. Cytosolic extracts were prepared as described under Materials and Methods. Thirty micrograms of cytosolic protein was resolved on 12% SDS–PAGE and then transferred to nitrocellulose, and probed with specific anti-cytochrome *c* antibody, or with anti-Hsp70 antibody to serve as control for the loading of protein level. (D) Fragmentations of genomic DNA in cells were treated for 24 h with indicated the 20 nM PMA, 40 nM okadaic acid and 20 nM PMA plus 40 nM okadaic acid. Fragmented DNA was extracted and analyzed on 2% agarose gel.

DISCUSSION

Okadaic acid is the most commonly used inhibitor of protein phosphatases 1 and 2A. Okadaic acid, a toxic polyether compound of a C_{38} fatty acid, was first isolated from two sponges, *Halicondria okadai* and *H. melanodocia* (25). Okadaic acid has been reported to exert a variety of biological effects including tumor promotion, differentiation and apoptosis (5–8, 25). Although okadaic acid seems to have a wide range of potential targets, the underlying mechanisms of apoptosis induction are not well understood. Recently, several results were reported on the induction of apoptosis by okadaic acid (9–11).

In this study, we evaluated the possibility that okadaic acid induces apoptotic cell death in U937 cells. Okadaic acid-induced apoptosis is mediated by caspase 3 activation and downregulation of IAP family proteins, but not Bcl-2 family proteins. Furthermore, PMA cotreatment attenuates okadaic acid-induced

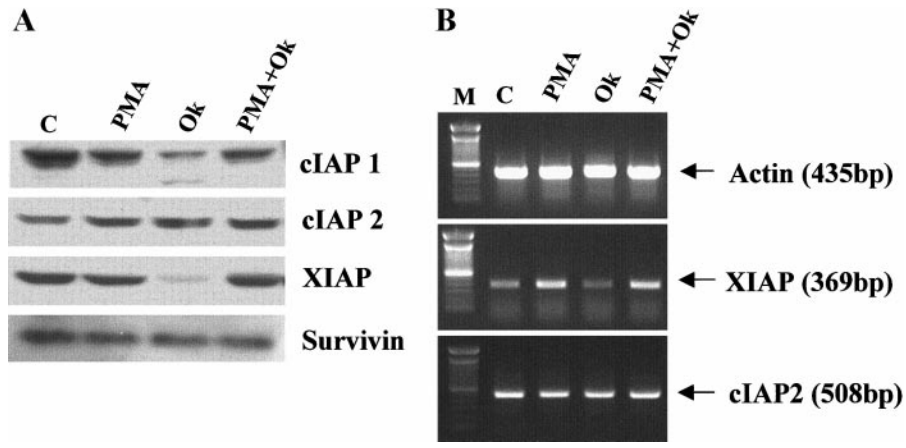

FIG. 4. Effect of PMA on IAPs protein and mRNA expression. (A) U937 cells were treated with the 20 nM PMA, 40 nM okadaic acid and 20 nM PMA plus 40 nM okadaic acid. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-cIAP1, anti-cIAP2, anti-XIP, and anti-survivin). A representative study is shown; two additional experiments yielded similar results. (B) XIAP and cIAP2 mRNA expression in PMA treatment. Cells were incubated for 24 h with the 20 nM PMA, 40 nM okadaic acid and 20 nM PMA plus 40 nM okadaic acid. Total RNA was isolated and RT-PCR analysis was performed. The cycle numbers used for XIAP, cIAP2 and actin were 30, 30, and 25, respectively. Results are representative of three independent experiments.

apoptosis in U937 cells by inhibition of caspase 3 activity and sustained expression of the IAP caspase inhibitors.

One reason for inhibition of caspase 3 activity in PMA treated U937 cells may be blocked release cytochrome *c* from mitochondria. The release of cytochrome *c* induces the activation of caspase 3 and fragmentation of DNA (24). My data showed that PMA partly blocked okadaic acid-induced release of cytochrome *c* from the mitochondria into the cytoplasm. PMA treatment may rescue cells from apoptosis by regulating the permeability of the cellular membranes, blocking the release cytochrome *c* from mitochondria and maintaining membrane integrity. The other reason for caspase 3 inactivation in PMA treated U937 cells may be increased IAP expression. Human IAP proteins, including XIAP, c-IAP1, c-IAP2, NAIP, and survivin, are characterized by the presence of one to three copies of a 70-amino-acid motif, the BIR domain, which bears homology to sequences found in the baculovirus IAP proteins (15). IAPs have been reported to inhibit apoptosis due to their function as direct inhibitors of activated effector caspases, caspase 3 and caspase 7. Furthermore, cIAP1 and cIAP2 are also able to inhibit cytochrome *c*-induced activation of caspase 9 (15–17). In the present study, I also demonstrate that PMA cotreated with okadaic acid recover expression levels of XIAP and cIAP1 in U937 cells. In addition, PMA-mediated XIAP expression was regulated in part at the transcriptional level.

In summary, phosphatase inhibitor okadaic acid induces apoptosis in U937 cells via a mechanism that appears to involve caspase 3 activation, but not modulation of Bcl-2, Bax and Bcl-X_L expression levels. Moreover, PMA, protein kinase C (PKC) activator, protected U937 cells from okadaic acid-induced apoptosis, abrogated okadaic acid-induced caspase 3 activation, and specifically inhibited downregulation of XIAP by okadaic acid. PMA cotreated U937 cells exhibited less cytochrome *c* release and sustained expression levels of the IAP proteins during okadaic acid-induced apoptosis. The results presented indicated that the cell survival signal imparted by PKC signal pathway can be diverted to cell death signals by inhibition of PP1 and PP-2A activities. Thus, the PP1 and PP2A and their associated signaling molecules may be potential targets for human cancer treatments.

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