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Activation of protein kinase C for protection of cells against apoptosis induced by the immunosuppressor prodigiosin

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Abstract

Prodigiosin (PG) is a red pigment produced by *Serratia marcescens* with immunosuppressive activity. We had recently shown that PG-induced apoptosis in several cancer cell lines including Jurkat-T cells, while acting rapidly, potently and with no marked toxicity in non-malignant cells. Here we examine the role of protein kinase C (PKC) in the regulation of apoptosis triggered by PG. We evaluated the use of phorbol-myristate acetate (PMA) in the inhibition of apoptosis induced by PG in Jurkat-T cells by using FACS analysis of the phosphatidylserine externalisation, Hoechst 33342 staining and fragmentation pattern of DNA as well as proteolysis of poly-(ADP) ribose polymerase (PARP). The anti-apoptotic effect of PMA was accompanied by phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). Pretreatment of cells with MEK inhibitor PD98059 inhibited PMA-induced phosphorylation of ERK1/2 and the cytoprotective ability of PMA. These results suggest that activation of PKC in Jurkat-T cells confer protection against apoptosis induced by PG and that ERK1/2 mediate anti-apoptotic PKC signaling.

Keywords: Apoptosis; Cancer; Chemotherapy; Protein kinase C; Phorbol ester; Prodigiosin

1. Introduction

Apoptosis, also known as programmed cell death, is responsible for the removal of cells from healthy tissue during development and tissue homeostasis [1,2]. Apoptosis malfunctions can have health implications, as in cancer. Radiation and chemotherapy have been shown to cause cell death by apoptosis [3,4].

Several bacterial pathogens have been identified as mediators of apoptosis [5]. A family of natural red pigments called PG, which are synthesized from different bacteria characterized by a common pyrrolylpyrromethane skeleton have been seen to possess interesting immunosuppressive properties [6–9] and apoptotic effects in cancer cell lines [10–17].

PKC is a well known serine/threonine protein kinases, consisting of three groups, cPKC (α, β, β_2 and γ), nPKC (δ, ε, η and θ) and aPKC (ζ, ι and λ), and it is involved in many cellular functions, such as proliferation and differentiation. PKC also participates in the regulation of apoptosis induced by many stimuli, such as tumor necrosis factor α (TNFα), ionising irradiation and anti-tumor drugs [18–20]. However, the regulatory effect of PKC on apoptosis is still controversial. Several reports demonstrate that activators of PKC such as the tumor-promoter PMA inhibit apoptosis [20–24], whereas others have shown that pharmacological inhibition of PKC activity inhibits apoptosis [25–28]. As with other molecules, the role of PKC depends on the cell type, the state of activation of the cell, the phase of the cell cycle and the nature of the agent being used. Further studies demonstrated that ERK1/2 mediates the anti-apoptotic signaling of PKC [29–31].

In this study, we investigated the role of ERK signaling in the regulation of apoptosis induced by PG in Jurkat-T cells. Cells treated with PG underwent phosphatidylserine translocation to the external leaflet of the cell membrane and DNA fragmentation characteristic of apoptosis. PMA, a phorbol ester that stimulates PKC activity, was protective

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Abbreviations: cPrG·HCl, cycloprodigiosin hydrochloride; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly-(ADP) ribose polymerase; 4α-PDD, 4α-phorbol-12,13-didecanoate; PG, prodigiosin; PI, propidium iodide; PKC, protein kinase C; PMA, phorbol-myristate acetate; UP, uncedylprodigiosin.

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against PG-induced apoptosis and this apoptotic effect was reversible when PMA was used in conjunction with the MEK inhibitor, PD98059. Therefore, we conclude that the anti-apoptotic effects of PMA in Jurkat cells are mediated in part through ERK activation.

2. Materials and methods

2.1. Chemical and reagents

PG was isolated and stored at -80° as described [15]. PMA and 4α-phorbol-12,13-didecanoate (4α-PDD) were purchased from Sigma (St Louis, MO, USA), and PD98059 was obtained from Alexis (Alexis Biochemicals, San Diego, USA).

2.2. Cells and cell culture

Human T cell acute leukemia cells (Jurkat clone E6-1) from ATCC (VA, USA) were maintained in RPMI-1640 medium (Biological Industries, Beit Haemek, Israel), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin (all from GIBCO BRL, Paisley, UK), and 2 mM L-glutamine (Sigma), and grown at 37° in 5% CO_2.

2.3. Detection of apoptosis by flow cytometric analysis

Jurkat cells were exposed to 55 and 275 nM PG in the absence or presence of PMA (50 nM) for 4 hr. PMA was added 30 min before PG administration. Exposure of phosphatidylserine was quantified by surface annexin V-FITC (Bender MedSystems, Boehringer Mannheim, Mannheim, Germany) staining as described previously [15]. Briefly, cultured cells (5×10^5 cells/mL) were washed in PBS and resuspended in 200 μL binding buffer (BB), containing 10 mM Hepes/NaOH pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$, plus 0.6 μL of annexin V-FITC Kit and incubated for 30 min at room temperature (RT) in the dark. After incubation, we added 200 μL BB and propidium iodide (PI) to a final concentration of 5 μg/mL. Cells were analyzed using a Becton Dickinson FACS Calibur flow cytometer (Mountain View, CA). Samples were acquired and analyzed using Cell Quest software and data were analyzed with the Paint-a-gate Pro software (Becton Dickinson).

2.4. Detection of apoptosis by DNA fragmentation

Jurkat cells (5×10^5 cells/mL) were exposed to 200 nM PG in the absence or presence of PMA (50 nM) and/or PD98059 (100 nM) for 4 hr. PMA and/or PD98059 were added 30 min before PG administration. Briefly, cells were incubated in ice-cold lysis buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.2% Triton X-100) for 15 min at 4°. Then, cell lysates were centrifuged at 14,000 *g* for 15 min and the supernatants were treated with 0.2 mg/mL of proteinase K in a buffer containing 150 mM NaCl, 10 mM Tris–HCl pH 8.0, 40 mM EDTA and 1% SDS, for 4 hr at 37°. The DNA preparations were phenol/chloroform extracted and DNA was precipitated. DNA pellets were resuspended in TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA) and treated with DNase-free RNase (Boehringer Mannheim, Mannheim, Germany) for 1 hr at 37°. Finally, samples were electrophoresed in a horizontal 1% agarose gel containing ethidium bromide and visualized under UV illumination [15].

2.5. Electrophoresis and Western blot analysis

For detection of ERK1/2, the phosphorylation of ERK1/2 and the PARP cleavage by Western blot, cells were exposed to 200 nM of PG in the absence or presence of PMA (50 nM) and/or PD98059 (100 nM) for 3 hr. PMA and/or PD98059 were added 30 min before PG administration. Cells were washed in PBS and lysed with Laemmli sample buffer [32] and 50 μg protein extracts were electrophoresed on 12% polyacrylamide gel and transferred to Immobilon-P (Millipore, MA, USA) membrane. Blots were blocked in 5% dry milk diluted in TBS-T (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 hr and then incubated overnight with mouse monoclonal antibody (mm) or rabbit polyclonal antibody (rp) antiserum according to the manufacturer's instructions: anti-phospho-ERK1/2 (New England BioLabs Inc., E10-9106, mm, diluted 1:1000), anti-ERK1 (Santa Cruz Biotechnologies (SCB), CA, USA, K23 sc-94-G, rp, diluted 1:200), anti-ERK2 (SCB, C14 sc-154-G, rp, diluted 1:200), and anti-PARP (Boehringer Mannheim, ref. 1-835-238, rp, diluted 1:1000). Secondary antibodies conjugated to HRP were goat anti-rabbit IgG (BioRad, UK, ref. 170-6515) or goat anti-mouse (BioRad, ref. 170-6516), diluted 1:1000 in 5% dry milk-TBS-T for 1 hr at RT. Between each incubation, blots were washed 3×5 min in 5% dry milk-TBS-T. After washing blots in TBS-T 3×5 min, peroxidase was then developed by incubating the membrane with 2.25 mM luminol (Sigma) and 0.45 mM *p*-iodophenol (Fluka, Buchs, Switzerland) in 50 mM Tris–HCl pH 9.0 plus 0.03% H_2O_2 for 1 min at RT and enhanced chemiluminescence in an autoradiography film.

2.6. Hoechst staining

Cell morphology was evaluated by fluorescence microscopy following Hoechst 33342 DNA staining. Jurkat cells (5×10^5 cells/mL) were exposed to 200 nM PG in the absence or presence of PMA (50 nM) and/or PD98059 (100 nM), or in presence of 4α-PDD (50 nM) for 4 hr. PMA, 4α-PDD and PD98059 were added 30 min before PG administration. Cells were then washed in PBS and

incubated with Hoechst 33342 (2 μg/mL) for 30 min at 37° in the dark. Finally, cells were washed again and the sections were examined with a Leitz Diaplan microscope and photographed with a Wild MPS 45 Photoautomat system.

3. Results

It has been reported that the alteration of the PKC activity is associated with modulation of apoptosis induced by a variety of stimuli [30,33]. We were interested in determining whether PKC is involved in the regulation of apoptosis induced by PG.

Preliminary experiments determined that the IC_{50} was 225 nM of PG that induced apoptosis in Jurkat cells [15]. We incubated Jurkat cells with 0, 55 and 275 nM of PG for 4 hr. The mean values of the early apoptotic populations (IP^-/annexin V^+ cells) were 3 ± 1, 28 ± 4 and $64 \pm 2\%$, respectively (Fig. 1). Afterward, in order to demonstrate the effect of PKC on PG-induced apoptosis, we first examined the effect of PMA, a potent PKC activator. When cells were incubated with 0, 55 and 275 nM of PG in the presence of 50 nM of PMA, the mean values of the early apoptotic population were 2 ± 1, 3 ± 0.4 and $6 \pm 2\%$, respectively (Fig. 1). These results show that PMA inhibits the apoptotic action of PG.

Since PMA activates PKC and stimulates the Raf/MEK/ERK signal transduction pathway, we postulated that this pathway contributes to PMA ability to inhibit PG-induced apoptosis. Fig. 2 shows that cells treated for 4 hr with 200 nM of PG produced internucleosomal DNA fragments or "ladders" (Fig. 2A, lane 3), whereas cells treated for 30 min with 50 nM PMA did not (Fig. 2A, lane 4).

Since the activation of ERK has been reported to play an anti-apoptotic role in several systems [29,30,33], we wondered whether the anti-apoptotic action conferred by PMA was due to the activation of ERK pathway. To test this possibility we investigated the effect of the specific MEK inhibitor, PD98059. This inhibitor did not induce internucleosomal DNA fragmentation when used alone (Fig. 2A, lane 6). As seen in lane 4 of Fig. 2A, PMA inhibited the appearance of PG-induced DNA fragmentation whereas this inhibition was reversed in cells that were pretreated with PD98059 (Fig. 2A, lane 7).

To investigate the signaling mechanisms responsible for PD98059's effects on apoptosis we used Jurkat cells untreated or treated with PG in presence or absence of PMA. While control cells showed an imperceptible baseline ERK1/2 phosphorylation (Fig. 2B, lane 1) pretreatment of cells with PMA-induced the phosphorylation of ERK1/2 (Fig. 2B, lane 2). This phosphorylation was abolished by MEK inhibitor PD98059 (Fig. 2B, lane 7), and partially abolished by PG (Fig. 2B, lane 4). These results indicate that activation of ERK1/2 is required for mediating the anti-apoptotic effect of PKC.

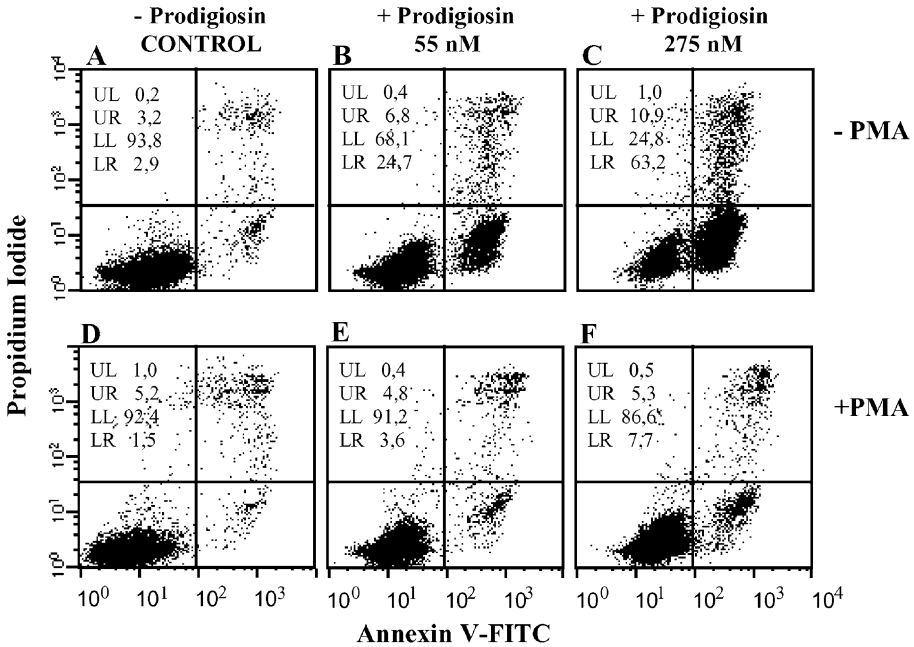

Fig. 1. Involvement of PKC in the regulation of apoptosis induced by PG. A representative annexin-V/IP assay of three independent experiments with Jurkat is shown. Cells staining was analyzed using the Becton Dickinson FACS Calibur flow cytometer as described in Section 2. (A) Control cells. (B, C) Cells were incubated with 55 and 275 nM PG, respectively. (D) Cells were incubated with 50 nM PMA. (E, F) Cells were incubated with 55 and 275 nM PG, respectively in the presence of 50 nM PMA. Cells (5×10^5 cells/mL) were exposed to 55 and 275 nM PG in the absence or presence of PMA (50 nM) for 4 hr. PMA was added 30 min before PG administration. Exposure of phosphatidylserine was quantified by surface annexin V-FITC staining.

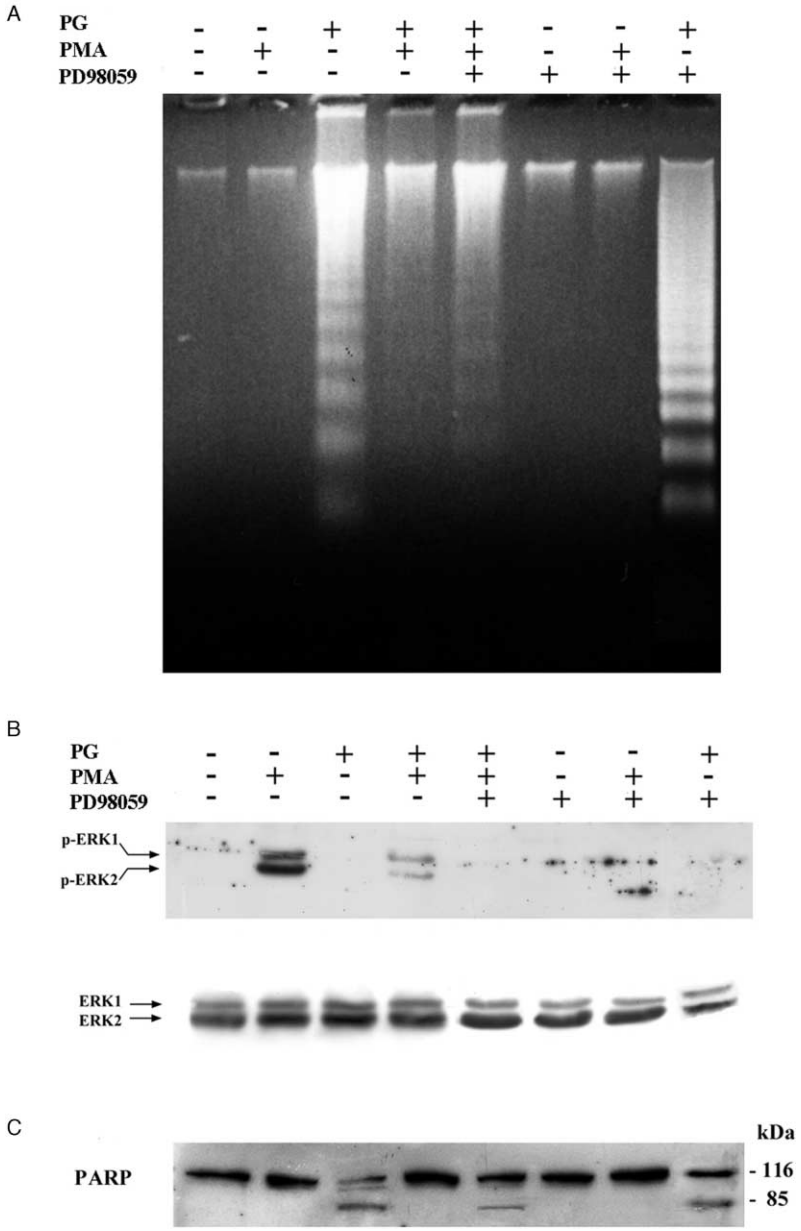

Fig. 2. Activation of PKC by PMA protects cells from PG-induced apoptosis. PD98059 inhibits PMA mediated ERK1/2 phosphorylation and abrogates the anti-apoptotic effect of PMA. (A) DNA fragmentation induced by PG was observed in the agarose gel electrophoresis. For this assay, 5×10^5 cells/mL were untreated, or treated with 200 nM of PG in the absence or presence of PMA (50 nM) and/or PD98059 (100 nM) for 4 hr. PMA and/or PD98059 were added 30 min before PG administration. (B) Phosphorylation of ERK1/2 or ERK1/2 was analyzed by Western blotting. Equal amounts of whole cell lysates (50 μg) were electrophoresed. (C) Effect of PG on PARP cleavage. The positions of native PARP (116 kDa) and the proteolytic fragment (85 kDa) are indicated. In (B) and (C), cells (1×10^6 cells/mL) were untreated, or treated with 200 nM of PG in the absence or presence of PMA (50 nM) and/or PD98059 (100 nM) for 3 hr. PMA and/or PD98059 were added 30 min before PG administration. Results are representative of three independent experiments.

We corroborated these results with the PARP cleavage analysis on protein extracts by Western blot as an early specific marker of caspase activity. Jurkat cells were incubated with the dose of PG indicated above, which produced a cytotoxic effect, and with PMA and PD98059 alone or in combination. The result of this experiment was that only cells treated with PG showed the PARP cleavage (Fig. 2C). Furthermore, we confirmed these results at microscopic level using Hoechst 33342 staining. In contrast to untreated cells, the apoptotic nuclei of Jurkat cells gave stronger blue fluorescence and were condensed and fragmented showing the typical apoptotic bodies (Fig. 3). However, when the cells were preincubated with PMA prior to the incubation with PG we did not observe any morphological changes indicative of apoptosis. In order to verify PKC involvement, prior to the incubation with PG we treated Jurkat cells with 4α-PDD, a phorbol ester derivative which does not activate PKC. The data in Fig. 3 show the typical apoptotic bodies in these cells.

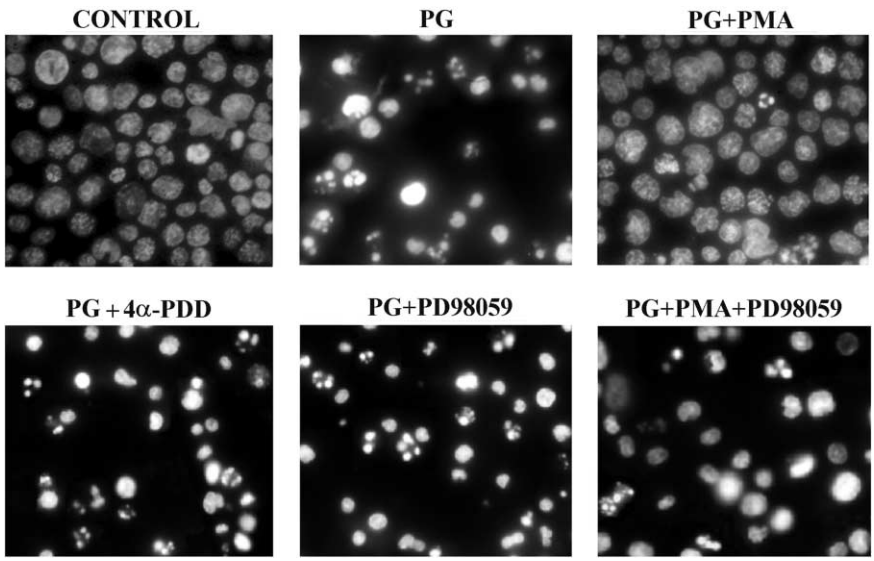

Fig. 3. Involvement of PKC in the regulation of apoptosis induced by PG. Fluorescence microscopic analysis of Jurkat nuclei with Hoechst 33342 staining. Cells (5×10^5 cells/mL) were untreated (control), treated with 200 nM of PG (PG) for 4 hr, treated with 200 nM of PG in the presence of PMA (50 nM) PG + PMA), treated with 200 nM of PG in the presence of 4α-PDD (50 nM) (PG + 4α-PDD), treated with 200 nM of PG in the presence of PD98059 (100 nM) (PG + PD98059) or treated with 200 nM of PG in the presence of PMA (50 nM) and PD98059 (100 nM) (PG + PMA + PD98059). PG-treated cells showed apoptotic signs with chromatin condensation and nuclei fragmentation. The inactive phorbol ester 4α-PDD had no effect in PG treated cells. The specific MEK inhibitor PD98059 inhibited the anti-apoptotic effect of PMA in PG treated cells. These results were reproduced three times.

4. Discussion

PG is produced by *S. marcescens* following a bifurcated biosynthesis pathway, in which mono- and bipyrrole precursors are obtained separately and then coupled to form the linear tripyrrole red pigment [34] during the stationary phase of bacterial growth [35]. Recently, screening for anticancer agents *in vitro* in our laboratory led to the discovery that PG triggered apoptosis in different cancer cell lines, while acting rapidly, potently and with no marked toxicity in non-malignant cell lines [15–17]. In this study, we have shown that activation of PKC by PMA suppresses PG-induced apoptosis in Jurkat and that the cytoprotective ability of PMA is associated with activation of ERK1/2.

The cytosolic pH in transformed or cancerous cells is generally regulated at neutral or even slightly more alkaline levels than normal cells [36] by a variety of pHi homeostatic machinery, including Na^+/H^+ exchanger, Na^+-dependent and -independent Cl^-/HCO_3^- exchangers, V-ATPase, and others [37]. Moreover, Matsuyama *et al.* [38] hypothesized that changes in intramitochondrial and cytosolic pH may be the early events that modulate caspase activation during apoptosis. Therapeutic strategies exploiting the membrane proton gradient mentioned above have been proposed for specific tumor cell death [39,40]. Recent studies support the hypothesis that PG promote H^+/Cl^- symport and induce neutralization of the acidic compartment of cells which in turn results in acidification of the cytoplasm and thus cell cycle arrest and eventually apoptosis [11,13,41,42]. This apoptosis triggering action by PG family members may explain why apoptosis can be inhibited by pretreatment of Jurkat cells with PMA, which prevents intracellular acidification through PKC-induced activation of the Na^+/H^+ antiport [39]. Moreover, incubation of cells with imidazol, a cell permeable base, before apoptotic exposure to cycloprodigiosin hydrochloride (cPrG·HCl) prevents intracellular acidification and suppresses apoptosis [13].

Our results presented here demonstrate that PMA inhibits PG-induced apoptosis in Jurkat cells through an ERK-dependent pathway. However, though cPrG·HCl suppresses Jurkat proliferation as a result of apoptotic cell death, when the cells were stimulated by PMA the percentage of dead cells increased [10]. Given these contradictory results it is tempting to speculate that the differences in the chemical structures of PG and cPrG·HCl are responsible for these different effects in these cells and thus the results shown by Kawauchi *et al.* [10] with cPrG·HCl plus PMA require further investigation.

It has been proposed that activation of the ERK pathway prevents apoptosis and promotes cellular survival [29,31]. This hypothesis has been supported by the results presented here, which demonstrate that PMA inhibits the PG-induced apoptosis in Jurkat cells through an ERK-dependent pathway. Furthermore, the expression of p21 selectively inhibits the stress-activated protein kinases (SAPK) group of MAP kinases activated in response to a variety of cellular stresses, including DNA damage. Interestingly, p21 does not inhibit the ERK group of MAP kinases involved in proliferation, differentiation, etc. Jurkat cell activation by PMA was accompanied by the induction

of p21. This induction was almost completely abolished by PG (data not shown). Taking these results together, we considered the possibility that PMA not only activates ERK1/2 but may also induce p21, and that p21, by inhibiting SAPK, may participate in promoting cell survival.

In conclusion, the studies presented here illustrate the importance of MAP kinase signaling cascades in the differential response to mitogens and apoptotic inducers. Specifically, ERK activity confers protection from apoptosis in the presence of an apoptotic inducer. This was evidenced by the fact that the anti-apoptotic effects of PMA were abrogated by inhibiting PKC or MEK1, two enzymes necessary for PMA-induced ERK activation.

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