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Albumin prevents mitochondrial depolarization and apoptosis elicited by endoplasmic reticulum calcium depletion of neuroblastoma cells

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Abstract

Serum albumin protects against cell death elicited by various cytotoxic agents; however, conflicting views on the protective mechanism still remain. Hence, we have studied the ability of serum albumin to prevent apoptosis of human neuroblastoma SH-SY5Y cells elicited by four compounds known to release Ca^{2+} from the endoplasmic reticulum, i.e. dotarizine, flunarizine, thapsigargin and cyclopiazonic acid. Spontaneous basal apoptosis, after 24 h incubation in Dulbecco's Modified Eagle Medium (DMEM) containing 10% serum, was 5%. Dotarizine (30–50 μM) enhanced basal apoptosis to 18–43%, flunarizine (30–50 μM) to 15%, thapsigargin (1–10 μM) to 21–35%, and cyclopiazonic acid (100 μM) to 10%. Serum deprivation augmented basal apoptosis to 20%. Under serum-free medium, 30 μM dotarizine or flunarizine drastically enhanced apoptosis to 63% and 68%, respectively; the increase was milder with 1 μM thapsigargin (37%) and 30 μM cyclopiazonic acid (27%). In serum-free medium, albumin (29 or 49 mg/ml) fully prevented the apoptotic effects of dotarizine, flunarizine and cyclopiazonic acid. The four compounds increased the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in fluo-4 loaded cells; such increase developed slowly to reach a plateau after several minutes, followed by a slow decline. Albumin did not modify the kinetic parameters of such increase. In the absence of serum, dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid caused mitochondrial depolarization in tetramethylrhodamine ethyl ester (TMRE)-loaded cells; depolarization was inhibited by cytoprotective concentrations of albumin. These results suggest that albumin protects cells from entering into apoptosis by preventing mitochondrial depolarization. They also suggest that inhibition of mitochondrial depolarization might become a target to develop new antiapoptotic compounds with therapeutic neuroprotective potential in stroke, Alzheimer's disease, and other neurodegenerative diseases.

Keywords: Reticular stress; Apoptosis; Mitochondria; Albumin; Neuroprotection

1. Introduction

Few reports in the recent literature describe a cytoprotective effect for serum albumin. For instance, Moran et al. (2002) show that albumin protects against the spontaneous apoptosis of chronic lymphocitic leukemia cells in cultures, Tabernero et al. (2002) show that it prevents apoptosis and promotes neuronal survival, and Matsui et al. (1993) and Belayev et al. (2001) show that albumin cause neuroprotection in acute ischemic stroke.

At present, the mechanism of the cytoprotective effects of albumin remains unclear. Tabernero et al. (2002) found that albumin promotes the synthesis and the release of

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glutamate that might exert neurotrophic effects by activating a tyrosine receptor kinase A (TrkA). Albumin also increases the $[Ca^{2+}]_c$ in neurons and glia of rat cerebellar cultures (Núñez and García-Sancho, 1996) and augments the $[Ca^{2+}]_c$ and DNA synthesis in astrocytes (Nadal et al., 1995). Finally, some reports describe an antioxidant effect for albumin (Emerson, 1989; Huh et al., 1998; Belayev et al., 2001; Moran et al., 2002; Gum et al., 2004), being even more efficient than vitamin E, likely due to its strong copper binding capacity (Belayev et al., 2001).

Alteration of intracellular Ca^{2+} movements is critical in apoptosis (Rizzuto et al., 2003). On the other hand, Ca^{2+} released from the endoplasmic reticulum into the cytosol is rapidly taken up by mitochondria (Rizzuto et al., 1993, 1998; Montero et al., 2000) that in few seconds can reach near millimolar intramitochondrial Ca^{2+} elevations, $[Ca^{2+}]_M$ (Montero et al., 2000). Although these elevations of $[Ca^{2+}]_M$ are transient and of physiological importance (Montero et al., 2000), if sustained they may start a chain of biochemical events leading to apoptosis. Hence, the prolonged elevation of $[Ca^{2+}]_M$ leas to the opening of the transition pore and the mitochondrial membrane potential collapses (Schinder et al., 1996; Tornero et al., 2002). This will cause the release of apoptogenic factors (i.e. cytochrome *c*) and cell death (Zoratti and Szabo, 1995; Bernardi and Petronilli, 1996).

Actually there is growing interest in the study of the relationship between endoplasmic reticulum Ca^{2+} depletion and apoptosis. The term "reticular stress" has been described as a modification of intracellular Ca^{2+} dynamics that leads to apoptotic cell death. In fact, reticular stress has been implicated as a primary event in apoptotic neuronal death occuring in Alzheimer's disease and stroke (Paschen, 2001). A way to deplete the endoplasmic reticulum Ca^{2+} consists in the inhibition of the sarcoplasmic reticulum Ca^{2+} ATPase, reversibly by cyclopiazonic acid (Demaurex et al., 1992) or irreversibly by thapsigargin (Kijima et al., 1991; Lytton et al., 1991). Particularly thapsigargin has been used to cause apoptosis in a pleyade of cell systems (Wei et al., 1998; Nguyen et al., 2002; Arias et al., 2004). We have found that two "wide-spectrum" calcium channel blockers, dotarizine and flunarizine (Villarroya et al., 1995), also release endoplasmic reticulum Ca^{2+}, thereby increasing the $[Ca^{2+}]_c$ and cell death (Novalbos et al., 1999a,b).

Data on the potential protective effects of albumin in neuronal cultures subjected to reticular stress are not available. Neither, we are aware of any study addressing the issue of mitochondrial "protection" exerted by albumin. Hence, we planned the present study to explore the effects of bovine serum albumin on: (i) apoptosis of human neuroblastoma SH-SY5Y cells, subjected to reticular stress produced by thapsigargin, cyclopiazonic acid, dotarizine or flunarizine; (ii) apoptosis caused by serum deprivation, alone or combined with those four agents; (iii) the changes of $[Ca^{2+}]_c$ elicited by the four agents; and (iv) mitochondrial membrane potential.

2. Materials and methods

2.1. Materials and solutions

Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum, L-glutamine, non essential amino acids, penicillin, streptomycin, trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) and proteinase K were obtained from Invitrogen Life Tecnologies, Spain. Dotarizine was obtained from Grupo Ferrer, Barcelona, Spain. Flunarizine, thapsigargin, cyclopiazonic acid, bovine serum albumin minimum 98% purity, ovalbumin (98% purity), RNase A, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and probenecid were obtained from Sigma Aldrich, Spain. Agarose was obtained from Bio-Rad, Spain. Fluo-4 AM, pluronic acid, Hoechst 33342 and tetramethhylrhodamine (TMRE) were purchased from Molecular Probes (Eugene OR, USA).

Dotarizine, flunarizine, and cyclopiazonic acid were dissolved in dimethylsulphoxide (DMSO) at 10^{-2} M, thapsigargin was diluted in DMSO at 10^{-3} M and then diluted in DMEM or in Tyrode-*N*-2-hydroxyethylpiperazine-*N*′-2′-ethanesulfonic acid (*Tyrode*-HEPES) to the desired concentration.

2.2. Cell culture

Human neuroblastoma SH-SY5Y cells were a generous gift of Dr. Hugo Geerts, from the Janssen Research Foundation. Cells were cultured in DMEM supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 1% non essential amino acids, 25 U/ml penicillin and 25 μg/ml streptomycin, and maintained in a humidified incubator aerated with 95% air and 5% CO_2 at 37 °C. Culture medium was changed three times weekly.

2.3. Detection of apoptosis by flow cytometry

Cells were plated on 60 mm plates at a density of 175×10^4 cells per plate and used 24 h after plating. Drugs were added to the cells for 2 h in the case of thapsigargin (followed by 22 h in DMEM free of thapsigargin), or 24 h for dotarizine, flunarizine, or cyclopiazonic acid. Apoptosis was measured after this 24-h incubation period by flow cytometry analysis after DNA staining with propidium iodide (PI) (Robinson et al., 1997). Supernatants containing floating cells, and attached cells removed by tripsinization were collected together and centrifuged at $200 \times g$ for 5 min at 4 °C. Supernatants were discarded and pellets suspended in 0.5 ml cold phosphate buffer saline (PBS), by pipetting thoroughly to avoid cell clumping. The cell suspension

was transferred to 4.5 ml of 70% cold ethanol and kept in this fixative for a minimum of 2 h; then, cells were centrifuged at $200 \times g$ for 5 min at 4 °C and washed once with 10 ml cold PBS. Finally, the cell pellet was resuspended in 1 ml PI/Triton X-100 staining solution (0.1% Triton X-100, 20 μg/ml PI, 20 mg/ml RNase A in PBS) and incubated for 15 min at 37 °C. Samples were analyzed by flow cytometry (FACSCalibur, Beckton Dickinson). The analysis of the samples included a first selection (gate 1) in which events with the appropriate size (forward scatter) and complexity (side scatter) were selected. Then, selected events were analyzed to discard doublets using a PI intensity-width versus PI intensity-area dot plot (gate 2). Finally, events (cells) that were contained in gates 1 and 2 were plotted in a histogram representing the number of events (cells) containing a specific PI intensity-area (e.g. specific amount of DNA) (Álvarez-Tejado et al., 2001). Apoptosis was measured as the percentage of cells with a sub G0/G1 DNA content in the PI intensity-area histogram plot (Robinson et al., 1997).

2.4. DNA laddering

Untreated or drug-treated cells were removed by trypsinization and collected into a centrifuge tube. Cells were then centrifuged at $200 \times g$ for 5 min at 4 °C, the supernatant discarded, and the cell pellet suspended in 0.5 ml cold PBS. The cell suspension was transferred into 4.5 ml 70% cold ethanol and kept for a minimum of 2 h (Robinson et al., 1997). Cells were then centrifuged at $800 \times g$ for 5 min and ethanol was thoroughly removed. The cell pellets, in 1.5 ml tubes, were resuspended in 25 μl phosphate-citrate buffer (192 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid, pH 7.8), at 37 °C, for 30 min on the shaker. After centrifugation at $1000 \times g$ for 5 min, the supernatant was transferred to a new tube, and incubated with 5 μl RNase A (2 mg/ml) at 37 °C for 30 min. After this incubation, 5 μl proteinase K (2.5 mg/ml) was added and the extract incubated for an additional 30 min at 37 °C (Gong et al., 1994). 5 μl of 6 × loading buffer was added to each tube and the entire tube content was transferred to one well of 1% agarose horizontal gel containing ethidium bromide. Electrophoresis was run for 12–16 h at 2 V/cm, and DNA in the gel was visualized under UV light.

2.5. Nuclear staining of DNA

Cells were plated on 24-well plates at a density of 3×10^5 cells per well and used the day after plating. Cells were incubated with the drugs for 24 h. Then, cells were incubated with the dye Hoechst 33342 (5 μg/ml) for 30 min at 37 °C in the dark. Cells were viewed in a Nikon Diaphot inverted epifluorescence microscope with a 40× objective (excitation 355 nm, emission 465 nm). Cells showing condensed or fragmented nuclei (apoptotic cells) were identified from an average of 300 cells per treatment and cell batch. Samples were examined by blinded counting; samples were taken randomly from different fields. Data were expressed as percentage of apoptotic cells with respect to the total amount of cells counted.

2.6. Monitoring of $[Ca^{2+}]_c$ changes

Cells were plated at a density of 10^4 cells per well into 96-well plates, and the experiments performed 48 h later with confluent cultures. Cells were loaded with Tyrode-HEPES (in mM: 145 NaCl, 2.7 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 D-glucose, 10 HEPES, pH 7.4) containing 10 μM fluo-4 AM, 0.02% pluronic acid, and 1 mM probenecid for 45 min at 37 °C in the dark. After this incubation period, cells were washed with Tyrode-HEPES for 30 min at room temperature in the dark. Changes in fluorescence (excitation 485 nm, emission 520 nm) were measured using a fluorescent plate reader (Fluostar, BMG Labtechnologies).

Basal levels of fluorescence were monitored before adding drugs using an automatic dispenser. After stimulation, changes in fluorescence were measured for 6 s during 15 min. To normalize fluo-4 signals, responses from each well were calibrated by measuring maximum and minimum fluorescence values. At the end of each experiment, addition of 2% Triton X-100 (F_{max}) was followed by addition 10 mM ethyleneglycol-*bis*(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, F_{min}). Data were calculated as a percentage of $F_{max} - F_{min}$.

2.7. Assessment of mitochondrial membrane potential

Mitochondrial membrane potential was monitored with TMRE, a positive charged fluorescent indicator (excitation 550 nm, emission 590 nm) in quenching mode. SH-SY5Y cells were loaded in Tyrode-HEPES containing 3 μM TMRE and 1 mM probenecid for 15 min in the dark. Cells were then washed with Tyrode-HEPES and the fluorescence measured in a fluorescence plate reader. Basal levels of fluorescence were monitored before adding drugs to each well and measurements were taken for a total of 60 min. Data were calculated as F/F_0 fluorescence after drug treatment (F) with respect to basal value (F_0). Depolarization induced by each drug treatment was represented as percentage respect to control (cells without any treatment).

2.8. Statistical analysis

Averaged data are means ± S.E.M. Analysis of variance (ANOVA) was applied to see differences between groups. When significant differences were found, the Least Significant Difference (LSD) test was applied. The level of significance was taken as $P < 0.05$. Analysis was performed using the SPSS version 11.5.

3. Results

3.1. Apoptotic cell death elicited by dotarizine, flunarizine, thapsigargin, or cyclopiazonic acid, in the presence of foetal bovine serum

Initial experiments were planned to establish the parameters to quantitate reliably the proportion of cells undergoing apoptosis, after a defined treatment, using flow cytometry. Cells were incubated 24 h in DMEM containing 10% foetal bovine serum, with or without 50 μM dotarizine.

Fig. 1A shows an example on the distribution of cells at different stages of the cell cycle; the proportion of cells undergoing spontaneous apoptosis was 3.5%. This basal percentage rose to 31.6% in cells incubated with dotarizine (Fig. 1B).

Fig. 1C shows pooled data on the concentration-dependent pro-apoptotic effects of dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid. In these experiments cells were incubated with increasing concentrations of these compounds for 24 h (dotarizine, flunarizine and cyclopiazonic acid) or 2 h (thapsigargin). The fraction of cells undergoing spontaneous apoptosis under basal conditions was around 5% in all cases (Basal in Fig. 1C). The threshold concentration of dotarizine that enhanced apoptosis above basal levels was 10 μM. At 30 and 50 μM, dotarizine enhanced apoptosis to 18% and 43%, respectively (Fig. 1C). Flunarizine caused a significant increase in apoptosis at 30 and 50 μM, around 15%. Cyclopiazonic acid had a poorer effect (10% apoptosis at 100 μM). Because of its irreversible inhibition of the endoplasmic reticulum Ca^{2+} ATPase (Kijima et al., 1991; Lytton et al., 1991), cells were incubated for 2 h with various concentrations of thapsigargin and then with fresh DMEM during the following 22 h. At 1 μM, under these experimental conditions thapsigargin elicited 21% apoptosis, a figure that rose to 35% at 10 μM (Fig. 1C).

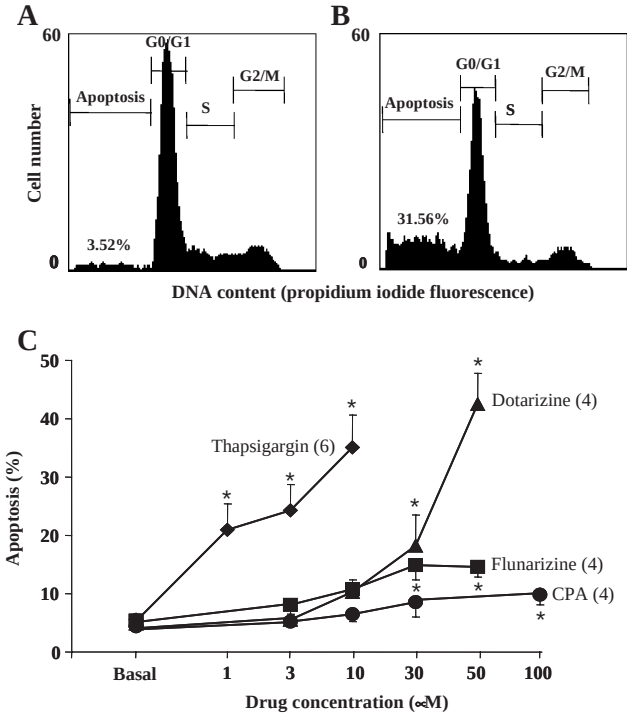

Fig. 1. Apoptotic cell death elicited by increasing concentrations of dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid (CPA). Panel A shows an original trace of the fluorescence signal (abscissa) of a flow cytometer, generated by control SH-SY5Y neuroblastoma cells, incubated in DMEM for 24 h. Panel B shows original traces from cells incubated for 24 h with DMEM containing 50 μM dotarizine. The percentage of apoptotic cells is shown by the horizontal bar in each trace. Panel C shows the concentration–response curves of the apoptotic effects of thapsigargin, dotarizine, flunarizine, and cyclopiazonic acid. Data are means ± S.E.M. of the number of experiments shown in parentheses. *$P < 0.05$, compared to basal.

3.2. Apoptotic cell death elicited by dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid: enhancement by serum deprivation and inhibition by serum albumin

Serum deprivation elicits apoptosis in several cell systems (Zhong et al., 1993; Howard et al., 1993; Kulkarni and McCulloch, 1994; Di Jeso et al., 1995; Dermitzaki et al., 2000). Hence, we performed experiments to estimate how the removal of serum from the DMEM bathing the cells affected apoptosis, both in the absence or the presence of the four endoplasmic reticulum-depleting compounds.

Fig. 2A shows that incubation of the cells in DMEM-containing serum elicited apoptosis in 5% of the cells (basal conditions). Serum deprivation enhanced apoptosis to 20%. Dotarizine (30 μM) caused 14% apoptosis in the presence of serum and as much as 60% in its absence. In the case of flunarizine (30 μM) apoptosis was 13% in the presence of serum and 65% in its absence. Thapsigargin (1 μM) elicited 15% apoptosis with serum and 39% in its absence. Finally, cyclopiazonic acid (30 μM) caused 7% apoptosis with serum, and 27% without serum. In summary, the four compounds caused a greater enhancement of apoptosis in the absence of serum. Upon subtraction of apoptosis obtained in basal conditions it was estimated that serum deprivation enhanced by 5.7-fold the dotarizine apoptotic effects, and by 4.7-fold those of flunarizine; with thapsigargin the enhancing apoptotic effect of serum deprivation was 1.5-fold and with cyclopiazonic acid, 4-fold. The drastic enhancement by serum deprivation of the apoptotic effects of dotarizine and flunarizine could be due either to some specific effect (i.e. the absence of some neurotrophic factor present in the serum) or simply to deprivation of serum proteins. Therefore, we decided to test whether albumin (the most abundant protein in serum) could mimick the neuroprotective effects of serum.

The three experimental conditions shown in Fig. 2B were run in parallel: cells were incubated with DMEM-containing serum, with DMEM deprived of serum, or with DMEM deprived of serum but containing bovine serum albumin, in the same proportion of total proteins contained in the serum

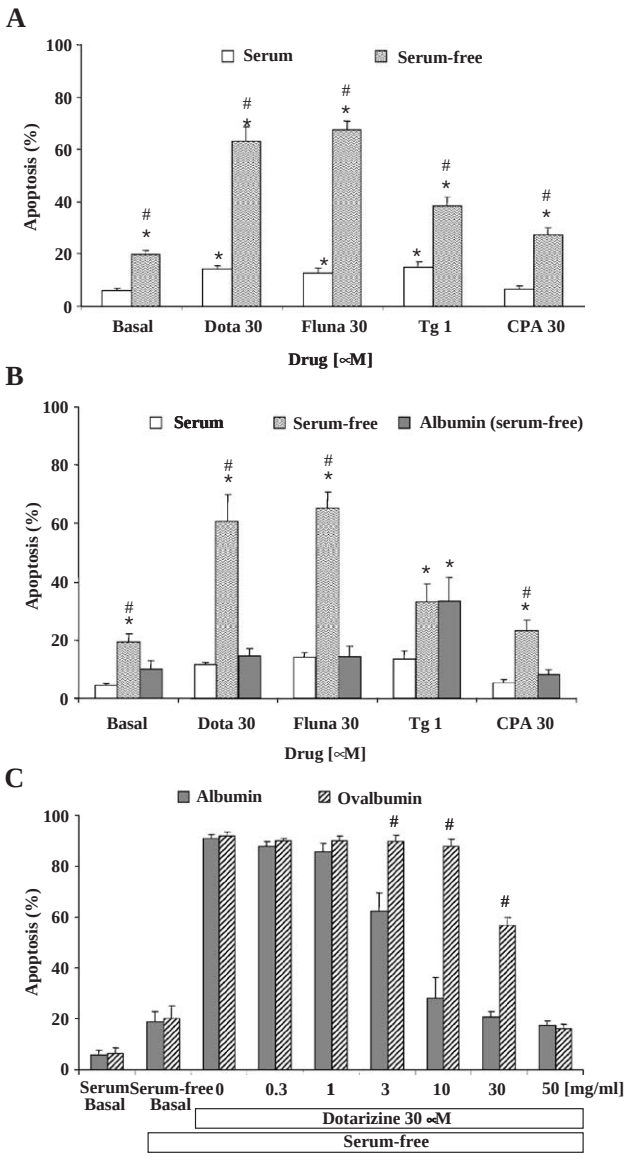

Fig. 2. Panel A shows the effects of serum deprivation on the apoptotic effects of 30 μM dotarizine (Dota 30), 30 μM flunarizine (Fluna 30), 1 μM thapsigargin (Tg 1), and 30 μM cyclopiazonic acid (CPA 30), in the presence (Serum) or in the absence of fetal bovine serum (Serum-free). Apoptotic cell death, expressed as percentage in the ordinate, elicited by serum deprivation, in the absence (Basal) or the presence of Dota, Fluna, Tg, or CPA, during a 24-h incubation period. Data are means ± S.E.M. of 11–15 experiments, performed with different cell batches. $^{*}P<0.05$ comparing to control data in the presence of serum, $^{\#}P<0.05$ comparing serum versus serum-free. Panel B shows that the enhanced apoptosis elicited by serum deprivation was inhibited by bovine serum albumin, in the case of dotarizine (Dota 30), flunarizine (Fluna 30), and cyclopiazonic acid (CPA 30), but not in the case of thapsigargin (Tg 1). Experiments were run in parallel, using three variables, i.e., cell incubation 24 h in the presence (Serum) or the absence of serum (Serum-free), and substituting serum by albumin (4.9%) (Albumin, serum-free). Apoptotic cell death is expressed as percent in the ordinate. Data are means ± S.E.M. of 4–6 experiments performed with different batches of cells. $^{*}P<0.05$, with respect to cells incubated with serum; $^{\#}P<0.05$ compared with cells incubated with albumin. Panel C shows an experiment with a design similar to that of panel B, comparing the effects of increasing concentrations of albumin and ovalbumin, on apoptosis elicited by 30 μM dotarizine in the absence of serum. Data are from 3 to 5 experiments done with different batches of cells. $^{\#}P<0.05$ compared to albumin.

(4.9 g/100 ml). In the presence of serum, basal apoptosis was 4%; serum deprivation enhanced apoptosis to 19%, and albumin decreased it back to 10%. In the absence of serum, dotarizine (30 μM) increased apoptosis from 12% to 61%; albumin reduced the rate of dotarizine-elicited apoptosis to 15%. Similarly, flunarizine (30 μM) enhanced apoptosis from 14% in the presence of serum to 65% in its absence; once more, albumin reduced the apoptotic effects of flunarizine back to 14%.

Thapsigargin (1 μM) also augmented apoptosis to 14% in the presence of serum, and to 33% in its absence; it is surprising that albumin did not reduce the apoptotic effects of thapsigargin. Finally, cyclopiazonic acid (30 μM) enhanced apoptosis from 5% in the presence of serum to 23% in its absence; albumin reduced the apoptotic effects of cyclopiazonic acid to 8%. In two experiments, a concentration of albumin similar to that found in serum was used (2.9 g/100 ml); the results were similar to those found with 4.9 g/100 ml (data not shown).

It was of interest to know whether as albumin, ovalbumin protected neuroblastoma cells against apoptosis. Hence, an experiment was run in which albumin and ovalbumin were compared in a wide range of concentrations. Fig. 2C shows that albumin had a cytoprotectant threshold concentration of 3 mg/ml; ovalbumin begun to protect at 30 mg/ml, a concentration about 10-fold higher. However, full protection against apoptosis elicited by

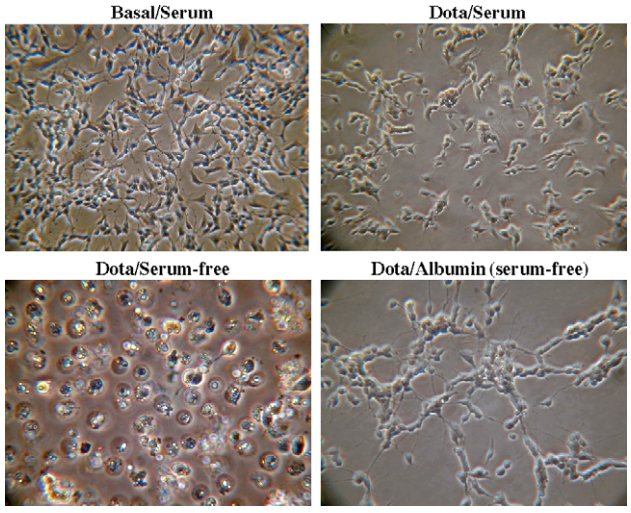

Fig. 3. Phase contrast microphotographs of neuroblastoma cells incubated during 24 h with the following treatments: A, DMEM containing 10% foetal bovine serum; B, same medium as A, but adding 30 μM dotarizine; C, serum deprivation plus 30 μM dotarizine; and D, serum substituted by 4.9% bovine serum albumin plus 30 μM Dota. Observe the bright birrefringency of control cells in panel A, which also shows a homogeneous cytosol and the emission of abundant cell processes. In panel B cells accumulate in islets, with their plasma membrane less nitid, and the loss of birrefringency and cell processes. Cells in panel C (Dota plus serum deprivation) show a round-shaped appearance and abundant cell debris, indicating cell death. Finally, cells in panel D, treated with albumin, partly recovered their healthy appearance, although they did not reach the state of control cells (panel A).

dotarizine in serum-free medium was achieved by albumin at 10 mg/ml and ovalbumin at 50 mg/ml. Thus albumin was about 5-fold more potent than ovalbumin in causing neuroprotection.

Fig. 3 shows phase contrast photomicrographs of neuroblastoma cells under various incubation conditions. Panel A shows control cells after a 24-h incubation period in DMEM-containing serum. Note the nitid birrefringency, the cell processes and the homogeneous cytoplasm. Panel B shows cells incubated for 24 h in serum-free medium containing 30 μM dotarizine; note the cell aggregation in islets, the less nitid plasma membrane contours, and the poor birrefringency and lack of homogeneity of the cytosol. Cells incubated with dotarizine in the absence of serum were round shaped, the cytoplasm was granular and abundant cell debris were also present (panel C). This drastic cell damage was prevented in the presence of albumin (panel D).

An additional proof of the apoptotic effect of dotarizine in serum-deprived medium, and the cytoprotection elicited by albumin was obtained by visualizing DNA laddering (Gong et al., 1994). Fig. 4 shows the DNA gel electrophoresis obtained from five groups of cells treated under the same conditions than those of Fig. 2B. Line 1 shows the standard of DNA fragments (100 base pairs). Lines 2, 3 and 4 show the DNA from control cells incubated during 24 h in serum-containing medium in the absence of serum or in the presence of albumin. The next 12 lines correspond to cells incubated 24 h with thapsigargin, dotarizine, flunarizine, and cyclopiazonic acid in the presence of serum, in its absence or with albumin, as indicated. Note the clear DNA ladder, a sign of apoptosis, when cells were incubated with dotarizine or flunarizine in the absence of serum; the ladder disappeared when albumin was added to the serum-free medium.

A last paradigm of apoptosis studied was the condensation of chromatin that we measured using the dye Hoechst 33342. See in Fig. 4B the nuclear chromatin in control cells (24 h in DMEM containing 10% serum) or after 24 h treatment with 30 μM dotarizine in serum-free medium (panel C). Note the condensed chromatin of the 2 cells shown. Fig. 4D shows pooled results; as with flow cytometry, the number of cells suffering apoptosis drastically increased upon serum removal. Thus, in the case of dotarizine the number of apoptotic nuclei rose from 8% in the presence of serum to 50% in its absence; albumin decreased back to 20% the apoptotic nuclei. In the case of flunarizine, the absence of serum increased the apoptotic nuclei from 7% to 41%; albumin decreased this number to 22% (Fig. 4D).

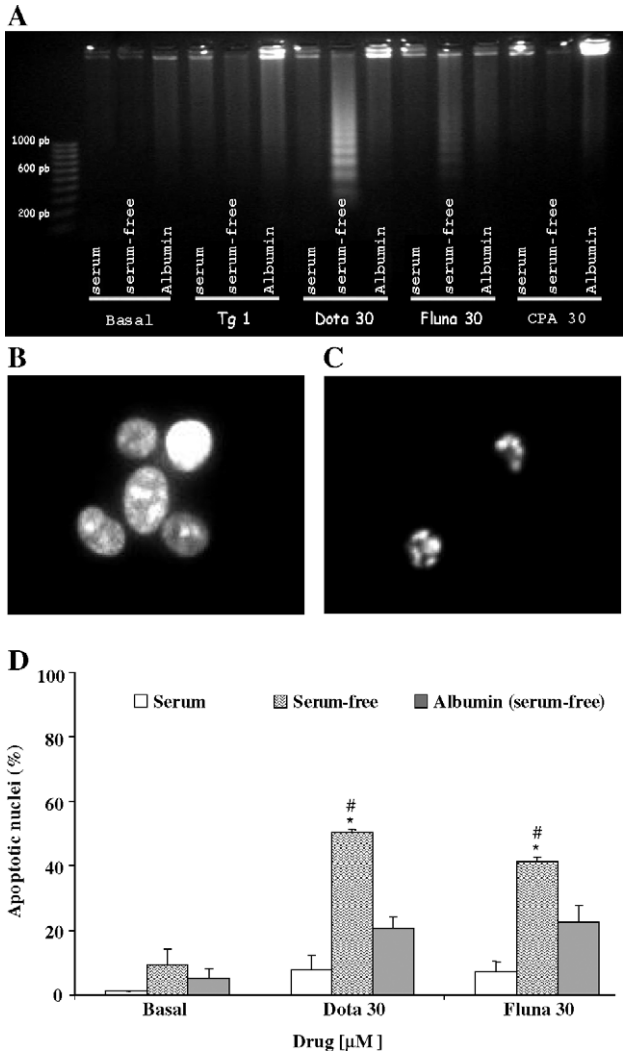

Fig. 4. Panel A shows the gel electrophoresis patterns of DNA from cells subjected to treatments similar to those of Fig. 2B (see Materials and methods for details). Line 1 shows standard DNA fragments; lines 2, 3 and 4 show DNA from control cells that were incubated for 24 h in serum-containing DMEM; in the absence of serum or in the absence of serum but in the presence of albumin (10 mg/ml). The next four triplets of lines show the DNA patterns from cells incubated 24 h with 1 μM thapsigargin (Tg 1), 30 μM dotarizine (Dota 30), 30 μM flunarizine (Fluna 30), or 30 μM cyclopiazonic acid (CPA 30). Panels B and C show images of neuroblatoma cells stained with the fluorescence probe Hoechst 33342 to visualize apoptotic fragmented nuclei after a 24-h incubation in DMEM containing 10% serum (control, panel B) or with dotarizine 30 μM in the absence of serum (panel C). Panel D shows averaged results of experiments performed following the protocol of panel C, in control conditions or after incubation with 30 μM dotarizine (Dota 30) or 30 μM flunarizine (Fluna 30), in the presence or the absence of serum or with albumin, as indicated. Data are means ± S.E.M. of 3 experiments from different batches of cells. $^{*}P<0.05$ compared to serum; $^{\#}P<0.05$ compared to albumin.

3.3. Effects of dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid on $[Ca^{2+}]_c$

As documented in Introduction, these four agents cause the release of Ca^{2+} from the endoplasmic reticulum into the cytosol, thus rising the $[Ca^{2+}]_c$. Hence, we thought of interest to test whether the changes of $[Ca^{2+}]_c$ somehow differed between the effects of the four agents on apoptosis. Fig. 5 displays four traces on the time course of the changes of $[Ca^{2+}]_c$ elicited by 30 μM each of dotarizine, flunarizine or cyclopiazonic acid, or by 1 μM thapsigargin, in fluo-4-loaded SH-SY5Y cells. The concentrations of thapsigargin and cyclopiazonic acid were

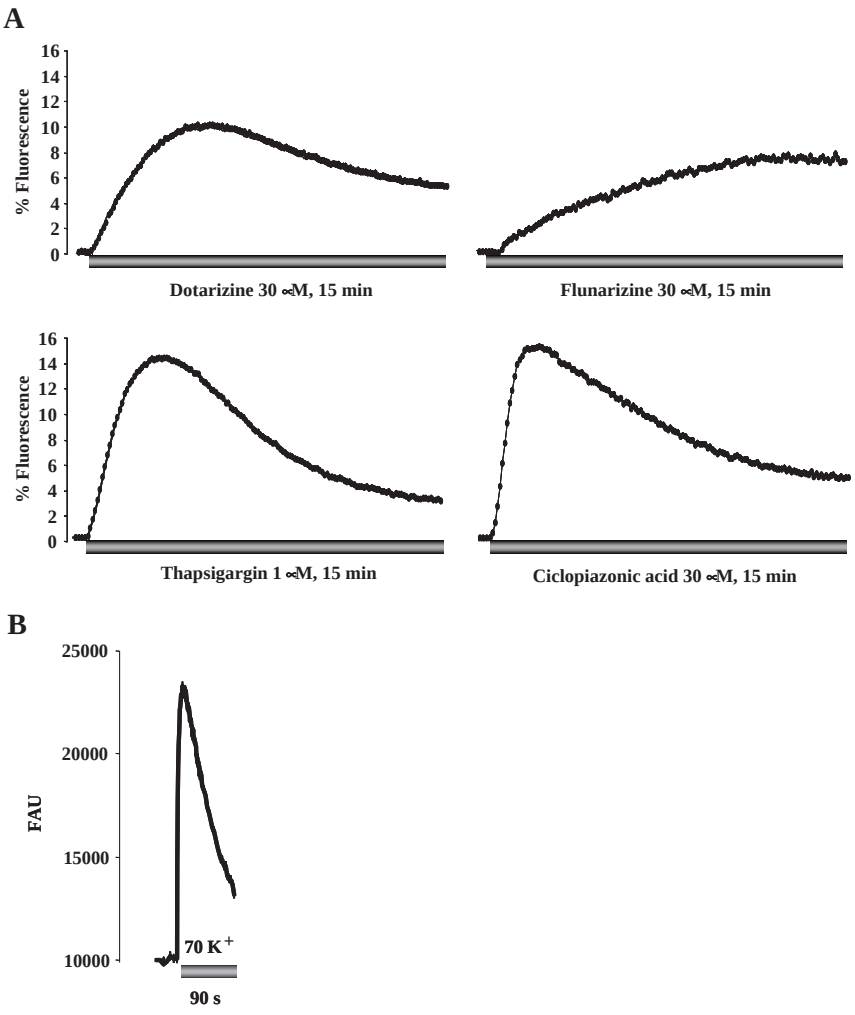

Fig. 5. Panel A. Time course of the elevation of $[Ca^{2+}]_c$ elicited by dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid at the concentrations shown in the horizontal bars. The records are from a representative experiment performed in SH-SY5Y cells loaded with fluo-4. The traces were obtained along a 15-min recording period, and the fluorescence signal was recorded every 6 s. Fluorescence is expressed as % of $F-F_0/F_{max}-F_{min}$ (see Materials and methods). Panel B shows a trace of the fluorescence generated by high K^+ (70 mM). Note that this response is considerably faster than that evoked by the four drugs applied in panel A.

supramaximal to block the endoplasmic reticulum Ca^{2+}-ATPase (Kijima et al., 1991; Lytton et al., 1991; Demaurex et al., 1992). Those of dotarizine and flunarizine cause the release of Ca^{2+} from the endoplasmic reticulum, as demonstrated with endoplasmic reticulum-targeted aequorin (Novalbos et al., 1999a). Dotarizine caused a slow, gradual elevation of $[Ca^{2+}]_c$ (T_{max}, 8.5 min) that reached a plateau and then declined very slowly (Fig. 5A). The $[Ca^{2+}]_c$ increase evoked by flunarizine was even slower (T_{max}, 12.5 min); it reached a plateau at around 15 min. Thapsigargin evoked a faster increase of $[Ca^{2+}]_c$ (T_{max}, 4 min) that reached a plateau and then gradually declined to near basal levels. The fastest increase of $[Ca^{2+}]_c$ was evoked by cyclopiazonic acid (T_{max}, 3 min); this signal also reached a transient plateau and then gradually declined. On a quantitative base, we tried to measure the total amount of Ca^{2+} released from the endoplasmic reticulum into the cytosol, by calculating the area under the curve (AUC) obtained along the 15 min of the recording period (Table 1). The sizes of the areas were thapsigargin>cyclopiazonic acid>dotarizine>-flunarizine.

For comparative purposes, a record on the $[Ca^{2+}]_c$ changes evoked by a 70-mM K^+ solution is shown in Fig. 5B. Note the faster activation of the K^+-evoked $[Ca^{2+}]_c$ rise (T_{max}, 10 s) and also its fast decline to near basal levels; this rapid decrease of $[Ca^{2+}]_c$ is likely due to inactivation of voltage-dependent Ca^{2+} channels (Hernández-Guijo et al., 2001).

3.4. Effects of serum and albumin on the changes of $[Ca^{2+}]_c$ evoked by dotarizine, flunarizine, thapsigargin and cyclopiazonic acid

These experiments tested whether albumin and serum affected the changes of $[Ca^{2+}]_c$ produced by these compounds.

Table 1
Kinetic parameters of the $[Ca^{2+}]_c$ changes elicited by dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid at the concentrations indicated in parentheses

Drug		Serum	Serum-free	Albumin (serum-free)
Basal	AUC (FUxs)	1819±479	459±445[a]	715±288[a]
	E_{max} (% F)	3.1±0.7	1±0.8[a]	1.1±0.4[a]
	T_{max} (s)	774±106	267±214[a]	398±141[a]
Dotarizine (30 μM)	AUC (FUxs)	6415±465	5479±595	5007±678
	E_{max} (% F)	10.8±1.2	8.4±1	7.9±1.2
	T_{max} (s)	338±76	517±133	487±127
Flunarizine (30 μM)	AUC (FUxs)	5212±1283	4686±479	2308±799[b]
	E_{max} (% F)	550±163	7.3±0.8	3.8±1.3[b]
	T_{max} (s)	8.4±2	753±82	612±129
Thapsigargin (1 μM)	AUC (FUxs)	10104±1352[c,d]	7552±954[d]	8606±1181[d]
	E_{max} (% F)	16.6±2.6[c,d]	16.1±2.1[c,d]	16.3±2.6[d]
	T_{max} (s)	244±38	245±15[d]	354±82
Cyclopiazonic acid (30 μM)	AUC (FUxs)	7557±1174	6536±781	7484±879[d]
	E_{max} (% F)	12.7±1.8	15.3±1.7[c,d]	16.7±2[d]
	T_{max} (s)	176±20[d]	184±14[d]	226±18[d]

AUC, area under the curve expressed in flourescence units per second; E_{max}, peak flourescence, expressed in % F (see Fig. 5 legend); T_{max}, time to peak in seconds. Data are means±S.E.M. of 3–8 experiments from different cell cultures. [a]$P<0.05$ compared to serum, [b]$P<0.05$ compared to serum free, [c]$P<0.05$ compared to dotarizine, [d]$P<0.05$ compared to flunarizine.

In basal conditions (no drugs added) and the presence of serum, the AUC of the $[Ca^{2+}]_c$ curve was 1819 FUxs (Fluorescent Units per second), the E_{max} 3.1% and the T_{max} 774 s (Table 1). These values decreased by 60–70% in the absence of serum; albumin (10 mg/ml) did not restore those values to the basal levels in the presence of serum.

In the presence of serum, dotarizine augmented the basal levels of $[Ca^{2+}]_c$ to 6415 FUxs, about 3.5-fold ($P<0.05$). Such increment was similar in serum-free, with or without albumin. The values of E_{max} and T_{max} were also similar in the three conditions. Flunarizine elicited similar responses in serum and serum-free media; however, in albumin (serum-free) flunarizine produced AUC and E_{max} responses that were about 40% of those in serum or serum-free ($P<0.05$).

Thapsigargin caused the greatest increment of $[Ca^{2+}]_c$, 10104 FUxs and 16.6% F in the presence of serum; the T_{max} was shorter (244 s), indicating a faster response. Similar values were obtained in serum-free and albumin. Finally, cyclopiazonic acid produced effects essentially similar to those of thapsigargin (Table 1).

3.5. Changes of the mitochondrial membrane potential elicited by dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid: effects of serum and albumin

Mitochondrial depolarization is a crucial event in the apoptosis cascade (Schinder et al., 1996; Tornero et al., 2002). Therefore, we explored the effects of dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid, in the presence or the absence of serum and albumin, on the mitochondrial membrane potential of TMRE-loaded cells. Fig. 6 shows the rapid and drastic mitochondrial depolarization caused by 1 μM of the potent protonophore FCCP, a reference control. The mitochondrial membrane potential of control cells remained unaltered

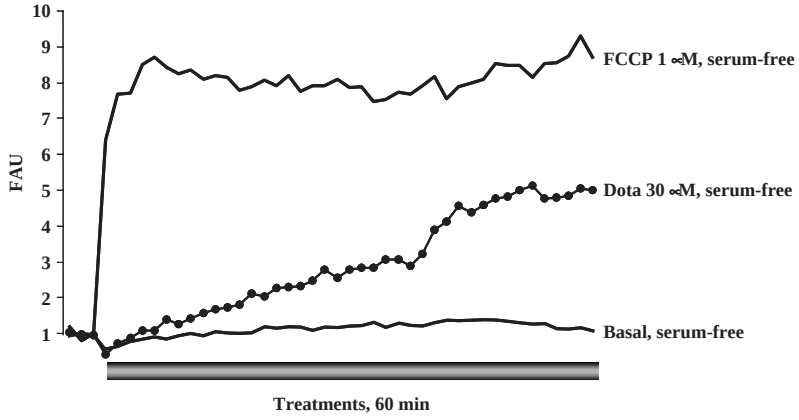

Fig. 6. Changes of the mitochondrial membrane potential evoked by FCCP (1 μM) or dotarizine in the absence of serum (Dota 30 μM, serum-free) in TMRE loaded cells. The trace labelled Basal belongs to untreated cells. Mitochondrial depolarization causes the release of TMRE into the cytosol, with the corresponding increase of fluorescence, which is expressed in arbitrary units (FAU, ordinate). The fluorescence was monitored along a 60-min period.

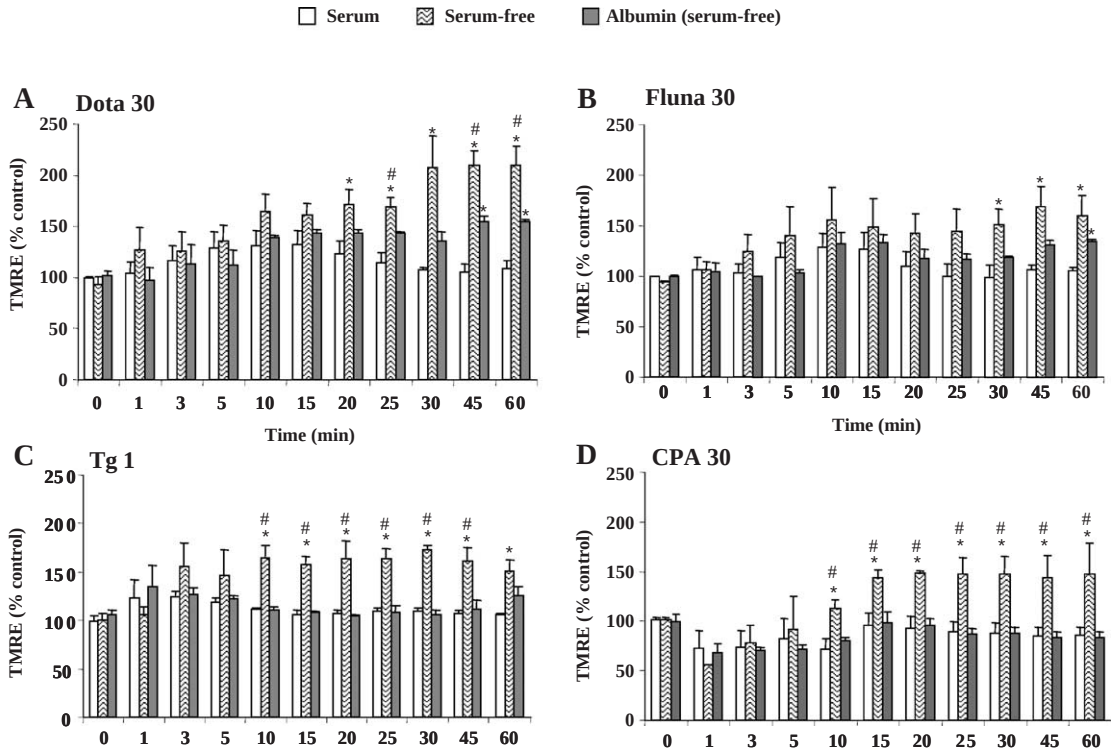

Fig. 7. Changes of the mitochondrial membrane potential produced by 30 μM dotarizine (Dota 30, panel A), 30 μM flunarizine (Fluna 30, panel B), 1 μM thapsigargin (Tg 1, panel C) or 30 μM cyclopiazonic acid (CPA 30, panel D), in the presence of serum, in its absence (Serum-free), or in the absence of serum with 10 mg/ml bovine serum albumin added (Albumin/serum-free). TMRE-loaded neuroblastoma cells were incubated under the indicated conditions, and fluorescence was continuously monitored along a 60-min period, as indicated in Fig. 6. For statistical analysis, fluorescence values were averaged at the times indicated in the abscissae; the initial values were normalized to 100% and the subsequent fluorescence changes were expressed as percentage of those initial values in every individual experiment (ordinates). Data are means ± S.E.M. of 3–4 experiments from different batches of cells. $^{*}P<0.05$ compared to Serum; $^{\#}P<0.05$ compared to albumin.

along the 1-h recording period (basal line in Fig. 6). In the absence of serum, dotarizine (30 μM) caused a gradual mitochondrial depolarization beginning at around the minute 10th of incubation, and gradually increased to reach a plateau.

Quantitative data on the changes of mitochondrial membrane potential are represented in Fig. 7. Note in panel A that in the absence of serum, dotarizine significantly increased mitochondrial depolarization from the 10th to the 60th minute; note also that in the presence of albumin (serum-free) the depolarizing effects of dotarizine were reduced. In the case of flunarizine the increase of mitochondrial depolarization upon serum removal was seen at the 10th minute, becoming stable until the end of the experiment; again, the presence of albumin significantly reduced such depolarization (panel B). Thapsigargin in serum-free caused a faster mitochondrial depolarization that lasted for the remaining 1-h period; albumin significantly counteracted such depolarizing effect (panel C). Mitochondrial depolarization caused by cyclopiazonic acid in the absence of serum followed a particular pattern; at the first minute we observed a hyperpolarization followed by a gradual depolarization that reached a maximun at the 15th minute and remained stable until the end of the experiment. In the presence of serum or albumin the net depolarization evoked by cyclopiazonic acid was significantly reduced.

4. Discussion

We have found that dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid caused apoptotic death of human neuroblastoma SH-SY5Y cells. Although the ability of thapsigargin and cyclopiazonic acid to elicit apoptosis is well established (Wei et al., 1998; Nguyen et al., 2002; Arias et al., 2004), this is the first report showing that dotarizine and flunarizine are also capable of eliciting apoptotic cell death. Previous reports measuring LDH release as an indicator of cell death could not establish whether the effects of dotarizine and flunarizine were due to necrosis or apoptosis (Novalbos et al., 1999a,b). Here we have measured cell death elicited by dotarizine using three different markers of apoptosis, i.e. propidium iodide staining combined with flow cytometry, DNA laddering and nuclei staining with Hoechst 33342. Using these three markers we have demonstrated that dotarizine and flunarizine indeed elicited apoptotic cell death.

There is no obvious relationship between the effects on $[Ca^{2+}]_c$ and apoptosis. We found minor differences between

the four drugs as far as their intracellular Ca^{2+}-releasing effects in concerned. Thus, the greater apoptotic effects of dotarizine and flunarizine, as compared to thapsigargin and cyclopiazonic acid are apparently unrelated to their Ca^{2+}-mobilizing actions. The mechanism of the Ca^{2+}-depleting effects of thapsigargin and cyclopiazonic acid is the inhibition of endoplasmic reticulum Ca^{2+}-ATPase (Kijima et al., 1991; Lytton et al., 1991; Demaurex et al., 1992); that for dotarizine and flunarizine remains to be established. These possible differences could explain the different ability to evoke apoptosis between the four compounds. Some differences may also rely on the property of albumin to bind fatty acids, some drugs and their metabolites, and various anions and cations, including Ca^{2+} (Emerson, 1989). The fact that albumin did not affect the $[Ca^{2+}]_c$ changes evoked by dotarizine, thapsigargin and cyclopiazonic acid suggest that binding of those drugs to albumin is not altering their effects. In the case of flunarizine, there was a 50% decrease of AUC and E_{max}, and T_{max} was delayed with respect to other drugs, suggesting that flunarizine might bind to albumin more strongly.

Whatever the mechanism involved, it is plausible that mitochondria will sense the slow but progressive $[Ca^{2+}]_c$ increments caused by the four agents, to reach large intramitochondrial Ca^{2+} concentrations, as visualized with targeted aequorins (Montero et al., 2000). Hence, opening of the transition pore causes mitochondrial depolarization and cytochrome *c* release, leading to activation of the apoptotic cascade (Schinder et al., 1996; Tornero et al., 2002). ATP depletion causes inhibition of Ca^{2+}-ATPases further enhancing $[Ca^{2+}]_c$, the activation of phospholipases and nitric oxide synthase and free radical production (Coyle and Puttfarcken, 1993).

Several reports suggest that albumin exhibits cytoprotective effects (Emerson, 1989; Matsui et al., 1993; Huh et al., 1998; Belayev et al., 2001; Moran et al., 2002; Tabernero et al., 2002; Gum et al., 2004). One report shows that in chronic lymphocytic leukaemia cells, serum albumin protects against spontaneous apoptosis. Albumin also decreases reactive oxidant-induced apoptosis (Moran et al., 2002; Gum et al., 2004). The cytoprotective effect of albumin was observed at concentrations of 10–20 mg/ml, 2–4-fold lower than in plasma, and 1–2-fold higher than in culture medium containing 10% serum. In our experiments, albumin exerted significant protection at 10 mg/ml and afforded full cytoprotection at 29 mg/ml and 49 mg/ml, concentrations in the range of those present in the blood, about 40 mg/ml (Andersson, 1979).

Albumin is capable of binding free radicals and prevents their accumulation inside the cells (Emerson, 1989; Huh et al., 1998; Belayev et al., 2001; Moran et al., 2002; Gum et al., 2004). Albumin is an antioxidant more efficient than vitamin E, due to its ability to bind copper, thus delaying the formation of free radicals (Belayev et al., 2001). These antioxidant properties of albumin could explain our present results. In fact, we have recently shown that the cytotoxic effects of dotarizine are reduced in the presence of *N*-acetylcysteine, suggesting that they are mediated by free radical production (unpublished preliminary results). Free radicals cause mitochondrial depolarization (Aon et al., 2003); by sequestering them, albumin could prevent mitochondrial depolarization and the activation of the apoptotic cascade.

In conclusion, we have shown that endoplasmic reticulum Ca^{2+} depletion elicited by dotarizine, flunarizine, thapsigargin, and cyclopiazonic acid cause apoptosis of SH-SY5Y human neuroblastoma cells, that is augmented upon serum deprivation, and that albumin protects against such apoptotic effects by preventing mitochondrial depolarization. Our findings might stimulate the search for a small molecular weight drug having the properties of albumin with neuroprotective effects in Alzheimer's disease and stroke. In fact, the 4-amino acid terminal sequence of human albumin is a metal binding site that may play a prominent role in its neuroprotective effects (Bar-Or et al., 2001) and serve as a model for the design of new smaller molecular weight antioxidant drugs.

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