A Requirement for Protein Kinase C Inhibition for Calcium-triggered Apoptosis in Acute Lymphoblastic Leukemia Cells[1]

De-Min Zhu, Wei-Hua Fang, Rama Krishna Narla, and Fatih M. Uckun[2]

Departments of Immunology [D-M. Z., W-H. F.], Experimental Oncology [R. K. N., F. M. U.], and Drug Discovery Program [F. M. U.], Hughes Institute, St. Paul, Minnesota 55113

ABSTRACT

We have evaluated the cytotoxicities of the combinations of calcium mobilizers and PKC inhibitors against human acute lymphoblastic leukemia (ALL) cells. Here we report that calcium mobilizers alone or PKC inhibitors alone do not induce apoptosis in human ALL cells. However, the combinations of calcium mobilizers with potent inhibitors of PKC cause significant apoptosis in ALL cells. Our results provide experimental evidence that PKC blocks Ca^{2+}-triggered apoptosis in human ALL cells. Thus, PKC inhibitors can be used to enhance the antileukemic activity of chemical or biological agents that trigger an apoptotic calcium signal in ALL cells. The exquisite sensitivity of ALL cells to calcium-dependent apoptosis in the presence of PKC inhibitors could provide the basis for new treatment programs against ALL.

INTRODUCTION

The identification and development of new potent anti-ALL[3] drugs are focal points in translational leukemia research. We have reported recently that the dual-function calcium mobilizer calphostin C (C44H38014, UCN-1028), a naturally occurring lipophilic perylenequinone antibiotic from the wood fungus *Cladosporium cladosporiodes* FERM BP-1285 with potent PKC inhibitory activity, induced apoptosis in human ALL cells, whereas other calcium mobilizers did not (1). In biochemical studies, calphostin C was discovered to induce rapid calcium mobilization from intracellular stores of ALL cells, and its cytotoxicity against ALL cells was correlated with the magnitude of this calcium signal. Calphostin C-induced apoptosis was markedly suppressed by bis-(O-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl)-ester, a cell-permeable Ca^{2+} chelator as well as $NiCl_2$, an inhibitor of Ca^{2+}/Mg^{2+}-dependent endonucleases (1). Inhibition of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin with perfluoreperazine dimadeate, a calmodulin antagonist, or cyclosporin A, a specific inhibitor of calcineurin, also reduced the magnitude of calphostin C-induced apoptosis in leukemia cells. Taken together, these results provided evidence that calphostin C triggers a Ca^{2+}-dependent apoptotic signal in human ALL cells (1). The antileukemic potency of the dual function calcium mobilizer/PKC inhibitor calphostin C, taken together with the inability of other calcium mobilizers to induce apoptosis in human ALL cells, prompted the hypothesis that the PKC inhibitory function of calphostin C also contributes to its Ca^{2+}-dependent antileukemic activity.

The purpose of the present study was to examine the ability of calcium mobilizers and PKC inhibitors to induce apoptosis in human ALL cells. Here we report that calcium mobilizers alone or PKC inhibitors alone do not induce apoptosis in human ALL cells. However, the combination of calcium mobilizers with a potent PKC inhibitor caused significant apoptosis in NALM-6 cells. Thus, Ca^{2+} mobilizers can trigger apoptosis in ALL cells only when PKC is inhibited. These results provide experimental evidence that PKC has a protective function in ALL cells and blocks Ca^{2+}-triggered apoptosis. PKC inhibitors can be used to enhance the antileukemic activity of chemical or biological agents that trigger an apoptotic calcium signal in ALL cells.

MATERIALS AND METHODS

Cell Lines. NALM-6 human pre-B ALL cells were maintained in tissue culture at 37°C in a humidified 5% CO_2 atmosphere using RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Summit Biotech, Ft. Collins, CO), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.).

Reagents. Thapsigargin, ionomycin, phorbol 12-myristate 13-acetate, and PKC inhibitors, including Gö 6976 and Gö 6983, were purchased from Calbiochem (San Diego, CA). B43, a monoclonal anti-human CD19 antibody, was produced as described previously (2).

Cell Viability Assay. The viability of ALL cells treated with calcium mobilizers and PKC inhibitors was evaluated using the mixture of calcien/AM and ethidium homodimer (3) from Molecular Probes, Inc. (Eugene, OR), according to the manufacturer's recommendations. Calcien/AM is a cell membrane permeable ester form of calcien. The hydrolysis of calcien/AM by cytosolic esterase in the cells releases free calcien molecules, which accumulate intracellularly and emit green fluorescence at 488 nm excitation. Thus, green fluorescence is an indicator of living cells characterized by esterase activity as well as an intact membrane to retain the esterase product. Ethidium homodimer is a high affinity, red fluorescent nucleic

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[2] To whom requests for reprints should be addressed, at Hughes Institute, 2665 Long Lake Road, Suite 330, St. Paul, MN 55113. Phone: (612) 697-9228; Fax: (612) 697-1042.

[3] The abbreviations used are: ALL, acute lymphoblastic leukemia; PKC, protein kinase C; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end labeling.

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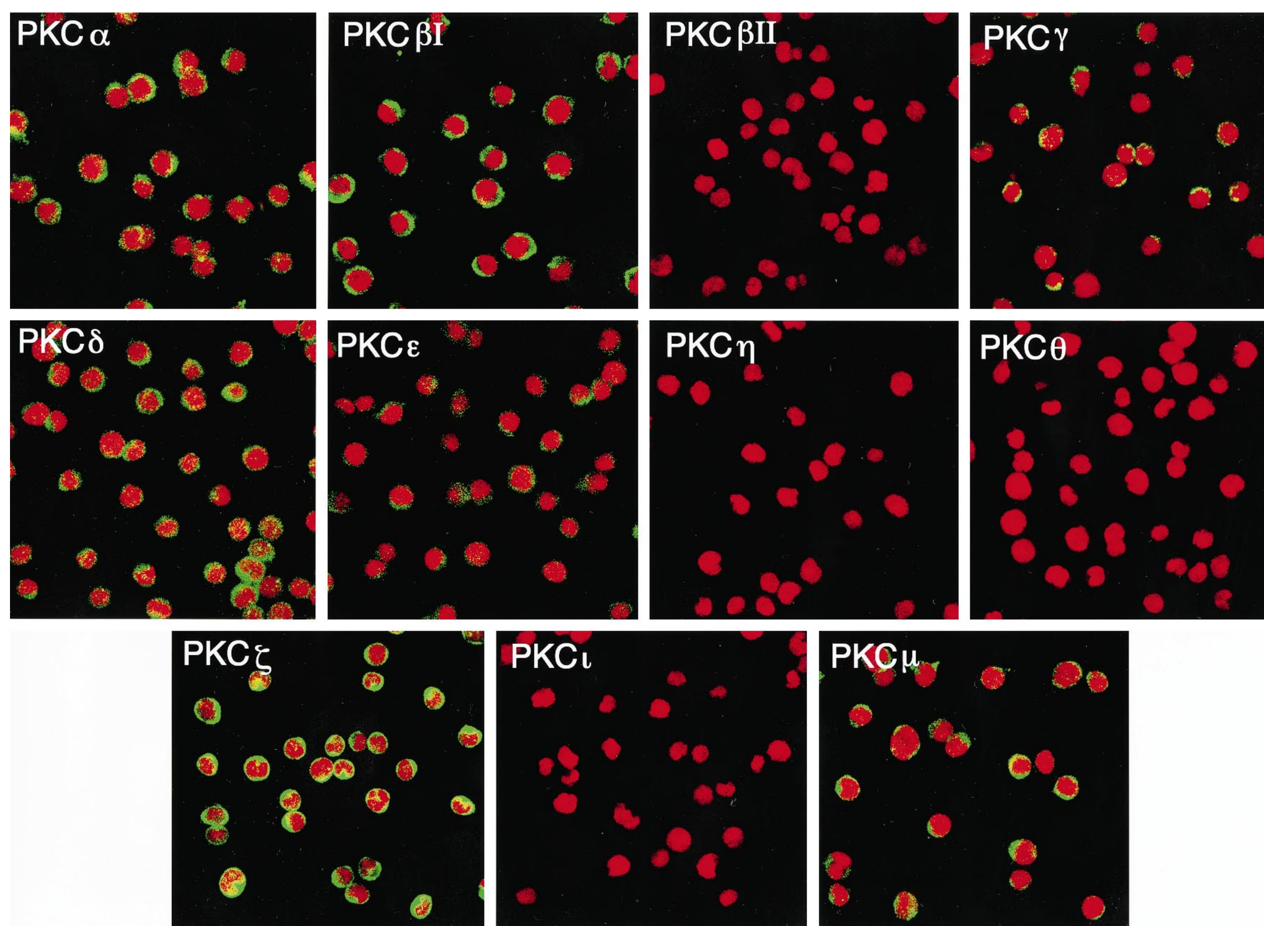

Fig. 1 PKC isoform expression of NALM-6 Cells. NALM-6 cells were stained by immunofluorescent staining techniques with various isoform-specific anti-PKC antibodies and then imaged using a confocal microscope, as described in "Materials and Methods."

acid stain. It is only able to pass through the compromised membranes of dead cells. The molecules of ethidium homodimer bound to nuclear DNA emit strong red fluorescence at 514 nm excitation. Therefore, the red fluorescence is an indicator of dead cells. In our experiments, ALL cells treated by the reagents were centrifuged at 850 × *g* for 3 min and resuspended in HEPES buffer [25 mM HEPES (pH 7.4), 121 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 5 mM D-glucose] containing 2 μM calcien/AM and 4 μM ethidium homodimer at a density of 5×10^6 cells/ml. After a 20-min incubation at room temperature, the viability was determined using a fluorescence microscope (Nikon ECLIPSE TE300). The percentage of cell death was calculated by the formula: % Cell Death = [number of dead cells/(number of dead cells + number of living cells)] × 100%. To get valid cell viability values, >500 cells from each sample were counted.

Apoptosis Analysis. The demonstration of apoptosis was performed by the TUNEL assay using the ApopTag *in situ* detection kit (Oncor, Gaithersburg, MD) according to the manufacturer's recommendations, as reported previously (4). In brief, exponentially growing ALL cells were treated by the reagents in a cell culture incubator for 24 h. The cells were then centrifuged at 850 × *g* for 5 min. Cells were resuspended in 50 μl of PBS, transferred to poly-L-lysine-coated coverslips, and allowed to attach for 15 min. The cells were washed once with PBS and were fixed with 4% paraformadehyde in PBS for 10 min. The cells were washed again with PBS and incubated for 1 h at 37°C with the reaction mixture containing TdT and FITC-conjugated digoxigenin-11-UTP for labeling of exposed 3′-hydroxyl ends of fragmented nuclear DNA according to the manufacturer's instructions. After washing the cells with PBS, the coverslips were mounted onto slides with Vectashield containing propidium iodide (Vector Labs, Burlingame, CA) and viewed with a confocal laser scanning microscope (MRC 1024; Bio-Rad, Inc., Richmond, CA). Nonapoptotic cells do not incorporate significant amounts of dUTP because of the lack of exposed 3-hydroxyl ends and consequently have much less fluorescence than apoptotic cells, which have an abundance of exposed 3′-hydroxyl ends. In control reactions, the TdT enzyme was omitted from the reaction mixture.

PKC Profiling of Leukemia Cells. Immunofluorescence was used to examine the expression of various isoforms of PKC in Nalm 6 cells. The cells were plated on Superfrost Plus slides, and we allowed the cells to adhere for 15 min and then fixed them in methanol at −20°C for 10 min. The cells were washed with PBS and permeabilized, and nonspecific binding

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sites were blocked with 2.5% BSA in PBS containing 0.1% Triton X-100 for 30 min. To localize the PKC isoforms, we used anti-PKC α, βI, βII, δ, ϵ, η, γ, ι, θ, μ, and ζ antibodies. All antibodies were purchased from Transduction Labs except for anti-PKCβI (Calbiochem), anti-PKCβII (Calbiochem), and anti-PKCμ (Santa Cruz). The cells were incubated with anti-PKC antibodies at a dilution of 1:50 for 1 h at room temp. The cells were washed with PBS and then incubated with FITC-conjugated donkey anti-mouse or donkey anti-rabbit IgG (Amersham Corp., Arlington Heights, IL) for 1 h at room temp. Cells were washed in PBS and counterstained, and the coverslips were mounted with Vectashield containing propidium iodide (Vector Labs) and viewed with a laser scanning confocal microscope (MRC 1024; Bio-Rad) equipped with krypton/argon mixed gas laser mounted in a Nikon upright microscope (Nikon, Tokyo, Japan). Digital images were saved on a Jaz disc and processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA).

RESULTS AND DISCUSSION

We have used immunofluorescence and confocal laser scanning microscopy to determine the PKC isoform expression profile of NALM-6 human pre-B ALL cells. As shown in Fig. 1, NALM-6 cells expressed abundant levels of PKCα, PKCβ1, PKCγ, PKCμ, and PKCζ, as well as low levels of PKCδ and PKCϵ, but lacked the other isoforms. We hypothesized that the expressed PKC isoforms may have a protective survival-promoting function. In a systematic effort to test this hypothesis, we first examined the antileukemic activity of different PKC inhibitors and calcium mobilizers that cause cytosolic Ca^{2+} elevation as well as their combinations against NALM-6 cells. The staurosporin analogues Go 6983 (5, 6) and Go 6976 (7–9) were used as PKC inhibitors. The calcium mobilizers selected for our studies were ionomycin, a Ca^{2+} ion carrier (10–13), thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+} pump (Ca^{2+}-ATPase; Refs. 14–24), and B43, an anti-CD19 monoclonal antibody (2, 25, 26). As shown in Fig. 2*A,* neither the PKC inhibitors nor the calcium mobilizers exhibited significant cytotoxicity against NALM-6 cells. However, when combined with the PKC inhibitor Go 6976 (but not Go 6983), the calcium mobilizers were cytotoxic.

We next set out to determine whether the cytotoxicity of the calcium mobilizer/PKC inhibitor pairs was due to induction of apoptosis using TUNEL assays and confocal laser scanning microscopy. The results of the TUNEL assays are shown in Fig. 2*B*. Neither the calcium mobilizers nor the PKC inhibitors, when used alone, triggered apoptosis in NALM-6 cells. However, the combination of the calcium mobilizers with the PKC inhibitor Go 6976 (but not Go 6983) caused significant apoptosis in NALM-6 cells. Fig. 3 shows the confocal images of the apoptotic nuclei labeled by FITC-conjugated digoxigenin-11-UTP in the presence of TdT. These results support the hypothesis that PKC protects ALL cells from calcium-triggered apoptosis, and certain PKC inhibitors can be used to enhance the cytotoxicity of the drugs that trigger an apoptotic calcium signal.

As discussed above, PKC inhibitors Go 6976 and Go 6983 were not equivalent in their ability to promote the calcium-dependent apoptotic death of ALL cells. Go 6983 is a potent inhibitor of PKCα (IC_{50}, 7 nM), PKCβ (IC_{50}, 7 nM), PKCγ (IC_{50}, 6 nM), PKCδ (IC_{50}, 10 nM), and PKCζ (IC_{50}, 60 nM; Ref. 5). This inhibitor did not cause apoptosis in NALM-6 cells either alone or in combination with calcium mobilizers, indicat-

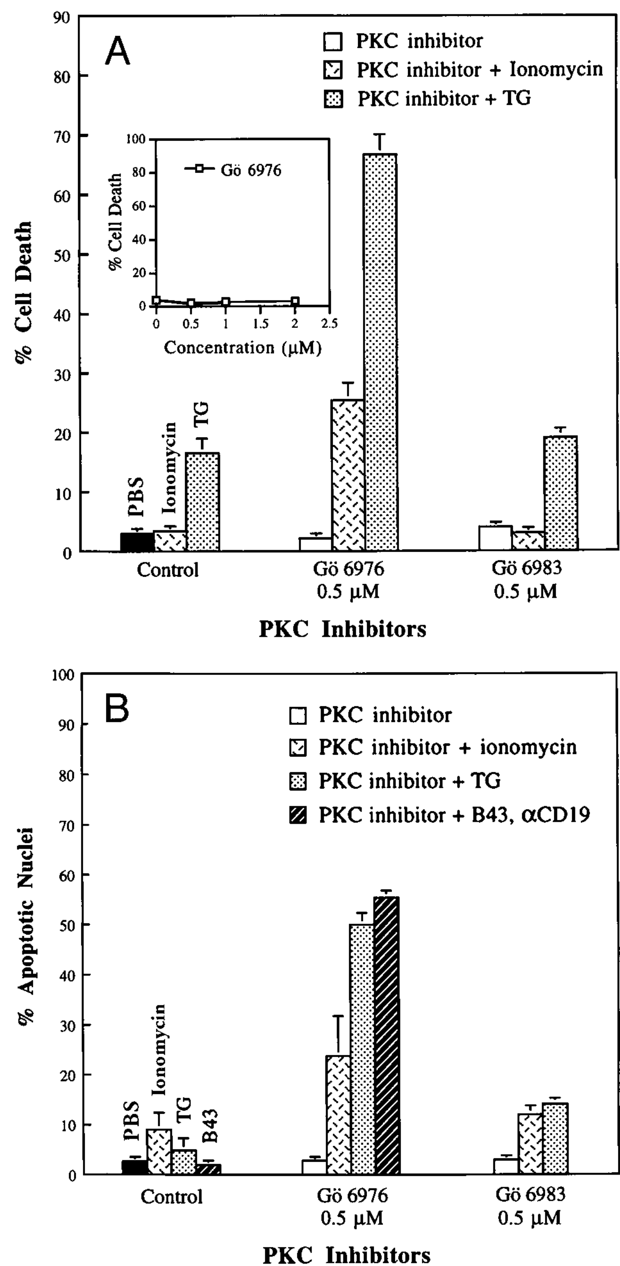

Fig. 2 *A,* cytotoxicity of the combinations of calcium mobilizers and PKC inhibitors against human ALL cells. NALM-6 cells were incubated with the reagents at the given concentrations for 24 h, and the percentage of cell death was determined by viability assays, as described in "Materials and Methods." *Inset,* cytotoxicity of PKC inhibitor Gö 6976. Each data point represents the percentage of dead cells (means) of three independent experiments; *bars,* SE. *B,* apoptosis in ALL cells induced by calcium mobilizers and PKC inhibitors. The percentage of apoptotic nuclei of NALM-6 cells were obtained from TUNEL assays. Each data point (means) was from three independent experiments; *bars,* SE.

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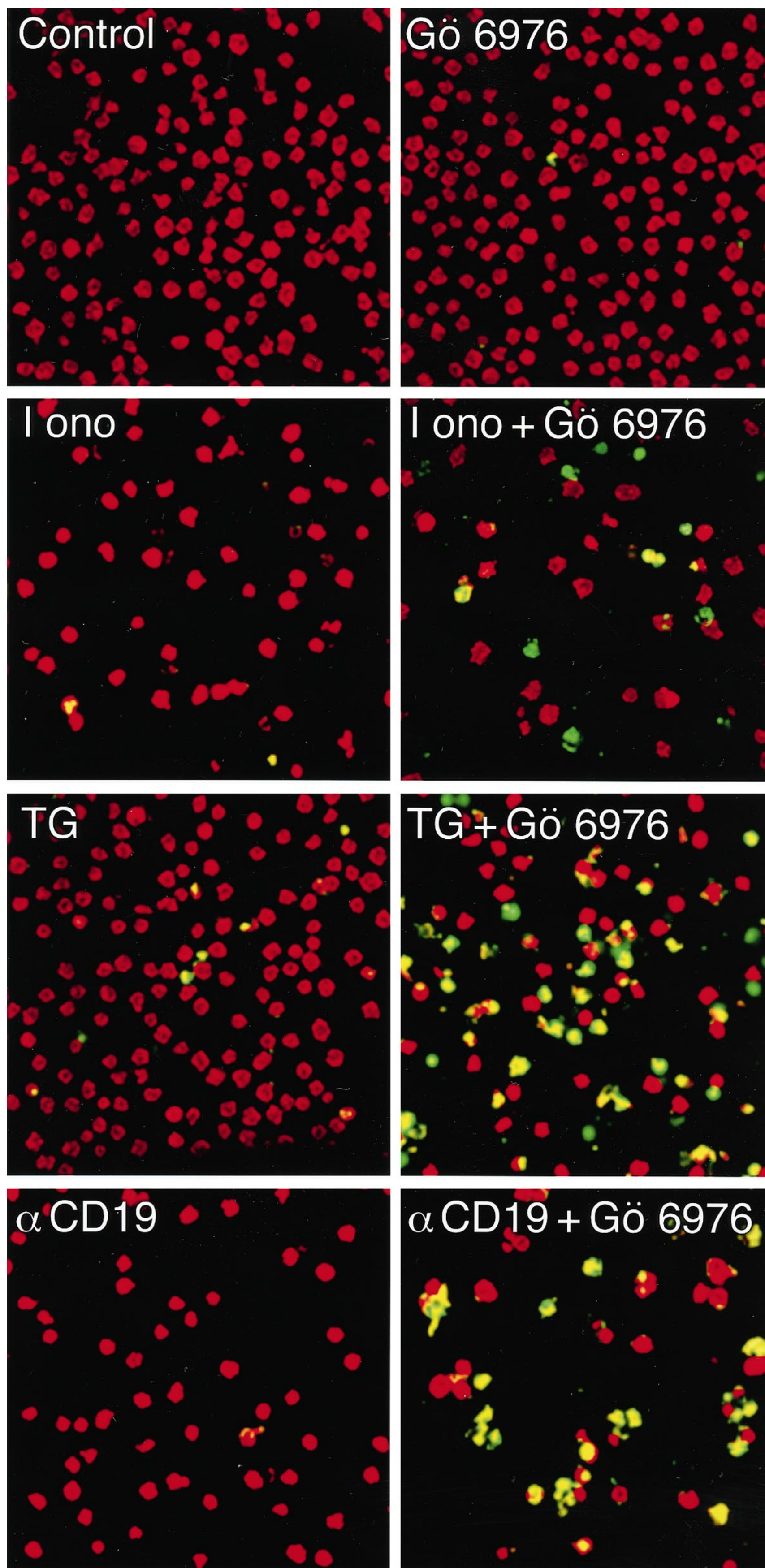

Fig. 3 Apoptosis in ALL cells induced by calcium mobilizer/PKC inhibitor pairs. Confocal images are of apoptotic nuclei. The reagents used were described in the image frames. NALM-6 cells were incubated with the reagents for 24 h and processed for the *in situ* apoptosis assay (TUNEL assays) as described in "Materials and Methods." The images were taken with a laser scanning confocal microscope. Red fluorescence represents nuclei that stained, and green fluorescence shows apoptotic nuclei. Note the increased numbers of apoptotic nuclei when calcium mobilizers were combined with Gö 6976.

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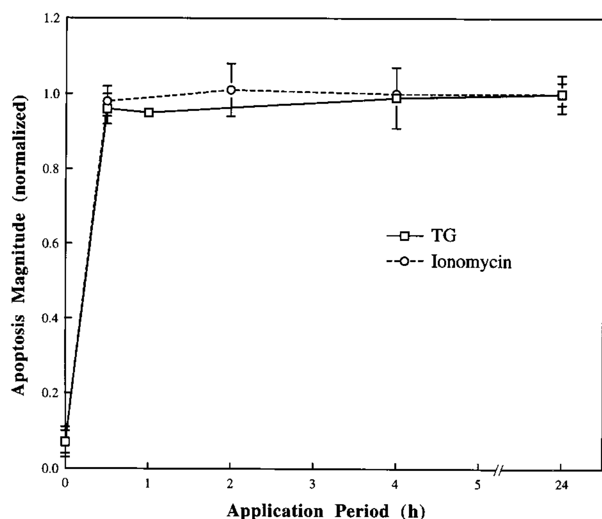

Fig. 4 Initiator role of calcium mobilizers in calcium mobilizer/PKC inhibitor-induced apoptosis. NALM-6 cells were first exposed to the mixtures of ionomycin (2 μM)/Gö 6976 (0.5 μM; ○) and thapsigargin (2 μM)/Gö 6976 (□). The cells were then washed twice at different incubation time points and resuspended in the medium containing Gö 6976 only. The total incubation time was 24 h. The apoptosis magnitude was analyzed by TUNEL assays and normalized against that the cells incubated with calcium mobilizer and Gö 6976 mixture for 24 h. The data at time zero represent the apoptosis magnitude in the absence of the calcium mobilizers. Each data point (means) was from three independent experiments; *bars,* SE.

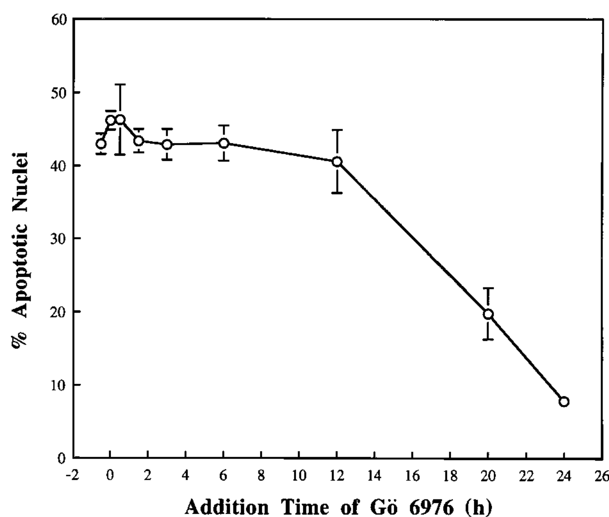

Fig. 5 Downstream enhancement of PKC inhibitors to calcium-triggered apoptosis. NALM-6 cells were exposed to 2 μM thapsigargin, and 0.5 μM Gö 6976 was then added to the cell suspensions at different time points. The negative time point indicates that Gö 6976 was incubated with the cells before thapsigargin. The incubation was terminated at 24 h, and apoptosis was analyzed by TUNEL assays. Each data point (means) was from three independent experiments; *bars,* SE.

ing that the PKC isoforms α, β, γ, δ, and ζ do not exert a significant antiapoptotic function in ALL cells. Therefore, the ability of Gö 6976 to promote calcium-dependent apoptosis in ALL cells cannot be explained by its effects on one or more of these PKC isoforms or PKCε, which it does not inhibit (7). The ability of Gö 6976, which inhibits PKCμ with a 1000-fold better IC_{50} than Gö 6983 (20 nM *versus* 20 μM), suggests that PKCμ may be the primary antiapoptotic PKC isoform in NALM-6 cells.

In the next series of experiments, NALM-6 cells were exposed to a mixture of the calcium mobilizers and Gö 6976 for 0.5, 1, 2, or 4 h; then the cells were washed and resuspended in medium containing only Gö 6976. As shown in Fig. 4, a 0.5-h exposure to calcium mobilizers was sufficient to induce apoptosis in NALM-6 cells. These results indicate that the Ca^{2+} signal is an initiator of apoptosis, and once initiated, the execution of apoptosis does not depend on the continued exposure to calcium-mobilizing agents. To test this hypothesis and further elucidate the role of PKC inhibition in calcium-induced apoptosis of human ALL cells, NALM-6 cells were treated with thapsigargin for calcium mobilization to trigger the apoptotic signal, and Gö 6976 was added to cell suspensions at different time points to eliminate the protective PKC function. The apoptotic events were analyzed by TUNEL assays. As shown in Fig. 5, when Gö 6976 was added to the cell suspensions either before or up to 12 h after the addition of the calcium mobilizer, the magnitude of apoptosis remained unchanged. In contrast, when Gö 6976 was added 20 h after the addition of the calcium mobilizer, apoptosis was reduced by >50%. These results demonstrate that PKC does not prevent the initiation of the apoptotic signal, which occurs within the first 12 h, but it blocks the downstream events of Ca^{2+}-triggered apoptosis.

In a recent study, we found that the naturally occurring perylenequinone antibiotic calphostin C with PKC inhibitory activity was capable of inducing calcium mobilization and apoptosis in human ALL cell lines as well as freshly obtained primary leukemic cells from children with ALL (1). Our results provided unprecedented evidence that calphostin C is a dual-function PKC inhibitor and triggers a Ca^{2+}-dependent apoptotic signal in human ALL cells. Here, we evaluated the ability of monofunction PKC inhibitors and calcium mobilizers to induce apoptosis in human ALL cells. We found that calcium mobilizers alone or PKC inhibitors alone do not induce apoptosis in human ALL cells. However, the combination of calcium mobilizers with a potent inhibitor of PKC caused significant apoptosis in ALL cells. Thus, Ca^{2+} mobilizers can trigger apoptosis in ALL cells only when PKC is inhibited. These results provide experimental evidence that certain PKC isozymes have a protective function in ALL cells and block Ca^{2+}-triggered apoptosis in ALL cells. Therefore, PKC inhibitors can be used to enhance the antileukemic activity of chemical or biological agents that trigger an apoptotic calcium signal in ALL cells. Alternatively, dual-function agents with calcium-mobilizing and PKC-inhibiting activities, such as calphostin C, are likely to cause calcium-dependent apoptosis in human ALL cells. The exquisite sensitivity of ALL cells to calcium-dependent apoptosis in the presence of PKC inhibitors could provide the basis for new treatment programs against ALL.

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