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Cytoskeletal disruption is the key factor that triggers apoptosis in okadaic acid-treated neuroblastoma cells

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Abstract Okadaic acid (OA) is a tumour promoter that induces apoptosis in several cell models. Following previous findings, the objective of this work was to elucidate the pathways involved in OA-triggered apoptosis in BE(2)-M17 cells by using a combination of pharmacological agents and apoptosis-related assays. OA-induced apoptosis involves disruption of F-actin cytoskeleton, activation of caspase-3, collapse of mitochondrial membrane potential, DNA fragmentation and decreased levels of monomeric Bcl-2 and Bax proteins. All the agents tested were unable to obliterate changes in F-actin levels, caspase-3 activation or DNA fragmentation, but all of them prevented OA-induced decrease of mitochondrial potential and changes in Bax/Bcl-2 levels. Taken together, these results demonstrate that collapse of mitochondrial membrane potential is accessory in the execution of apoptosis, which is directly dependent on cytoskeletal changes. Mitochondrial changes are mediated by complex associations among the Bcl-2 proteins. Cytochrome *c* release from mitochondria is a late event, occurring 24 h after OA exposure. Moreover, okadaic acid triggers activation of upstream caspases resembling the extrinsic pathway of apoptosis.

Keywords Apoptosis · Okadaic acid · Cytoskeleton · Mitochondria · Neuroblastoma cells

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Introduction

Apoptosis or programmed cell death is a multistep, evolutionary conserved process characterized by a set of biochemical and morphological changes that result in selective and highly regulated dismantling of cell structures. Cells committed to die are marked with signals that lead to recognition, engulfment and degradation by phagocytes as the final act in the programmed cell death, which suppress inflammatory and immune responses and controls tissue remodelling (Savil and Fadok 2000).

Two main apoptotic pathways have been described, a mitochondrial or intrinsic pathway and a death-receptor or extrinsic pathway (Bredesen 2000; Hengartner 2000), both of them leading to activation of caspases, a family of cysteine–aspartate proteases that are responsible for the disassembly of cells committed to die (Thornberry and Lazebnik 1998; Slee et al. 1999). Whereas the intrinsic pathway involves an active role for mitochondria, the extrinsic pathway can bypass mitochondria by involving a direct activation of caspases, although both pathways converge at the level of caspase-3 activation.

The mitochondrial pathway plays a central role in most apoptotic scenarios through the release of pro-apoptotic factors to the cytosol, such as cytochrome *c*, procaspases 2, 3, 9 and apoptosis-inducing factor (AIF) (Loeffler and Kroemer 2000). This mitochondrial-dependent pathway is tightly regulated by members of the Bcl-2 protein family. To date, over 20 members of the Bcl-2 family have been identified in humans, including proteins that suppress and proteins that promote cell death (Tsujimoto and Shimizu 2000; Ke et al. 2001). Mostly, pro-apoptotic members reside in the cytosol and, in response to a stimulus, translocate to the mitochondrial membrane where they interact with other proteins to achieve cytochrome *c* release from the mitochondrial intermembrane compartment into the cytosol (Tsujimoto and Shimizu 2000). The exact mechanism by which Bcl-2 proteins regulate cytochrome *c* release has not been proven, although three different

models have been proposed. Bcl-2 family proteins are capable of physically interacting, forming homo- or hetero-dimers, which function as agonists or antagonists of each other. Moreover, these proteins can display different phenotypes depending on the cellular context. (Desagher and Martinou 2000; Hengartner 2000).

To date, apoptosis research has mainly focused on mitochondria as the key executioner of the apoptotic cascade (Pedersen 1999; Desagher and Martinou 2000; Gottlieb 2000; Hengartner 2000). However, mitochondrial-independent pathways have gained an increasing interest as pivotal mechanisms of the apoptotic process induced by many cytotoxic drugs. In this context, there is an emerging view in the literature indicating that cytoskeletal components are involved in downstream signal transduction pathways closely related to cell survival/cell death (Bissell and Nelson 1999; Santini et al. 2000; van de Water et al. 2000). Furthermore, a specific form of apoptosis, termed anoikis, is mediated by different signal transduction pathways and has been identified in cells deprived of anchorage to the extracellular matrix (Frisch et al. 1996; Frisch and Ruoslahti 1997; Khwaja and Downward 1997). Changes in cell shape and anchorage are associated with reorganisation of actin filaments, mostly depending on their intrinsic ability to rapidly assemble and disassemble and thus indicating a key role of the actin cytoskeleton in the complex network that engages membrane-related events and signal transduction cascades. (Small et al. 1999; Moreau and Way 1999; Schoenenberger et al. 1999). Cytoskeletal changes have been extensively reported in apoptotic cells (reviewed in van de Water et al. 2000), although its role in the apoptotic cascade has not been completely elucidated. In fact, actin has initially been identified as a substrate of the caspase family (Mashima et al. 1997), although further works support evidence of caspase-independent disruption of the actin cytoskeleton that precedes activation of the apoptotic machinery (Maruyama et al. 2000; van de Water et al. 2000).

The assembly and disassembly of actin filaments is directly regulated by Ser/Thr protein phosphatases (Kreienbühl et al. 1992), a group of cytosolic enzymes that are potently inhibited by the marine toxin okadaic acid (OA) (Bialojan and Takai 1988). OA is a polyether fatty acid (Yasumoto et al. 1978) extensively reported to induce changes on F-actin levels, both in cultured cells (Baldacini et al. 1993; Macías-Silva and García-Sáinz 1994; Blankson et al. 1995; Yano et al. 1995; Niggli et al. 1999; Leira et al. 2000) and in vivo (Fiorentini et al. 1996). Furthermore, OA has been identified as a potent inducer of apoptosis in a wide variety of cell lines (von Zezschwitz et al. 1997; Yan et al. 1997; Nuydens et al. 1998; Riordan et al. 1998; Leira et al. 2001), although the mechanisms underlying OA-induced apoptosis have not been fully elucidated.

Previous work characterized apoptotic changes induced by OA in the neuroblastoma cell line BE(2)-M17, which involve both mitochondrial membrane potential decrease and disruption of F-actin cytoskeleton (Leira et al. 2002b). Also, a recent study proved that initiation of apoptotic cascade in neuroblastoma cells exposed to OA is mainly mediated through caspase-8 activation (Cabado et al. 2003). In an attempt to investigate further the mechanisms of OA-induced toxicity, we focused on cytoskeletal and mitochondrial events mediated by OA. In this report, we present evidence that OA induces apoptosis in neuroblastoma cells through a cytoskeletal-dependent pathway and that mitochondria play a secondary role.

Materials and methods

Materials, solutions and antibodies

N-Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), *N*-Acetyl-Asp-Glu-Val-Asp-Al (Ac-DEVD-CHO), bovine serum albumin (BSA), dithiothreitol (DTT), aprotinin, benzamidine, pepstatin, leupeptin, paraformaldehyde and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Okadaic acid, phorbol-12-myristate-13-acetate (TPA), chelerythrine chloride, rottlerin, forskolin, H89 and wortmannin were purchased from Alexis Corp. (Lausen, Switzerland). LY294002, KT5720, PD98059, herbymicin A were obtained from Calbiochem Corp. (Darmstadt, Germany), and amphotericin B from Biochrom KG (Berlin, Germany). Stock solutions of these drugs were made in ethanol or dimethyl sulfoxide (DMSO).

The fluorescent probes Mitotracker Red CMXRos, SYBR-Green, CBQCA protein quantitation kit and Oregon Green-514 phalloidin were obtained from Molecular Probes Inc. (Eugene, OR, USA). The caspase-3 activity assay was from Sigma-Aldrich. The enhanced chemiluminiscent detection kit (ECL) was obtained from Amersham-Pharmacia Biotech (Little Chalfont, UK).

Lysis buffer contained 10 mM Tris-HCl (pH 7.05), 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate (NaPPi), 1% Triton X-100, 5 mM $ZnCl_2$, 100 mM Na_3VO_4, 1 mM DTT, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenolphosphate (PNPP), 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.5 mM benzamidine, and 5 μg/ml pepstatin.

Monoclonal antibodies anti-Bcl-2, anti-citochrome C, fluorescein isothiocyanate (FITC)-conjugated secondary antibody and anti-Bax were purchased from Sigma (St. Louis, MO, USA). Anti-mouse IgG, peroxidase-linked was from Amersham Pharmacia Biotech.

Cell culture

The Neuroblastoma cell line BE(2)-M17 (ATCC Number CRL-2267) was purchased from the European Collection of Cell Cultures (Salisbury, UK) and seeded in 25-cm^2 flasks at a density of 4×10^4 cells/cm^2. Cells were cultured on Eagle's Minimum Essential Medium:Nutrient Mixture F-12 Ham (1:1) with 2 mM glutamine, 1% non-essential amino acids, 15% foetal bovine serum, 50 mg/l gentamicin and 50 μg/l amphotericin B. The culture medium was renewed on a 2-to 3-day schedule and cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. In order to carry out the microplate assays, the attached cells were trypsinized after reaching optimum confluence, and seeded in 96-well microtitre plates at 2500 cells/well (200 μl). Following an additional incubation of 48 h (37°C/5% CO_2), cells grown in microtitre plates were used for fluorimetric assays.

Mitochondrial membrane potential

Changes in mitochondrial membrane potential in BE(2)-M17 cells were evaluated using the fluorescent probe Mitotracker Red

CMXRos. Following 1- or 24-h exposure of cells to OA and/or simultaneously to the various pharmacological agents Z-VAD-FMK, Ac-DVDE-CHO, TPA, chelerythrine, rottlerin, forskolin, H89 wortmannin, LY294002, KT5720, PD98059 and herbymicin A, plates were centrifuged. Then, 50 µl culture medium was removed and 50 µl Mitotracker Red CMXRos diluted in the same medium was added to each well to give a final concentration of 1 mM. After an additional incubation for 45 min at 37°C, plates were centrifuged, the culture medium removed and cells were washed and centrifuged. Finally, fluorescence was measured in a LS-50B fluorescence microplate reader (Perkin-Elmer Corp, Norwalk, CT, USA) set at 579 nm excitation and 599 nm emission. Results of triplicate experiments were expressed as average percentages of fluorescence obtained in controls. The influence of cell detachment in the assay was prevented by centrifugation of microplates after each washing step. The reliability of this procedure was demonstrated in previous experiments showing no difference ($P < 0.01$) among the total protein content on each well after OA-treatment, as determined by the CBQCA fluorimetric protein quantitation assay, which guarantees the accuracy of the fluorescence measurements as previously reported (Leira et al. 2001).

Caspase-3 activation

The activation of caspase-3 was measured by using a fluorimetric caspase-3 activity assay. Due to the high number of cells required for this assay ($> 10^6$), incubation of neuroblastoma cells with OA alone or in combination with several agents, was carried out in 25-cm^2 flasks. All cells contained in the flask (both attached and detached) were harvested after 1, 6 or 24 h incubation with the agents. Caspase-3 was extracted from cellular lysates and the fluorescence obtained after addition of a fluorogenic caspase substrate was measured in a final volume of 200 µl with a fluorimetric microplate reader set at 400 nm excitation and 505 nm emission. Cleaved substrate in the assay is proportional to the amount of activated caspase-3, thus allowing quantitative comparisons with respect to control cells.

DNA fragmentation

The typical pattern of DNA fragmentation in apoptotic cells was evaluated after 24-h exposure of BE(2)-M17 cells to 1000 nM OA alone and in combination with different agents. Electrophoresis of extracted DNA from both attached and detached cells was carried out on 1.5% agarose gels, and DNA bands were visualized after SYBR Green staining.

F-actin measurements

The fluorimetric microplate assay for quantitative analysis of F-actin was performed as follows: 2500 cells/well were fixed by adding 50 µl of 18.5% formaldehyde to each well for 30 min at room temperature. Following a washing step with Hanks' balanced solution (200 µl), cells were permeabilized (except blanks) with 200 µl of 0.1% Triton X-100 for 15 min at room temperature. Triton X-100 was removed by washing twice and 500 nM Oregon Green-514 phalloidin solution was added to each well (50 µl). Cells were stained with the fluorochrome for 30 min at room temperature and finally washed twice. Fluorescence of stained F-actin was measured in a LS-50B fluorescence microplate reader (Perkin-Elmer, Beaconsfield, UK) set at 511 nm excitation and 528 nm emission.

The effect of OA as well as other drugs on F-actin levels of neuroblastoma was evaluated in triplicate experiments after 1- and 24-h exposure of the cells to these agents. From each well of the microplate, 100 µl culture medium was removed and 100 µl of OA diluted in culture medium was added to yield final concentrations ranging from 1 to 1000 nM per well. Following incubation, F-actin was measured as previously described, and results were expressed as percentages of fluorimetric values observed in controls after blank subtraction. The same procedure was followed to study the modulatory effect of different signal transduction agents on OA-induced depolymerization of F-actin.

Cells lysates preparation

Neuroblastoma cells were seeded in flasks at least 4 days before the experiment and grown to 80–90% confluence. Cells were incubated with OA alone or in combination with the different agents for 1 or 24 h in culture medium. After incubation, attached and detached cells were collected and centrifuged. Cell lysates were obtained by adding 80 µl lysis buffer to cells followed by incubation on ice for 15 min. Then, cellular lysates were centrifuged for 15 min and the resulting supernatant was resuspended in Laemmli sample buffer for electrophoresis.

Gel electrophoresis and Western blot analysis

Samples containing equal amounts of protein (50 µg), as measured by the CBQCA fluorimetric protein quantitation assay, were resolved under reducing conditions on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Hybond ECL). Membranes were blocked for 2 h with phosphate-buffered saline (PBS) containing 0.3% BSA, as reported (Cabado et al. 1996). Then, blots were exposed to one of the primary antibodies described above, anti-Bcl-2 (1:1000 dilution) or anti-Bax (1:2000 dilution) for 2 h at room temperature. In order to remove unbound antibody, membranes were washed three times with PBS-Tween. Then, blots were incubated for 2 h at room temperature with the appropriate peroxidase-conjugated secondary antibody (1:1000 dilution) and washed as before. Immunoreactive bands were visualized using enhanced chemiluminiscence and analysed with an Image Master VDS CL and TotalLab software (Amersham Pharmacia Biotech).

Fluorescent labelling of cytochrome *c*

Selective labelling of cytochrome *c* was carried out in neuroblastoma cells grown on glass culture slides at 60–70% confluence. Cells were fixed with 3.7% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 in PBS as described by Leira et al. (2003). Fixed cells were specifically labelled with an α-cytochrome *c* antibody and an IgG FITC-conjugated secondary antibody. Specimens were observed in a confocal Nikon Eclipse 800 microscope equipped with Ar:Kr laser, MRC-1024 software (Bio-Rad, Hercules, CA, USA), Plan Apo 40/Plan Apo 60 objectives and an epifluorescence module with B2A/G2A filters.

Statistical analysis

Results plotted in figures are representative of at least three independent experiments showing the average of all the experiments with the SD for each mean. Data were analysed using the Student's *t*-test for unpaired data. A probability level of 0.05 was used for statistical significance.

Results

Changes in F-actin cytoskeleton

In order to investigate the effect of okadaic acid on cytoskeletal disruption of neuroblastoma cells, we measured F-actin levels after 1 or 24 h incubation with OA

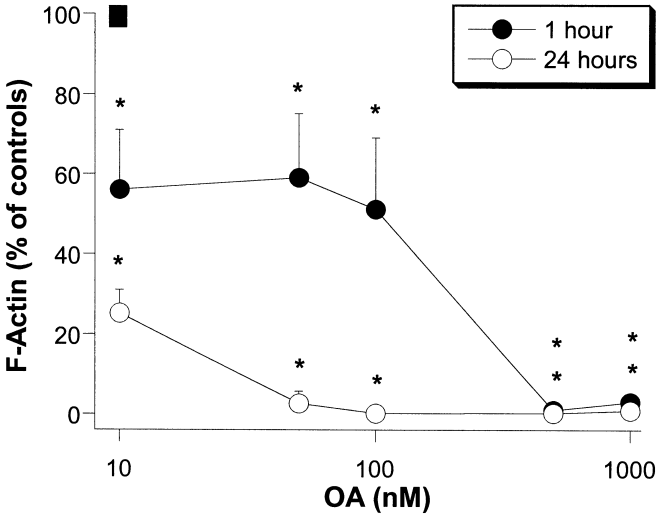

Fig. 1 Effect of okadaic acid (OA, 10–1000 nM) on F-actin levels of neuroblastoma cells after incubation for 1 or 24 h. Quantitative analysis of F-actin were carried out by fluorimetric microplate assay using Oregon Green-514 phalloidin to stain F-actin. The *square* represents the value in non-treated cells. Absolute values of control were 150 ± 5. Values are the means ± SD of three experiments performed in triplicate. *$P < 0.05$, significant difference with respect to control

(10–1000 nM). Figure 1 shows that F-actin pools significantly decreased after 1- or 24-h incubation with OA over the entire range tested.

To better understand the pathways used by OA to cause cytoskeletal disruption in neuroblastoma cells, we probed several agents known to interfere with different intracellular signalling pathways; F-actin levels were studied by treatment with the protein kinase C (PKC) inducer TPA and the PKC inhibitors chelerythrine and rottlerin (Fig. 2A) and by treatment with the protein kinase A (PKA) inducer forskolin, which increases cAMP levels, and the PKA inhibitor H89 (Fig. 2B). The same procedure was used to study F-actin levels by treatment with the phosphoinositide 3-kinase (PI3-K) inhibitor wortmannin (or LY294002 in other experiments) and genistein (or herbymicin A) as inhibitors of tyrosine kinase(s) (Fig. 2C). We measured F-actin levels in cells after incubation for 1 h or 24 h (data not shown) with those agents alone or in combination with OA (1000 nM) and compared the results with those from control cells (OA-untreated cells). Interestingly, all of the tested agents induced significant changes on F-actin levels with respect to controls after 1- and 24-h incubation, although none of them was able to prevent OA-induced depolymerization of F-actin. PKC inhibitors chelerythrine and rottlerin showed similar effects to those reported for OA, thus inducing a complete disruption of F-actin cytoskeleton after 1- or 24-h incubation.

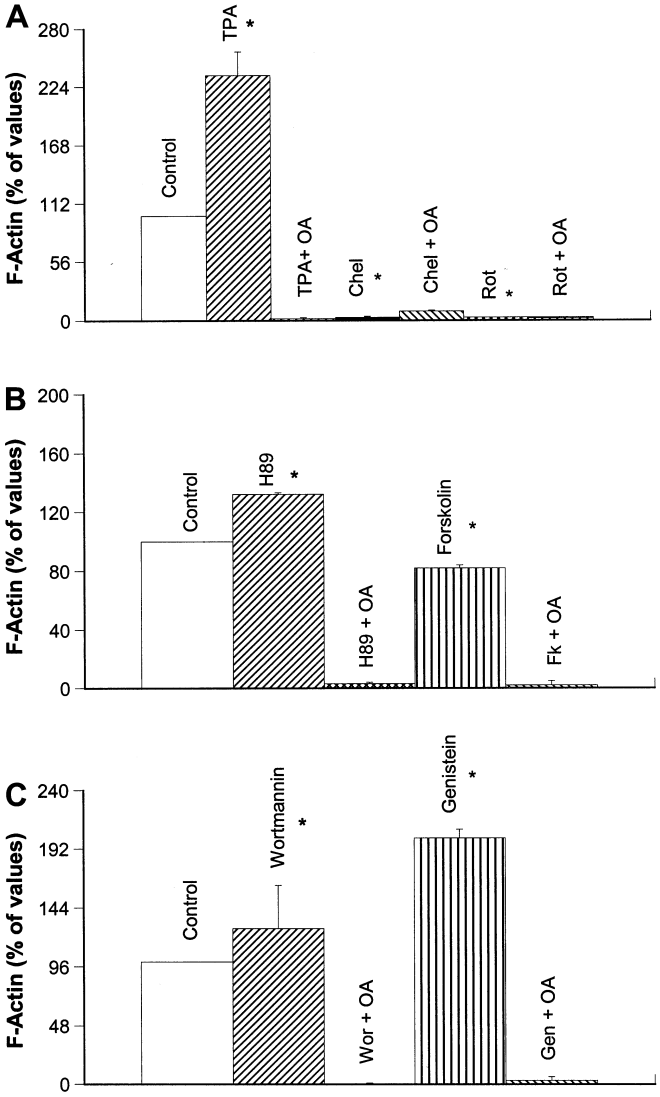

Fig. 2A–C Effect of different agents alone or in combination with okadaic acid (OA, 1000 nM) on F-actin levels in neuroblastoma cells after 1-h incubation. **A** TPA (100 nM), chelerythrine (10 μM) or rottlerin (1 μM). **B** H89 (100 nM) or forskolin (100 μM). **C** Wortmannin (100 nM) or genistein (10 μM). F-actin levels were measured by fluorimetric microplate assay using Oregon Green-514 phalloidin as a fluorochrome to stain F-actin. Control means cells without OA treatment. In all cases SDs of controls were lower than 5%. Values are the averages ± SD of three experiments performed in triplicate. *$P < 0.05$, significant difference with respect to control

Caspase-3 activation

Activation of caspase-3 has been defined as a major event in the execution phase of apoptosis. In Fig. 3 we analysed caspase-3 activity in cells exposed to OA (1000 nM) alone and combined with each of the agents mentioned above. Caspase-3 activation was evident after 1-h incubation with the toxin. None of the drugs tested, except the mitogen-activated protein kinase (MAPK) inhibitor PD98059, prevented OA-stimulated caspase-3 activation. On the contrary, several agents increased the level of caspase-3 activation reached by OA alone (Fig. 3). Similar results were obtained in cells incubated with the agents for 6 or 24 h (data not shown). The effect seen on caspase-3 activation after 1-h incubation with PD98059 was not advanced in longer incubations,

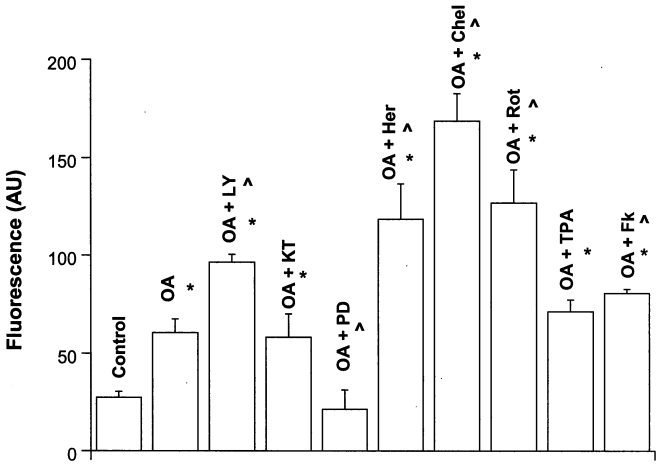

Fig. 3 Caspase-3 activation in neuroblastoma cells incubated with okadaic acid (OA, 1000 nM) alone or in combination with different agents(*LY* LY294002, *KT* KT5720, *PD* PD98059, *Her* herbymicin A, *Chel* chelerythrine, *Rot* rottlerin, *TPA* phorbol-12-myristate-13-acetate, *FK* forskolin). Caspase-3 activity was determined by fluorimetric microplate assay as described in methods (*AU* arbitrary units). Agents were incubated for 1 h with the cells. Control refers to non-treated cells. Values are the means ± SD of three experiments. *Significant difference with respect to control cells; ^ significant difference with respect to OA-treated cells

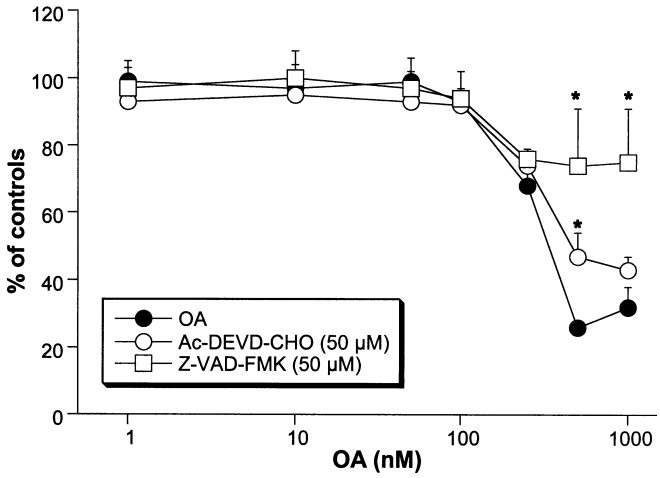

Fig. 5 Mitochondrial membrane potential in neuroblastoma cells treated with okadaic acid (OA). Changes in mitochondrial membrane potential were determined using the fluorescent probe Mitotracker Red CMXRos (1 mM). Effect of 50 μM Ac-DEVD-CHO, a caspase-3 inhibitor, and 50 μM Z-VAD-FMK, a pan-caspase inhibitor are shown. Incubation with OA (1–1000 nM) and the caspases inhibitors was carried out for 1 h. Values are the means ± SD of two experiments performed in triplicate. *Significant difference with respect to OA-treated cells

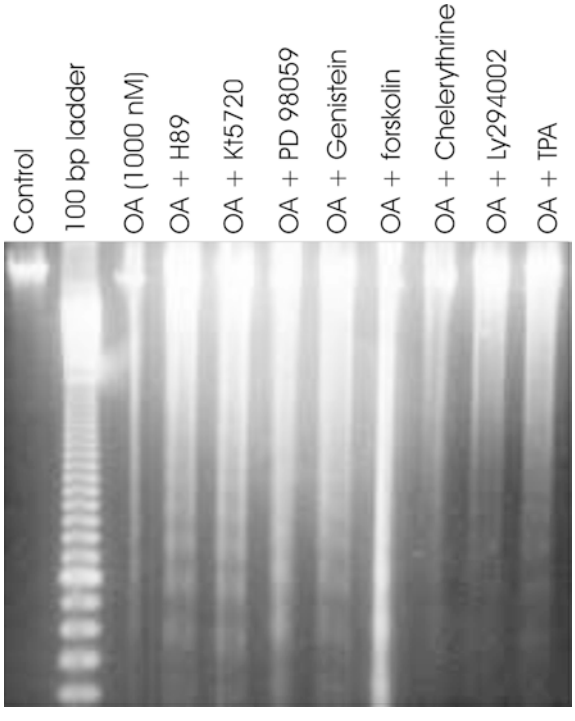

Fig. 4 DNA fragmentation induced by okadaic acid (OA) in neuroblastoma cells. DNA bands were visualized after electrophoresis on 1.5% agarose gel and SYBR Green staining. Effect of several drugs, H89 (100 nM), KT5720 (1 μM), PD98059 (10 μM), genistein (10 μM), forskolin (100 μM), chelerythrine (10 μM), LY294002 (30 μM) and phorbol-12-myristate-13-acetate (TPA, 100 nM), on the fragmentation of DNA caused by OA (1000 nM) is shown. Figure is representative of two independent experiments

indicating that the role of MAPK in the process of apoptosis occurs at a early step.

DNA fragmentation

One of the hallmarks of the late stages of apoptosis is the fragmentation of DNA. Therefore, we studied a potential modulation on OA-triggered DNA laddering with different agents. Cells incubated with OA (1000 nM) alone for 24 h showed the DNA fragmentation pattern characteristic of apoptotic cells, as illustrated in Fig. 4. None of the agents tested prevented OA-induced DNA fragmentation.

Mitochondrial membrane potential changes

Fall of mitochondrial membrane potential and activation of caspases occur in most cases of apoptotic cell death. Since previous studies have shown that OA decreases mitochondrial membrane potential, we investigated the potential involvement of caspases on mitochondrial changes using the caspase-3 inhibitor Ac-DEVD-CHO, and the pan-caspase inhibitor Z-VAD-FMK. Figure 5 shows that 50 μM Z-VAD-FMK significantly blocks the collapse of mitochondrial membrane potential caused by OA after 1-h and 24-h (data not shown) incubation, thus demonstrating a role for caspases on mitochondrial changes triggered by OA. In contrast, the caspase-3 inhibitor Ac-DEVD-CHO was not able to prevent mitochondrial membrane potential changes induced by 1-h or 24-h incubation with OA (1000 nM).

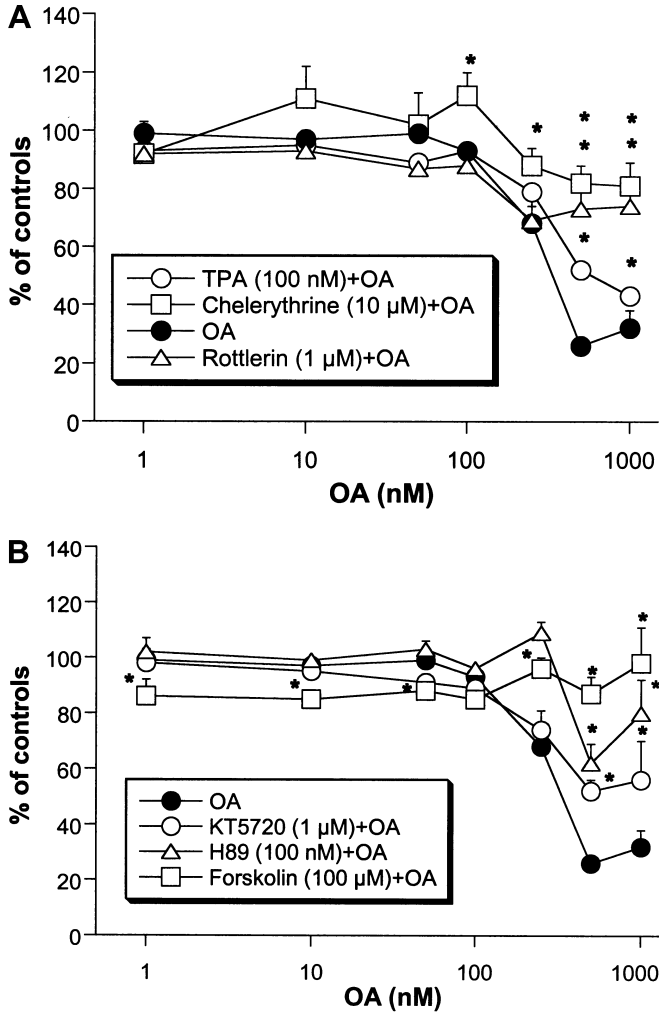

Fig. 6A,B Effect on mitochondrial membrane potential of neuroblastoma cells after 1-h incubation with okadaic acid (OA) in combination with **A** phorbol-12-myristate-13-acetate (TPA), chelerythrine and rottlerin, and **B** KT5720 (1 μM), H89 (100 nM) and forskolin (100 μM). Mitochondrial membrane potential changes were evaluated using the fluorescent probe Mitotracker Red CMXRos (1 mM). Values are the means ± SD of three experiments performed in triplicate. *Significant difference with respect to OA-treated cells

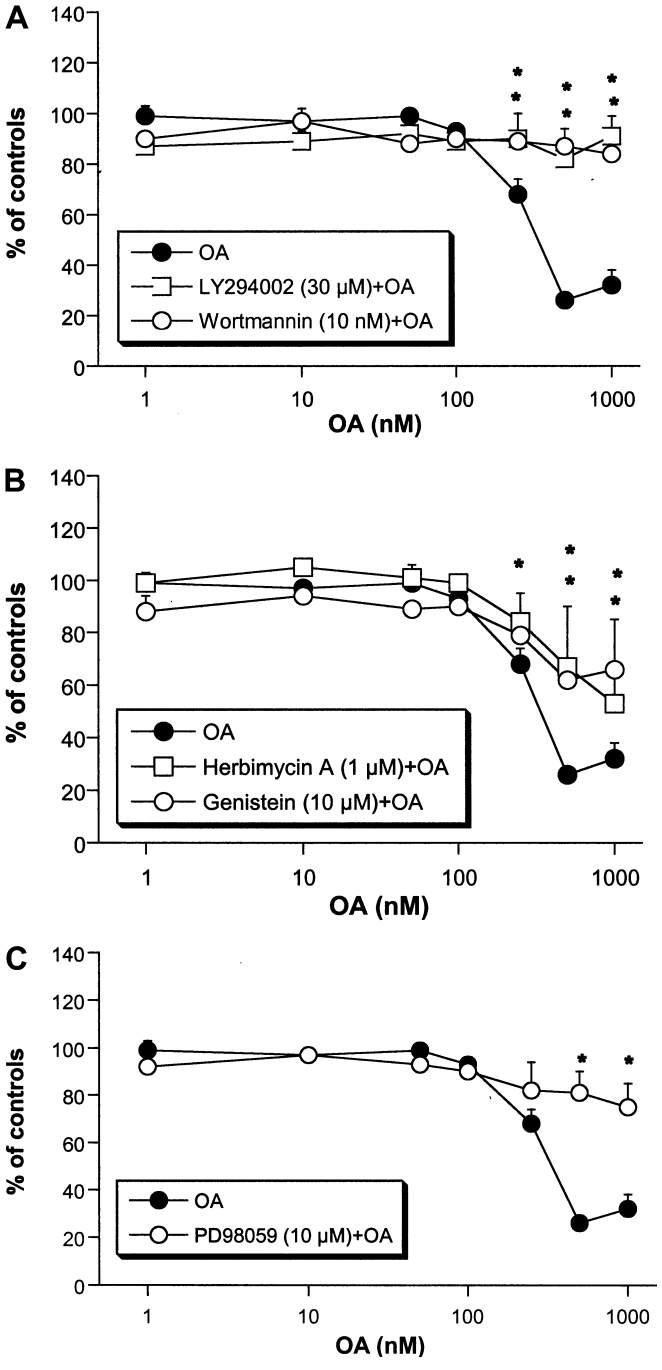

Fig. 7A–C Effect on mitochondrial membrane potential of neuroblastoma cells after 1-h incubation with okadaic acid (OA) in combination with: **A** LY294002 (30 μM) and wortmannin (10 nM); **B** herbymicin A (1 μM) and genistein (10 μM); **C** PD98059 (10 μM). Changes of mitochondrial membrane potential were analysed by using the fluorescent probe Mitotracker Red CMXRos (1 mM). Values are the means ± SD of three experiments performed in triplicate. *Significant difference with respect to OA-treated cells

The role that different protein kinases play in apoptosis is not clear and sometimes controversial depending on the cellular model, the stimulus and the cellular context. In order to determine which pathways are involved in apoptotic cell death driven by OA, we tried to block the loss of mitochondrial membrane potential triggered by OA. All the agents mentioned above were tested in combination with OA (1–1000 nM), and mitochondrial membrane potential was studied. As illustrated in Fig. 6A, stimulation of PKC with TPA significantly prevents the fall in membrane potential triggered by OA (500–1000 nM) after 1-h incubation. Paradoxically, the loss of membrane potential is also obliterated after 1-h incubation with the PKC inhibitors chelerythrine and rottlerin. Similar results were obtained after 24-h incubation (data not shown).

To study the role of PKA in OA-induced mitochondrial changes, we used forskolin to increase intracellular cAMP levels and two inhibitors of the kinase, KT5720 and H89, to block its activity. Incubation of cells for 1 or

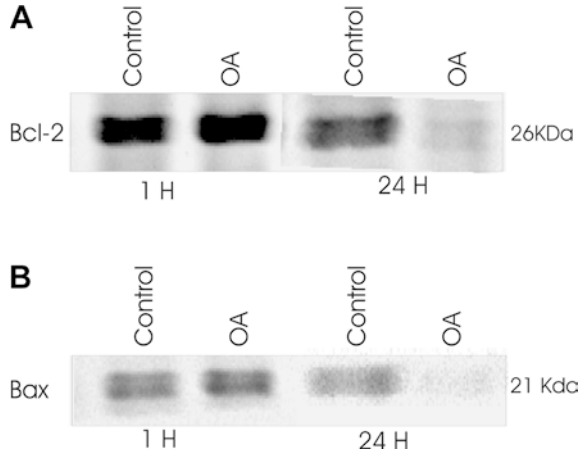

Fig. 8A,B Western blots of cells treated with okadaic acid (OA, 1000 nM) or without treatment (Control) after 1- or 24-h incubation. Samples of 50 μg protein were extracted from neuroblastoma cells, separated by 10% SDS-PAGE, blotted and probed with antibodies anti-Bcl-2 (**A**) or anti-Bax (**B**). Figures are representative of at least three experiments

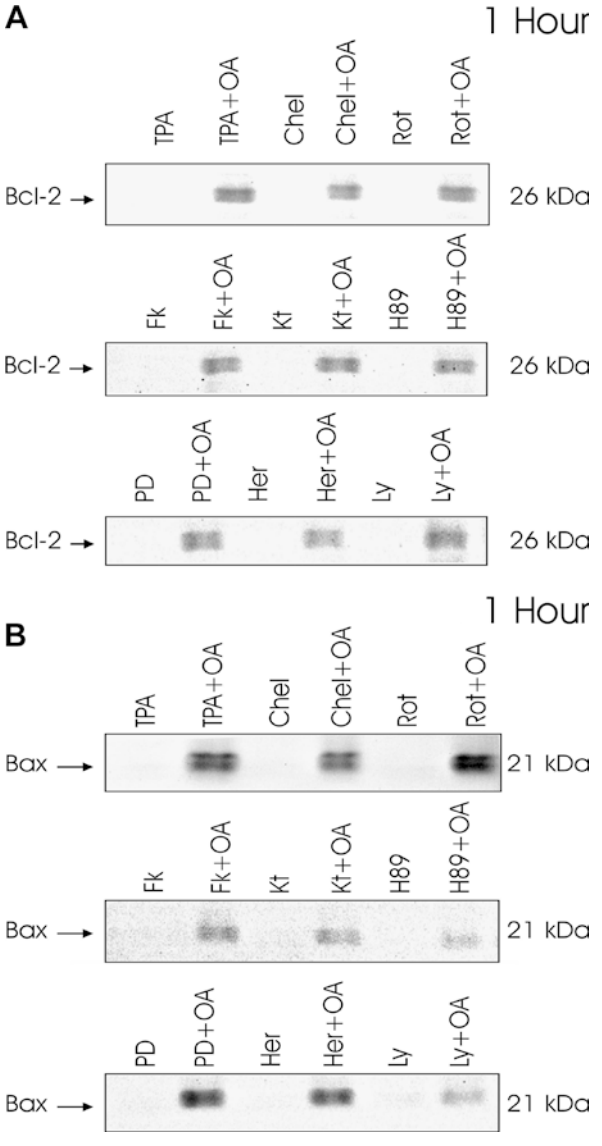

Fig. 9A,B Immunoblots of cell lysates obtained from neuroblastoma cell line treated with different agents alone or in combination with okadaic acid (OA, 1000 nM) for 1 h. Samples of 50 μg protein were extracted from neuroblastoma cell line and separated by 10% SDS-PAGE. Blots were probed with α-Bcl-2 (**A**) or α-Bax (**B**). The treatment consisted of phorbol-12-myristate-13-acetate (TPA, 100 nM), chelerythrine (Chel, 10 μM), rottlerin (Rot, 1 μM), forskolin (FK, 100 μM), KT5720 (KT, 1 μM), H89 (100 nM), PD98059 (PD, 10 μM), Herbymicin (Her, 1 μM), LY294002 (LY, 30 μM). Figures are representative of at least three experiments

24 h with those agents significantly prevented the loss of mitochondrial membrane potential caused by high concentrations of OA, as shown in Fig. 6B. However, at the concentration used, the protective effect of forskolin was not seen in longer incubations (24 h) with 1000 nM OA (data not shown).

The involvement of PI3-Ks on mitochondrial depolarization triggered by OA was evaluated by using LY294002 and wortmannin to block the activity of this kinase. As observed in Fig. 7A, both inhibitors abrogated changes in mitochondrial membrane potential induced by OA after 1-h incubation. The same result was obtained after 24 h (data not shown).

Tyrosine kinases are also possible targets of okadaic acid. Thus, two tyrosine kinase inhibitors, herbymicin A and genistein, were used in next experiment. As summarized in Fig. 7B, both inhibitors prevented the collapse of mitochondrial membrane potential triggered by OA (100–1000 nM) at 1-h incubation.

Finally, the role of MAPKs on OA-induced changes in membrane potential of mitochondria was also analysed. Similar to results previously described for most other agents used, the MAPK inhibitor PD98059 also blocked OA-induced decrease of mitochondrial membrane potential after 1 h of incubation (Fig. 7C). Similar results were obtained after 24-h incubation with OA for all agents described above (data not shown).

Western blots of Bcl-2 and Bax

In order to evaluate the role of Bcl-2 family members in apoptosis induced by OA, we carried out western blots in neuroblastoma cells after 1- or 24-h incubation in the absence or presence of OA (1000 nM). As shown in Fig. 8, blots were probed with anti-Bcl-2 (Fig. 8A) or anti-Bax (Fig. 8B). Both, Bcl-2 and Bax proteins, are present in control cells after 1 or 24 h of incubation, visualized as a band of 26 kDa for Bcl-2 and a band of 21 kDa for Bax. Similar results were obtained in OA-treated cells after 1-h incubation. However, Bcl-2 protein was almost undetectable in cells incubated for 24 h in the presence of OA (Fig. 8A) and Bax was not found after 24-h incubation with the phosphatase inhibitor (Fig. 8B).

To further analyze signal transduction pathways involved in OA-driven changes of Bcl-2 and Bax, western blots were carried out after cell incubation with different

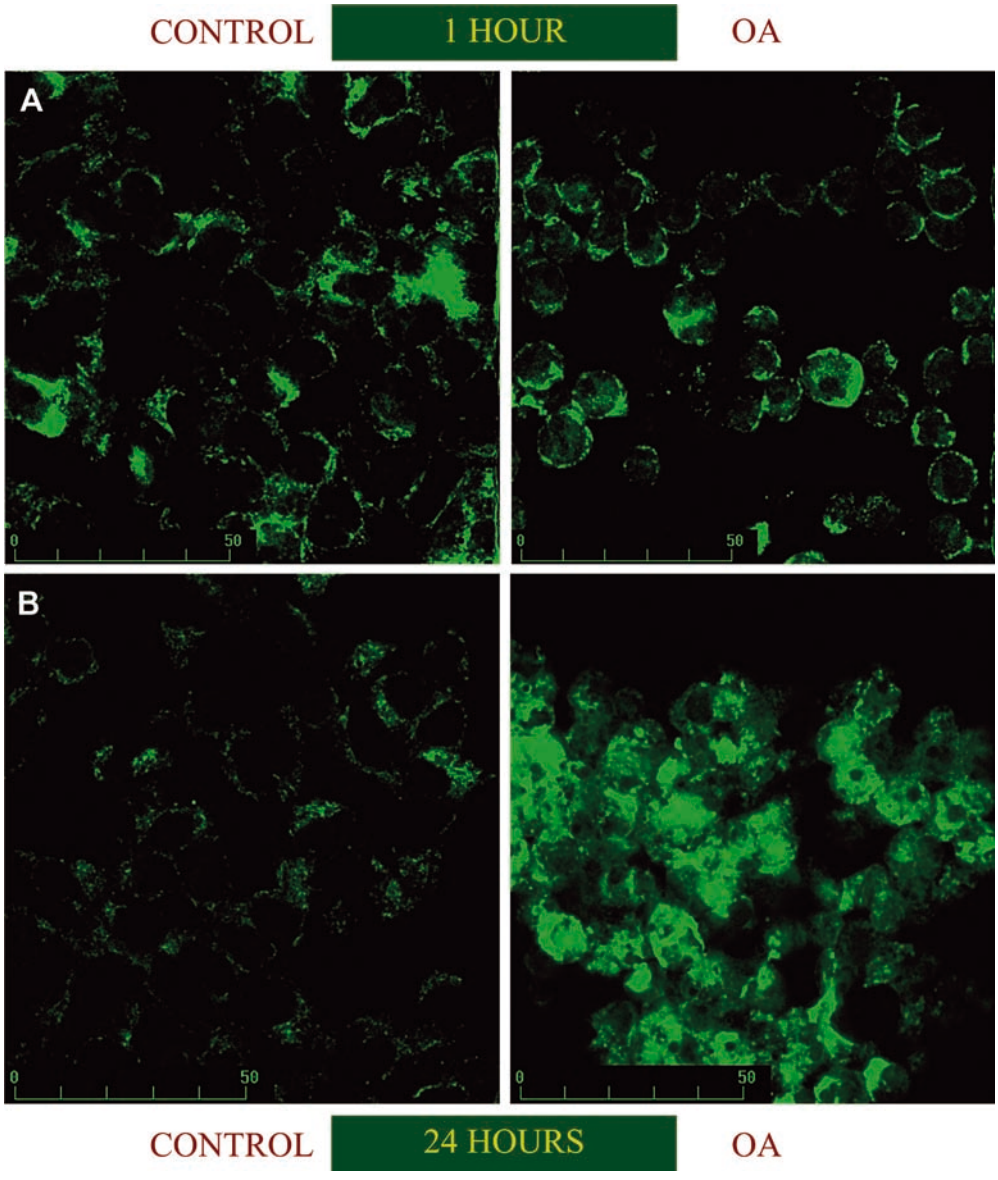

Fig. 10A,B Cytochrome *c* release from mitochondria in neuroblastoma cells after incubation with okadaic acid (1000 nM) for 1 h (**A**) or 24 h (**B**). Experiments were carried out by using an α-cytochrome *c* antibody and a FITC-conjugated secondary antibody. Specimens were observed in a confocal Nikon Eclipse 800 microscope. Photomicrographs are representative of two experiments

agents alone (TPA, chelerythrine, rottlerin, forskolin, KT, H89, herbymicin A and Ly), or in combination with OA, as summarized in Fig. 9. Surprisingly, similar results were found after 1- or 24-h incubation (data not shown), regardless of the protein analysed or agent considered, as observed in Fig. 9A for Bcl-2 and in Fig. 9B for Bax. When cells were incubated for 1 or 24 h with each of the agents described, Bcl-2 protein was not detectable in any case. However, when cells were simultaneously incubated with OA and each of those agents (Fig. 9A), Bcl-2 expression did not change, showing the same levels as in controls. Similar results were obtained in blots probed with anti-Bax, as shown in Fig. 9B. This protein could not be detected in lysates from cells treated for 1 or 24 h with each of the agents alone. In contrast, cells incubated with OA in combination with each agent showed the same expression pattern of Bax seen in controls.

Cytochrome *c* release from mitochondria

In order to further elucidate the involvement of mitochondria in OA-induced apoptosis, we studied the relevance of cytochrome *c* release from mitochondria that is known to be triggered by Bid. We monitored the time-course of cytochrome *c* release from mitochondria using a confocal laser microscope. Cells were stained with an antibody against cytochrome *c*, after treatment with OA. After 1 h of OA exposure there was almost no difference between control and treated cells, as shown in Fig. 10A.

In contrast, cytochrome *c* was strongly visualized 24 h after OA treatment, as seen in Fig. 10B, supporting the fact that Bid activation triggers cytochrome *c* release from mitochondria to cytosol (present results and Cabado et al. 2003).

Discussion

In addition to its physiological role, apoptosis is a common feature in cells exposed to many different xenobiotics, thus becoming an interesting subject in modern toxicological studies. In spite of this, molecular mechanisms controlling apoptotic commitment are still unclear (Gao and Dou 2000), which reinforces the relevance of studying apoptosis-related signal transduction pathways.

Apoptosis has been widely reported as a relevant mechanism of OA-mediated toxicity (Davis et al. 1996; Benito et al. 1997; Morimoto et al. 1997; Tergau et al. 1997; von Zezschwitz et al. 1997; Yan et al. 1997; Nuydens et al. 1998; Riordan et al. 1998; Leira et al. 2001), which is associated with its inhibitory activity on protein phosphatases. However, molecular pathways linking protein phosphatase 2A inhibition and OA-driven apoptosis still remain unclear. We have previously demonstrated that OA-triggered apoptosis in BE(2)-M17 cells involves changes in the transmembrane potential of mitochondria and cytoskeletal disruption (Leira et al. 2002a).

F-Actin cytoskeleton

The cytoskeleton can be considered as a complex network that assists in the formation of signal transduction complexes (Aplin et al. 1998, 1999; Aplin and Juliano 1999; Giancotti and Ruoslahti 1999). It has been recognized as one of the main cellular targets of OA (Baldacini et al. 1993; Macías-Silva and García-Sáinz, 1994; Blankson et al. 1995; Yano et al. 1995; Fiorentini et al. 1996; Niggli et al. 1999; Leira et al. 2000), comprising a complex of proteins, in many cases directly regulated by protein phosphatases (Kreienbühl et al. 1992). Moreover, cell-extracellular matrix and cell-cell interactions strongly influence cell survival through its association with the actin cytoskeleton (Bissell and Nelson 1999), which is directly regulated by PP2A-mediated mechanisms (Burridge and Chrzanowska-Wodnicka 1996; Marushige and Marushige 1998).

Results obtained in this work support these findings, since OA-treated neuroblastoma cells show a rapid and complete disruption of F-actin cytoskeleton, which was neither prevented nor even modulated by any of the agents used. Moreover, cytoskeletal changes play a key role in the apoptotic cascade triggered by OA, as demonstrated by the fact that neither caspase-3 activation nor DNA fragmentation is blocked by any of those agents. Furthermore, PKC blockers, tyrosine kinase inhibitors and forskolin, which also induce a significant decrease of F-actin pools by themselves, synergize with OA to enhance caspase-3 activity, thus also reinforcing the link between cytoskeletal disruption and execution of the apoptosis.

Activation of caspases

The role of actin on apoptosis was initially thought to be restricted to its involvement in morphological changes associated with caspase-3-mediated cleavage of cytoskeletal components (van de Water et al. 2000). However, further findings have demonstrated that caspase-independent cytoskeletal disruption may initiate the execution of apoptosis in different cell types (Maruyama et al. 2000; van de Water et al. 2000). In that study, cytoskeletal changes precede caspase-3 activation and DNA fragmentation, which renders the process irreversible. Results obtained in the present work confirm these findings, since F-actin seems to initiate the cascade of apoptotic events in OA-treated cells. Furthermore, the negligible effect of different agents on OA-induced F-actin disruption suggests a direct involvement of OA-sensitive protein phosphatases on cytoskeleton regulation and the relevance of cytoskeletal changes on OA-mediated cytotoxicity supporting previous findings (Cabado et al. 2003).

Mitochondrial membrane potential

Results confirm that caspase-3 is a key downstream caspase in OA-triggered apoptosis, as previously described (Fladmark et al. 1999; Leira et al. 2001; Rossini et al. 2001). However, in OA-treated cells, the activation of multiple caspase isoforms in several cell models has been recently reported (Rossini et al. 2001; Cabado et al. 2003). Our experiments with caspase inhibitors demonstrate that OA-signalling involves activation of upstream caspases. Indeed, the general caspase inhibitor Z-VAD-FMK (but not Ac-DEVD-CHO) abrogates OA-induced decrease of mitochondrial membrane potential, which is an early event in the apoptotic cascade triggered by OA (Fladmark et al. 1999; Leira et al. 2001; Rossini et al. 2001). In a recent report we show that caspase 8 activation is involved in mitochondrial changes observed during the execution phase of OA-induced apoptosis (Cabado et al. 2003), which resembles the death-receptor or extrinsic pathway of apoptosis (Hengartner 2000). Importantly, OA has been reported to mimic the effects of tumour necrosis factor α, one of the model agents of this apoptotic route in mammalian cells. On the basis of these findings we suggest that the extrinsic pathway is the main route activated during OA-induced apoptosis in BE(2)-M17 cells and is directly triggered by cytoskeletal changes. Recently, Parlato et al. (2000) have reported that ezrin-mediated association of CD95 cell membrane receptor with the actin cytoskeleton is a key

intracellular mechanism in human T-lymphocytes susceptibility to apoptosis, thus providing a direct link between the death receptor pathway and the actin cytoskeleton.

The relevance of cytoskeletal changes on OA-triggered apoptosis in BE(2)-M17 cells was further confirmed by the observation that mitochondria plays a secondary role in the process. Most apoptotic scenarios have been reported to involve changes in mitochondrial membrane potential and release of pro-apoptotic factors into the cytosol (Pedersen 1999; Desagher and Martinou 2000; Gottlieb 2000; Hengartner 2000), thus resulting in activation of downstream caspases. Results described in this work provide evidence of caspase-3 activation and DNA fragmentation in the absence of changes of mitochondrial membrane potential, thus demonstrating that that phenomenon is not a prerequisite for OA-induced cell commitment to death.

All the agents used in this work were able to block OA-induced decrease of mitochondrial membrane potential, although some differences were observed in the time-course or degree of this effect. Taking into account the opposite action on protein kinases of some of these agents (e.g. TPA and chelerythrine/rottlerin), the results obtained are quite unexpected. Furthermore, some of the agents used (rotlerin and genistein) induce by themselves a decrease in mitochondrial membrane potential, but all of them prevent OA-mediated changes. This phenomenon is supported by the fact that, in several cases, two different agents acting on the same specific target were used and a similar result was obtained. In agreement, decrease of mitochondrial potential induced by some agents alone is consistent with apoptosis induction, as reported previously (Jarvis et al. 1994; Séité et al. 2000; Yoon et al. 2000).

Bcl-2/Bax western blots analysis

The Bcl-2 family comprises proteins with pro- or anti-apoptotic activity (Desagher and Martinou 2000; Hengartner 2000) and the capability to form homo- or heterodimers to regulate each other (Frey 1997; Desagher and Martinou 2000; Tsujimoto and Shimizu 2000). Results obtained by western blot analysis strongly suggest that changes in mitochondrial membrane potential are regulated by the Bcl-2 family of proteins. OA-induced collapse of mitochondrial potential involves changes in at least three members of this family, Bax, Bid and Bcl-2 (Cabado et al. 2003). Decreased levels of monomeric Bax and Bcl-2 in OA-treated neuroblastoma cells are prevented by simultaneous incubation with each of the agents used in this work: TPA, chelerythrine, rottlerin, forskolin, KT5720, H89, PD98059, herbymicin and LY294002. This finding fully agrees with previously described data on mitochondrial membrane potential, thus pointing out the role of the Bcl-2 family in the regulation of mitochondrial membrane permeability. Moreover, all the agents induce by themselves a decrease of monomeric Bax and Bcl-2, but prevent OA-mediated downregulation of both proteins at 24 h. Taken together, these observations suggest that regulation of mitochondrial membrane potential in this cell line is based upon complex interactions of both pro- and anti-apoptotic Bcl-2 family proteins, rather than being linked to individual changes in their expression. For instance, inhibition of PI3-K would enhance OA-induced apoptosis since LY294002 increased Bcl-2 protein levels (Carbott and Duan 2002). Oligomerization between Bcl-2 proteins seems more likely to play a pivotal role on OA-induced mitochondrial changes. Subcellular localization of Bcl-2 determines whether Bcl-2 effectively increases apoptosis (Carbott and Duan 2002).The exact mechanism by which these interactions regulate mitochondrial permeability is far from being clear and different hypothesis have been suggested, although none of them has been definitively proven. Then, our results do not fit any described model to explain the modulation of mitochondrial changes by Bcl-2 family of proteins.

Cytochrome *c* release from mitochondria

In a previous paper we demonstrated that activation of Bid, a pro-apoptotic member of Bcl-2 family, is a late event occurring 24 h after OA exposure (Cabado et al. 2003). It is known that Bid assumes cytochrome *c*-releasing activity; however, this feature is only observed 24 h after OA exposure and is preceded by activation of caspases, suggesting that the involvement of mitochondria in initiation of apoptosis is unlikely. This fact supports previous findings in which Bid activation occurs 24 h after OA treatment (Cabado et al. 2003), which assumes cytochrome *c* release is also detected 24 h after OA treatment.

Surprisingly, all the agents used in this work were able to block OA-induced mitochondrial changes. Furthermore, OA and most of those agents, which act on different protein kinases, mutually counteracted pro-apoptotic events induced by themselves alone. It has been reported that PP2A is the major phosphatase in eukaryotic cells that downregulates activated protein kinases, and it is known that more than 30 kinases are modulated by PP2A in vivo (Millward et al. 1999). It has been described that Bcl2 proteins specifically interact with protein phosphatases (Ayllon and Cayla 2002), and indeed it has been described that Bcl-2 function is regulated by PP2A (Ruvolo and Clark 2002). This could partially explain most of results obtained, although identical modulation observed with agents known to exert opposite effects on the same pathway remain unexplained. In epithelial cells a complex apoptotic model involving two or more apoptotic pathways has been described (Kolb et al. 2002). In our view, the study of linear signal transduction pathways does not provide accurate information of such a complex mechanism as apoptosis. Thus, it seems clear that elucidation of apoptosis-related signal transduction pathways requires

new and integrated approaches to understand such a complex scenario.

In summary, cytoskeletal disruption is the key factor that could trigger the cascade of apoptotic events in OA-treated BE(2)-M17 cells, which involves activation of upstream caspases. Furthermore, activation of downstream caspases is not dependent on changes in mitochondrial membrane potential, thus suggesting that the observed mitochondrial changes are accessory in the execution phase of apoptosis. OA-induced collapse of mitochondrial membrane potential is mediated by inhibitable and complex associations among the Bcl-2 family of proteins, involving at least Bax and Bcl-2. However, the molecular mechanisms underlying Bcl-2 proteins-mediated regulation of mitochondrial membrane potential should be further clarified.

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