Regulation of T-cell survival is important as a physiological mechanism involved in determining the development of the immune response, and also as one of the mechanisms that can contribute to the control of expansion of T-cell tumors. Moreover, it is well accepted that apoptosis plays a relevant role at the thymus level where massive cell death occurs in a continuous selection process. Among different tumors, it has been reported that GCH can induce thymocyte apoptosis. However, the molecular mechanisms responsible for this GCH-induced death have not been clarified. In this work, the biochemical events associated with apoptosis induced by Dexamethasone (Dex), a synthetic GCH, in normal mouse thymocytes, have been analyzed. Results indicate that Dex-induced thymocyte apoptosis is attributable to an early ceramide generation caused by the activation of an acidic sphingomyelinase (aSMase). Caspase activity plays a crucial role in Dex-induced apoptosis and is downstream the aSMase activation in that inhibition of the early ceramide generation inhibits caspase activation and thymocyte death.

Glucocorticoid hormones (GCH) have been implicated as regulators of T-lymphocyte growth and differentiation. In particular, it has been reported that GCH can induce thymocyte apoptosis. However, the molecular mechanisms responsible for this GCH-induced death have not been clarified. In this work, the biochemical events associated with apoptosis induced by Dexamethasone (Dex), a synthetic GCH, in normal mouse thymocytes, have been analyzed. Results indicate that Dex-induced thymocyte apoptosis is attributable to an early ceramide generation caused by the activation of an acidic sphingomyelinase (aSMase). Caspase activity plays a crucial role in Dex-induced apoptosis and is downstream the aSMase activation in that inhibition of the early ceramide generation inhibits caspase activation and thymocyte death.

Moreover, Dex treatment rapidly induces diacylglycerol (DG) generation, through a protein kinase C (PKC) and G-protein-dependent phosphatidylinositol-specific phospholipase C (PI-PLC), an event which precedes and is required for aSMase activation. Indeed, PI-PLC inhibition by U73122 totally prevents Dex-induced aSMase activity, ceramide generation, and consequently, caspase activation and apoptosis. All these effects require Dex interaction with GCH receptor (GR), are countered by the GR antagonist RU486, and precede the GCH/GR-activated transcription and protein synthesis. These observations indicate that GCH activates thymocyte death through a complex signaling pathway that requires the sequential activation of different biochemical events.

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Materials and methods

Cell system and treatments. Thymocytes, from 4- to 6-week-old C3H/HeN mice, were enriched by passage through nylon wool columns. The effect of several agents on Dex-induced apoptosis, ceramide
In vitro acidic and neutral SSmase analysis. Aliquots of 6 × 10^6 cells/mL were treated for the indicated times with Dex (10^-7 M/mL). Treatment was stopped by immersion of samples in methanol/dry ice (−70°C) for 10 seconds followed by centrifugation at 4°C in a microfuge. To measure neutral SSmase (nSSmase), pellets were dissolved in a buffer containing 20 mmol/L HEPES (pH 7.4), 10 mmol/L MgCl₂, 2 mmol/L EDTA, 5 mmol/L diithiothreitol (DTT), 0.1 mmol/L Na₂VO₃, 0.1 mmol/L Na₃MoO₄, 30 mmol/L β-nitrophenylphosphate, 10 mmol/L β-glycerophosphate, 750 mmol/L ATP, 10 µmol/L PMSE, 10 µmol/L leupeptin, 10 µmol/L pepstatin, and 0.2% Triton X-100. After incubation for 5 minutes at 4°C, the cells were sonicated as described above and centrifuged for 30 minutes at 14,000 rpm at 4°C. The supernatants were then collected, and protein concentration was determined through the Pierce Micro BCA assay kit with bovine serum albumine standards.

Proteins (50 to 100 µg) were incubated for 2 hours at 37°C in a buffer containing 20 mmol/L HEPES (pH 7.4), 1 mmol/L MgCl₂, and 0.32 µL of N-methyl-14C SM (0.04 µCi/µL, specific activity 56.6 mCi/mmol; Amersham). To measure aSSmase, after treatment, the cells were washed, and the pellet was resuspended in 200 µL of 0.2% Triton-X-100 and incubated for 15 minutes at 4°C. The cells were sonicated, and the protein concentration was assayed. Fifty to one hundred micrograms of protein were incubated for 2 hours at 37°C in a buffer (50 µL final volume) containing 250 mmol/L sodium acetate, 1 mmol/L EDTA (pH 5.0), and 0.32 µL of N-methyl-14C SM (0.04 µCi/µL, specific activity 56.6 mCi/mmol; Amersham). The reaction was stopped by the addition of 250 µL chloroform:methanol (2:1, by volume). The lipids were extracted as described above. The organic phase, obtained in the different extraction steps, was collected and washed once with 1 mL chloroform:methanol:water (3:48:47, by volume), to totally remove free radioactive phosphorylcholine. The aqueous phases were collected, transferred to scintillation vials, and routinely counted by liquid scintillation counting. The counts/minute represented the choline phosphate generated from SM hydrolysis. The organic phase was analyzed on thin-layer chromatography (TLC) plates by using chloroform:methanol:ammonium hydroxide (7:5:1) as solvent (water (85:15:0.5:0.5, by volume). The hydrolysis of SM was quantitated by autoradiography and liquid scintillation and expressed as picomoles SM hydrolyzed/10^6 cells.

Ceramide synthesis assay. Assay of ceramide synthesis was performed as previously described. After treatment with Dex for different times (5 to 180 minutes), thymocytes were collected and resuspended in 300 µL of homogenization buffer (25 mmol/L HEPES (pH 7.4), 5 mmol/L EDTA, 50 mmol/L NaF, 10µg/mL leupeptin, and 100 µg/mL bovine serum albumin). Protein concentrations were determined in the supernatant using a Bio-Rad Protein Assay kit. Protein concentrations in the supernatants were then determined by the bicinchoninic acid (BCA) assay.

Ceramide mass measurement (diacylglycerol kinase assay). Aliquots of 5 × 10^6 cells were suspended in 1 mL of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mmol/L glutamine, and antibiotics, and treated for the indicated times with Dex in the presence or absence of inhibitors. Treatment was stopped by immersion of samples in methanol/dry ice (−70°C) for 10 seconds, followed by centrifugation at 4°C in a microfuge. To measure ceramide levels, pellets were dissolved in a buffer containing Tris-HCl 20 mmol/L pH 7.4, 1 µmol/L phenylmethylsulfonyl fluoride (PMSF), 10 µmol/L β-glycerophosphate, 750 mmol/L ATP, 10 µmol/L PMSE, 10 µmol/L leupeptin, 10 µmol/L pepstatin, and 0.2% Triton X-100. After incubation for 5 minutes at 4°C, the cells were sonicated as described above and centrifuged for 30 minutes at 14,000 rpm at 4°C. The supernatants were then collected, and protein concentration was determined through the Pierce Micro BCA assay kit with bovine serum albumine standards. Proteins (50 to 100 µg) were incubated for 2 hours at 37°C in a buffer containing 20 mmol/L HEPES (pH 7.4), 1 mmol/L MgCl₂, and 0.32 µL of N-methyl-14C SM (0.04 µCi/µL, specific activity 56.6 mCi/mmol; Amersham). To measure aSSmase, after treatment, the cells were washed, and the pellet was resuspended in 200 µL of 0.2% Triton-X-100 and incubated for 15 minutes at 4°C. The cells were sonicated, and the protein concentration was assayed. Fifty to one hundred micrograms of protein were incubated for 2 hours at 37°C in a buffer (50 µL final volume) containing 250 mmol/L sodium acetate, 1 mmol/L EDTA (pH 5.0), and 0.32 µL of N-methyl-14C SM (0.04 µCi/µL, specific activity 56.6 mCi/mmol; Amersham). The reaction was stopped by the addition of 250 µL chloroform:methanol (2:1, by volume). The lipids were extracted as described above. The organic phase, obtained in the different extraction steps, was collected and washed once with 1 mL chloroform:methanol:water (3:48:47, by volume), to totally remove free radioactive phosphorylcholine. The aqueous phases were collected, transferred to scintillation vials, and routinely counted by liquid scintillation counting. The counts/minute represented the choline phosphate generated from SM hydrolysis. The organic phase was analyzed on thin-layer chromatography (TLC) plates by using chloroform:methanol:ammonium hydroxide (7:5:1) as solvent (water (85:15:0.5:0.5, by volume). The hydrolysis of SM was quantitated by autoradiography and liquid scintillation and expressed as picomoles SM hydrolyzed/10^6 cells.

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vesicles, respectively, to generate DAG. Cells were treated for the indicated times with Dex (10^{-7} mol/L) in the presence or absence of the PC-PLC inhibitor, D609 (50 µg/mL), or the PI-PLC inhibitor, U73122 (2.5 µmol/L). Treatment was stopped by immersion of samples in methanol/dry ice (−70°C) for 10 seconds followed by centrifugation at 4°C in a microfuge. The pellets were then resuspended in 250 mmol/L Tris-HCl buffer (pH 7.4), containing 10 mmol/L PMSF, 100 mmol/L bacitracin, 1 mmol/L benzamidine, 1 mmol/L aprotinin, 10 mmol/L leupeptin, 10 mmol/L pepstatin, and 5 µg/mL soybean trypsin inhibitor. Cells were lysed by sonication with a cell sonifier. Radiolabeled PC or PI vesicles were prepared by sonication (5 minutes, 5W, and 80% output) to generate DAG. Cells were treated for the specific activity 56 mCi/mmol; Amersham) or L-3-phosphatidylinositol-1,2-diacylglycerol (14C) [3H]arachidonoyl for the detection of released DAG through PC-PLC or PI-PLC, respectively. Vesicles were resuspended at 10 µmol/L in the reaction buffer (50 mmol/L Tris-HCl [pH 7.4], 5 mmol/L CaCl2, 5 mmol/L MgCl2, and 0.01% fatty acid free--bovine serum albumin [BSA]). Whole cell lysate (50 to 100 µg proteins) was added to 250 µL reaction buffer containing the vesicles, incubated at 37°C for 1 hour, and the reaction buffer was stopped by the addition of 250 µL chloroform:methanol:acetic acid (4:2:1, by volume). To separate the organic from the aqueous phase, 250 µL of H2O, 250 µL of CHCl3, and 100 µL of KCl were added, and the mixture was centrifuged at 4,000 rpm in a microfuge for 5 minutes. The organic phase was removed, dried under nitrogen, resuspended in 200 µL chloroform, and applied to a silica gel TLC plate (Merck, Darmstadt, Germany), with an automatic applicator (Linomat IV; CAMAG, Muttenz, Germany). Samples were chromatographed in chloroform:methanol:acetic acid:water (100:60:16:8) to separate the parent phospholipids from the product of PC-PLC and PI-PLC, ie, DAG. Authentic standards were cochromatographed with the lipid extracts to locate the compounds of interest by exposure to iodine vapor. Radioactive spots, visualized by autoradiography and corresponding to standards, were scraped from the plate and counted by liquid scintillation. Radioactive measurements were converted to picomoles of product by using the specific activity of substrate. Blank values obtained from controls lacking cell proteins were subtracted from the experimental values. PC-PLC or PI-PLC activities were expressed as picomoles DAG produced/10^6 cells.

CPP32 activity assay. The CPP32/Caspase-3 protease activity was assayed by using the ApoAlert CPP32 Caspase-3 Colorimetric Assay Kit (Clontech Laboratories, Inc, Palo Alto, CA), based on spectrophotometric detection at 405 nm of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate Aasp-Glu-Val-Asp (DEVD)-pNA. The units of protease activity can be quantitated using a standard curve established with the chromogenic molecule. Briefly, the cells (2 × 10^6/mL), after treatment with Dex (10^{-7} mol/L) for 8 hours, in the presence or absence of inhibitors, were collected and resuspended in a lysis buffer. Cellular lysates were then incubated in a microplate in the presence of conjugated protease substrate for 1 hour at 37°C and then analyzed using a 96-well plate reader. To verify that the signal detected was attributable to protease activity, the effect of the irreversible CPP32 inhibitor DEVD-FMK on the Dex-induced caspase activity was also tested. Thus, the cell lysates from Dex-induced samples were treated with CPP32 inhibitor before incubation with the substrate, according to the manufacturer’s instructions. Caspase activity is expressed as nanomoles pNA/10^6 cells.

Statistical analysis. For in vitro data analysis, the Student’s t-test was performed by the STATPAC Computerized Program (STATPAC Inc, Minneapolis, MN), and a P value less than .05 was used as the significance criterion.

RESULTS

Dex treatment induces thymocyte apoptosis and ceramide generation. Dex treatment caused a dose-dependent increase in the concentration of endogenous ceramide, which correlated well with the dose-related Dex-induced apoptosis showed in Fig 1A. In Figs 1B and C the results are reported showing ceramide generation from thymocytes incubated for 15 minutes in the presence of various concentrations of Dex (ranging from 10^{-7} to 10^{-12} mol/L), obtained by treating lipid extracts with DAG kinase for quantitation of ceramide-1-phosphate amounts. A basal level of ceramide was evident in the untreated control and further significantly increased after Dex treatment (P < .001, 10^-7, 10^-8, and 10^-10 mol/L compared with untreated control). Moreover, the dose able to induce maximal ceramide generation was 10^{-7} mol/L and corresponded to Dex concentration able to induce maximal levels of apoptosis in these cells (Fig 1A). As for apoptosis, levels of ceramide declined almost to the basal level at the Dex concentrations of 10^{-12} mol/L, being not significantly different from control value (P = .33).

The kinetics of ceramide induction after Dex treatment were also evaluated. Thymus cells were treated with Dex for different times, and lipid extracts from cell suspensions were prepared and subjected to ceramide level determination through DAG kinase assay. Figure 2A shows that Dex caused a rapid ceramide level increase, which was evident within 5 minutes, reached a maximum at 15 minutes, and declined after 2 hours of treatment. Values relative to Dex-treated samples were significantly different from corresponding control values at all the analyzed times (P < .01).

In Fig 2B, the results are reported of a representative experiment showing that thymocytes undergo apoptotic death on exposure to cell-permeant synthetic C2-ceramide (10 µmol/L) for 18 hours. Similar results were obtained with thymocytes exposed to synthetic ceramide (10 µmol/L) for 1 hour, extensively washed, and cultured for 17 additional hours in fresh medium. In this experiment, the percentage of apoptosis was as follows: untreated controls, 17%; ceramide treated, 65%. By contrast, the structural analogue C2-dihydroceramide (10 µmol/L) was totally ineffective, thus supporting the stereospecificity of ceramide (not shown, Obeid et al^30). Moreover, apoptosis induced by exogenous ceramide was inhibited by treatment with mRNA (actinomycin-D) and protein synthesis (cycloheximide) inhibitors (Fig 2B).

Dex induces activation of both acidic and neutral SMases. The contribution of acidic and neutral SMase to Dex-induced ceramide generation in thymocytes was investigated. The two classes of SMases can be defined based on in vitro pH optima: aSMase requires pH of 4.5 to 5.5 and DAG for activation, whereas nSMase prefers pH of 7.0 to 7.5.50-55 In addition, aSMase, consistent with its subcellular localization in vivo, is particularly resistant to the action of proteases and phosphatases, unlike nSMase, which is in turn associated with the outer cell membrane or within the cytosol.53-55 nSMase does not require DAG for activation and may require Mg^2+. Moreover, the residual aSMase activity at neutral pH was totally abolished in the presence of ATP and β-glycerophosphate. Thus, the analysis of SMases in different buffers (see Materials and Methods) allows to discriminate between aSMase and nSMase activity. Cellular extracts from mouse thymocytes treated for different times (5 to 180 minutes) with Dex 10^{-7} mol/L were incubated with radiolabeled SM vesicles to detect aSMase or nSMase activity. Results of a representative experiment presented in Fig 2C show that both aSMase and nSMase activities could be detected in extracts from untreated cells and significantly increased after Dex treatment. In particular, the Dex-
induced augmentation of aSMase activity was indeed evident and statistically significant \((P < .001)\) as early as 5 minutes from Dex addition, was maximal at about 15 to 30 minutes, and returned to the basal level within 180 minutes. Augmentation of nSMase activity was detectable and statistically significant \((P, .02)\) at 30 minutes, reached a peak at 120 minutes after treatment, and then decreased.

Taken together, these data indicate that Dex treatment induces two different temporal waves of SM hydrolysis and consequent ceramide generation through the sequential activation of aSMase and nSMase.

Ceramide synthase was not influenced by Dex treatment. The possibility that Dex-induced ceramide level increase in thymocytes could also be caused by the activation of ceramide synthase was assessed. This enzyme, also known as sphinganine N-acyl transferase,\(^6\) catalyzes the condensation of sphinga-
nine and fatty acyl-coenzyme A to form dihydroceramide, which is rapidly oxidized to ceramide. This pathway seemed to be slightly activated in control thymocytes (data not shown). This constitutive activity (about 5 to 8 pmol ceramide/10^6 cells) did not change when cells were incubated for 180 minutes and could account, together with constitutive SMase activity (Fig 2C), for the basal ceramide generation observed in these cells. On the other hand, Dex treatment did not significantly change the ceramide synthase activity in our experimental conditions and during the analyzed time interval (5 to 180 minutes; not shown), thus indicating that the above described Dex-induced ceramide increase could not be attributable to a stimulation of this pathway.

Role of acidic compartment in Dex-induced ceramide generation and apoptosis. Because of the lack of a specific inhibitor, we used some agents able to indirectly inhibit aSMase through different mechanisms. In fact, to evaluate the possible contribution of aSMase and nSMase to Dex-induced apoptosis, thymocytes were treated for different times with Dex 10^{-7} mol/L in the presence of three different endolysosomotropic agents such as monensin (10 µmol/L), NH_4Cl (8 mmol/L), or bafilomycin A1 (1 µmol/L), able to raise, through different

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Fig 2. (A) Kinetics of ceramide generation induced by Dex treatment. Thymocytes were treated with Dex (10^{-7} mol/L) for different times. Lipids were then extracted, subjected to DAG kinase assay, and separated by TLC. Radioactive spots were visualized by autoradiography, scraped from the plate, and counted by scintillation counting. On the left, the quantitative results for ceramide-1-phosphate levels, expressed as picomoles/10^6 cells are reported (mean values of three different experiments in duplicate; standard deviations, less than 10% of the mean values, are omitted for clarity). On the right side, a representative autoradiography of a ceramide chromatogram is shown. (B) C2-ceramide-induced apoptosis. Thymocytes were incubated for 18 hours with 10^{-7} mol/L C2-ceramide in the presence or absence of actinomycin-D (Act-D; 2.5 µg/mL) and cycloheximide (CHX; 50 µg/mL) and analyzed for apoptosis. Percentage numbers of hypodiploid nuclei are reported for each condition. Data shown are representative of one out of two experiments. (C) Dex-induced SMase activation. Acidic (on the left) and neutral (on the right) SMase activity induced by Dex treatment at the indicated times. Hydrolyzed SM was quantitated and expressed as picomoles/10^6 cells. The mean values from two determinations are reported. Standard deviation values were lower than 3% of mean values. The results are representative of one out of three separate experiments.
mechanisms, the pH to neutrality in endolysosomal compartments, thus impeding the activation of lysosomal enzymes, including aSMase. As expected, pretreatment with monensin, a Na$^+$ ionophore able to form stable complexes with monovalent cations, prevented the Dex-induced activation of aSMase (Fig 3A, left panel) without affecting nSMase (Fig 3A, right panel), as assayed in vitro on radiolabeled SM vesicles at 15 and 120 minutes, respectively. Moreover, monensin treatment was able to inhibit the early (5 to 15 minutes) ceramide generation caused by the activity of the aSMase, but did not affect the ceramide level increase observed later (120 minutes) and caused by the activity of nSMase (Fig 3B). Similar results (not shown) were obtained with NH$_4$Cl, a weak base that increases vacuolar pH, and with the highly specific inhibitor of the H$^+$-ATPase pump, able to alkalinize lysosomes and late endosomes, bafilomycin A1 (1 µmol/L). Moreover, monensin, as well as bafilomycin A1, showed a strong protective effect on Dex-induced apoptosis, being able to totally abrogate thymocyte death (Fig 3C). Similar results were obtained with NH$_4$Cl (not shown).

These findings suggest that Dex-induced thymocyte death is caused by aSMase activation responsible for the early ceramide generation.

Dex-induced activation of aSMase is dependent on PI-PLC activity. It has been previously reported that aSMase, associated with the lysosomal intracellular compartment or the caveolae, requires DAG for activation. The possible effect of Dex on the activity of DAG-generating enzymes, such as PI-PLC or PC-PLC, was directly addressed by evaluating the enzymatic activities in vitro using appropriate radiolabeled substrates and TLC analysis of the reaction products. In Fig 4, results are reported showing that Dex induced PI-PLC activity

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**Fig 3. Effect of monensin on Dex-induced ceramide generation, aSMase and nSMase, and apoptosis.** Thymocytes were treated with Dex (10$^{-7}$ mol/L) for the indicated times in the presence or absence of monensin (10 µg/mL). (A) Effect of monensin on acidic (on the left) and neutral (on the right) SMase activity induced by Dex treatment for 15 or 120 minutes, respectively. Hydrolyzed SM was quantitated and expressed as picomoles/10$^6$ cells. Mean values of three different experiments in duplicate are reported. Standard deviations, less than 5% of the mean values, are omitted for clarity. (B) Ceramide levels in thymocytes treated for 15 minutes with Dex (10$^{-7}$ mol/L) in the presence or absence of monensin. The quantitative results for ceramide-1-phosphate levels, expressed as picomoles/10$^6$ cells, are reported (mean values of three different experiments in duplicate; standard deviations, less than 10% of the mean values, are omitted for clarity). (C) Effect of monensin or bafilomycin A1 (1 µmol/L) on Dex-induced thymocyte apoptosis at 18 hours. Percentage numbers of hypodiploid nuclei are reported for each condition. The results are representative of one out of three separate experiments.
in mouse thymocytes. In particular, augmentation of PI-PLC activity was evident and statistically significant ($P < 0.001$) at 1 minute, peaked at 5 minutes, and then declined within 15 minutes after Dex treatment (Fig 4A, left panel). Furthermore, Dex-induced PI-PLC activation could be totally blocked by pretreatment of cells with U73122, a selective PLC$\beta$ inhibitor able to interfere with the G-protein-PLC coupling. Figure 4A (right panel) shows the results of a representative TLC autoradiography relative to the U73122-inhibitory effect of Dex-induced DAG generation at 5 minutes. No induction of PC-PLC activity could be detected in extracts from thymocytes treated with Dex (not shown).

PI-PLC seemed to be crucial for Dex-induced aSMase activation. Indeed, the pretreatment of cells with U73122 totally blocked Dex-induced aSMase activation assayed at 15 minutes (Fig 4B, left panel), without affecting the nSMase assayed at 120 minutes (Fig 4B, right panel), suggesting that PI-PLC and aSMase activation are two sequentially related steps of the same pathway. In accordance, ceramide generation was strongly compromised in Dex-treated cells cultured in the presence of

Fig 4. PI-PLC activity involvement in Dex-induced thymocyte apoptosis. Effect of U73122 on (A) Dex-induced PI-PLC activity, (B) aSMase (left) and nSMase (right), (C) ceramide generation, and (D) apoptosis. Thymocytes were treated with Dex ($10^{-7}$ mol/L) for the indicated times in the presence or absence of U73122 ($2.5 \mu$mol/L) or D609 (50 $\mu$g/mL). (A) Dex-induced PI-PLC activity. Cell extracts were reacted with radiolabeled PI vesicles, and then DAG released was separated by TLC, visualized by autoradiography, scraped from the plate, and counted by scintillation counting. PI-PLC activity is expressed as picomoles DAG/10^6 cells (left side). Mean values of three different experiments in duplicate are reported. Standard deviations (SD), less than 3% of the mean values, are omitted for clarity. On the right side, a representative autoradiography of a DAG chromatogram is shown. (B) Acidic (on the left) and neutral (on the right) SMase activity induced after 15 or 120 minutes of Dex treatment, respectively. Hydrolyzed SM was quantitated and expressed as picomoles/10^6 cells. Mean values of three different experiments in duplicate are reported. SD, less than 5% of the mean values, are omitted for clarity. (C) Ceramide levels in thymocytes treated for 15 minutes with Dex ($10^{-7}$ mol/L) in the presence or absence of U73122. The quantitative results for ceramide-1-phosphate levels, expressed as picomoles/10^6 cells, are reported (mean values from three experiments in duplicate; SD values, less than 10% of mean values, are omitted for clarity). (D) Effect of U73122 and D609 on Dex-induced thymocyte apoptosis, as detected after an 18-hour culture. Percentage numbers of hypodiploid nuclei are reported for each condition. The results are representative of one out of three separate experiments.
U73122 (Fig 4C) as a result of inhibition of the early ceramide peak (15 minutes) attributable to aSMase. Of note, the presence of U73122 in Dex-treated cells totally prevented steroid-induced apoptosis, whereas D609, a PC-PLC inhibitor, did not (Fig 4D).

These results indicate that Dex treatment activates a G-protein-dependent PI-PLC, but not PC-PLC. PI-PLC activation leads to a sequence of events including DAG release, aSMase activation, ceramide generation, and apoptosis.

**Dex-induced DAG and ceramide generation is countered by PKC inhibitors H7 and calphostin-C.** Dex-induced apoptosis is also dependent on PKC activity, and previous studies indicated that PKC inhibitors, including the broad serine/threonine kinase inhibitor H-7 and the more specific calphostin-C, completely counter thymocyte apoptosis. To evaluate the role of PKC activity in the Dex-induced thymocyte apoptosis, we tested the possible effect of H7 and calphostin-C on DAG and ceramide generation. Results indicate that both H7 and calphostin-C, at concentrations able to inhibit Dex-induced apoptosis (Fig 5A), also prevented DAG (Fig 5B) and ceramide generation (Fig 5C). These results suggest that Dex treatment induces PKC activity that is then involved in the apoptotic transduction pathway upstream PI-PLC activation.

**Dex-induced proteases activity is dependent on the PI-PLC/aSMase pathway.** It has been previously suggested that several interleukin-1β-converting enzyme (ICE)-family cysteine proteases (caspases) play a prominent role in GCH-induced thymocyte apoptosis. To further evaluate the caspase role in the Dex-induced thymocyte apoptosis, we tested the possible effect of Z-VAD-FMK, a highly specific cell-permeable, irrevers-

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![Graph A](image1)

![Graph B](image2)

![Graph C](image3)
ible inhibitor of caspases including caspase-1, also able to inhibit the activation of ProCPP32 to its active form. Results indicate that Z-VAD-FMK (50 µmol/L) totally inhibited Dex-induced thymocyte apoptosis (Fig 6A), whereas ceramide generation was not influenced (data not shown).

We also performed experiments to evaluate the possible role of PI-PLC/aSMase pathway in protease activation. For this purpose, thymocytes were treated for 8 hours at 37°C with Dex (10^{-7} mmol/L) in the presence or absence of U73122 (2.5 µmol/L) or bafilomycin A1 (1 µmol/L), and then CPP32/caspase 3 activity was evaluated.

In vitro experiments showed that Dex significantly (P < .002) augmented the basal CPP32 activity, expressed as nanomoles pNA/10^6 cells, and that the PI-PLC inhibitor U73122 and the aSMase inhibitor bafilomycin A1 inhibited this augmented CPP32/caspase 3 activity (Fig 6B). The inhibition of CPP32 activity observed in the presence of the irreversible CPP32 inhibitor DEVD-FMK allowed to verify the assay specificity.

These results indicate the Dex-induced caspase activation, required for apoptosis, is downstream and dependent on the PI-PLC/aSMase pathway.

Dex-induced ceramide generation and caspase activation are dependent on binding to GR. It has previously been shown that Dex-induced apoptosis is dependent on GR binding and is inhibited by the receptor antagonist RU486.39,61 We performed experiments to evaluate the effect of RU486 on ceramide generation and apoptosis. Figure 7A shows the results of a representative experiment confirming that RU486 inhibits Dex-induced apoptosis. Furthermore, Dex-induced aSMase (Fig 7B, left panel), nSMase (Fig 7B, right panel), and PI-PLC (Fig 7C) activation as abrogated in the presence of RU486. As expected, RU486 also prevented ceramide generation (not shown) and CPP32 activity (Fig 6B) induced by Dex, indicating that signals responsible for ceramide generation and caspase activation require the Dex/GR interaction.

Dex-induced apoptosis, but not DAG and ceramide generation and caspase activation, is inhibited by mRNA and protein synthesis inhibitors. It has been previously reported that Dex-induced apoptosis is countered by mRNA and protein synthesis inhibitors.5,62 We performed experiments to evaluate the possible effect of mRNA and protein synthesis inhibitors on ceramide generation and CPP32 activity. For that purpose, thymocytes were treated with Dex, Dex plus actinomycin-D, or Dex plus cycloheximide. Results of a representative experiment indicate that both actinomycin-D and cycloheximide inhibited the Dex-induced apoptosis (Fig 8A), but not the Dex-induced DAG and ceramide generation (Fig 8B) and induction of CPP32 activity (Fig 8C). These data confirm previously reported results suggesting that transcription and protein synthesis are required for Dex-induced apoptosis and also show these events are downstream in the pathway after DAG and ceramide generation and caspase activation.

**DISCUSSION**

In the present study, we show results indicating that an early generation of ceramide, caused by the sequential activation of
PI-PLC and aSMase, is required for Dex-induced thymocyte apoptosis. In particular, both induction of ceramide and cell death are dose-dependent with doses ranging between $10^{-7}$ mol/L and $10^{-12}$ mol/L, being evident also at low Dex concentrations ($10^{-10}$ mol/L, Fig 1). Moreover, thymocytes are sensitive to the apoptosis induction by soluble cell permeant ceramide (Fig 2B). As for most of the GCH-mediated effects, treatment with the GR antagonist RU486 results in the complete inhibition of Dex-induced ceramide generation and apoptosis (Fig 7). Dex-induced ceramide generation is caused by SMase activation. Interestingly enough, both aSMase and nSMase, respectively, are activated, although at different times, within 5 minutes and 30 minutes after Dex treatment (Fig 2). However, the inhibition of aSMase activity through agents (monensin, NH4Cl, or bafilomycin A1) able to cause, by different mechanisms, alkalinization of endolysosomal compartment, counteracts Dex-induced apoptosis (Fig 3). These results indicate that the early ceramide generated by aSMase (5 minutes) is necessary and sufficient to signal Dex-induced cell death.

Our findings further indicate that nSMase and aSMase are independently activated by Dex treatment and that the ceramide generated from the action of each SMase may be involved in separate and distinct signaling events, as previously suggested (as a review, see Riboni et al). The different contribution of aSMase and nSMase in apoptosis induction could thus be attributed to the distinct cellular compartmentalization of these enzymes. Ceramide generated from nSMase seems to remain associated to or near the plasma membrane facilitating the targeting plasma membrane–associated proteins, whereas the ceramide generated through the action of the aSMase is associated with an endosomal/lysosomal compartment.

Experiments performed to analyze the possible mechanisms involved in Dex-induced aSMase activation, indicate that Dex
treatment rapidly induces DAG generation (Fig 4A), an event which precedes and is required for aSMase activation.\textsuperscript{50-52} Dex-induced DAG generation is caused by PI-PLC, but not by PC-PLC activation. This Dex-induced PI-PLC is prevented by U73122 (Fig 4), a G-protein–PLC coupling inhibitor,\textsuperscript{40} and by PKC inhibitors (Fig 5), thus suggesting that Dex may act by inducing the activation of PLC\textsubscript{β}, an enzyme regulated by α subunits of G proteins (G\textsubscript{q} family) and phosphorylation.\textsuperscript{63-65} Interestingly, PI-PLC inhibition through U73122 also totally prevented Dex-induced aSMase activity, ceramide generation, and apoptosis (Figs 4B, C, and D). Our preliminary data suggest that a pertussis-toxin insensitive G-protein, activated by Dex, is responsible for PLC\textsubscript{β} activation. Indeed, pertussis-toxin treatment of thymocytes did not influence either Dex-induced PLC\textsubscript{β} activity or apoptosis (unpublished results). Of note, GCH have in the past been shown to regulate G-protein level and activity in different cell systems, thus modulating several enzymes.\textsuperscript{56,67} In particular, GCH-induced G\textsubscript{q}-11-protein regulation modulates PLC signal transduction.\textsuperscript{68} Similar mechanisms could be involved in the Dex-induced activation of thymocyte death. Studies are in progress to further analyze the mechanisms underlying the proposed Dex-induced G-protein activation potentially responsible for PI-PLC activity.

Activation of proteases has been postulated as a general feature of apoptosis.\textsuperscript{69,70} Many proteins have been reported to be cleaved during apoptosis, including fodrin, terminin, the protein component of the U1 small nuclear ribonucleoprotein, and poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP).\textsuperscript{71} Ceramide has recently been shown to induce cleavage of PARP, a nuclear enzyme that converts nicotinamide adenine dinucleotide (NAD) to ADP-ribose polymers.\textsuperscript{72} At the onset of apoptosis, PARP is cleaved to an 85-kD apoptosis-specific fragment in different cells, including thymocytes. The protease pro-ICE (YAMA/CPP32b/apopain), a mammalian
homolog of the Caenorhabditis elegans death gene ced-3, has been identified as the protease that cleaves PARP during apoptosis. The finding that ceramide is able to induce PARP cleavage provides the first evidence of the signal transduction pathway leading to activation of the ICE-like proteases.

The role of ICE-like proteases as mediators of glucocorticoid-induced apoptosis, even if currently receiving great attention, has not been completely clarified (as a review, see Distelhorst77). A specific involvement of CPP32 has been recently proposed in Dex-induced thymocyte apoptosis. In our hands, horst77). A specific involvement of CPP32 has been recently proposed in Dex-induced thymocyte apoptosis. In our experiments, treatment with actinomycin-D or cycloheximide, although it completely inhibited Dex-induced thymocyte apoptosis, did not influence the signaling pathway including DAG release, ceramide generation, and caspase activation (Fig 8), thus suggesting that all these events precede the Dex-induced transcription regulation required for thymocyte apoptosis. Moreover, as shown in Fig 2B, apoptosis induced by exogenous ceramide is inhibited by mRNA and protein synthesis inhibition, further suggesting that transcription is a downstream event required for Dex-induced apoptosis. Interestingly enough, these observations indicate that both Dex and ceramide, which is also generated after Dex treatment, induce thymocyte apoptosis in a transcription/translation-dependent manner. However, whether ceramide pathway affects the Dex/GR-mediated transcription is a matter of debate and requires further studies.

In conclusion, although the role of transcription in Dex-induced apoptosis remains to be clarified, future studies will be addressed to analyze the molecular mechanisms linking the signaling pathway described here to transcription regulation.

The knowledge of the molecular events involved in the GCH-mediated apoptosis in thymocytes and T lymphocytes is important for the understanding of mechanisms contributing to the immune response regulation. Moreover, these observations can be informative for the development of new pharmacological approaches aimed to control neoplastic T-cell growth.

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Either transcription-dependent or transcription-independent GCH-induced apoptosis has been previously described. However, mRNA and/or protein synthesis inhibitors counteract thymocyte apoptosis, suggesting that transcription events, consequent to Dex/GR interaction, are required for apoptosis induction.

In our experiments, treatment with actinomycin-D or cycloheximide, although it completely inhibited Dex-induced thymocyte apoptosis, did not influence the signaling pathway including DAG release, ceramide generation, and caspase activation (Fig 8), thus suggesting that all these events precede the Dex-induced transcription regulation required for thymocyte apoptosis. Moreover, as shown in Fig 2B, apoptosis induced by exogenous ceramide is inhibited by mRNA and protein synthesis inhibition, further suggesting that transcription is a downstream event required for Dex-induced apoptosis. Interestingly enough, these observations indicate that both Dex and ceramide, which is also generated after Dex treatment, induce thymocyte apoptosis in a transcription/translation-dependent manner. However, whether ceramide pathway affects the Dex/GR-mediated transcription is a matter of debate and requires further studies.
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Dexamethasone-Induced Thymocyte Apoptosis: Apoptotic Signal Involves the Sequential Activation of Phosphoinositide-Specific Phospholipase C, Acidic Sphingomyelinase, and Caspases

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