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Extracellular Signal-Regulated Kinase (ERK) Activity Is Required for TPA-Mediated Inhibition of Drug-Induced Apoptosis[1]

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Leukemia cells respond to toxic stimuli by undergoing a form of programmed cell death known as apoptosis. However, the signaling events responsible for the execution of this form of death are poorly understood. Mitogen-activated protein kinase (MAPK) signaling cascades are involved in the cellular response to extracellular stimuli. Specifically, extracellular signal-regulated kinases (ERKs) have been associated with proliferation and differentiation, whereas the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs) have been implicated in cell arrest and death. We report the use of 12-O-tetradecanoylphorbol-13-acetate (TPA) in the inhibition of apoptosis in HL-60 cells stimulated with the JNK/SAPK activator anisomycin. This anti-apoptotic effect was accompanied by a sustained increase in ERK activity. Furthermore, the use of protein kinase C (PKC) inhibitors suggested that PKC was involved in the induction of ERK activity and in the inhibition of apoptosis by TPA since the inhibition of apoptosis was attenuated when cells were pretreated with PKC inhibitors. Lastly, we observed that the use of the MEK1 inhibitor PD98059 inhibited TPA-mediated ERK activity and abrogated the anti-apoptotic effects of TPA. However, apoptotic inhibition was not solely ERK-dependent since cells lacking JNK/SAPK stimulation did not undergo apoptosis. Therefore, we conclude that TPA inhibits the induction of apoptosis in anisomycin-treated HL-60 cells through an ERK-dependent pathway and that this effect can be reversed by the attenuation of ERK activity accompanied with the stimulation of JNK/SAPK activity.

Key Words: **ERK, JNK/SAPK; apoptosis; PD98059; anisomycin.**

Cultured leukemia cells treated with chemotherapeutic agents undergo a form of programmed cell death known as apoptosis (1-3). Common characteristics associated with apoptosis include internucleosomal DNA fragmentation (4), phosphatidylserine translocation to the external leaflet of the cell membrane (5), membrane blebbing (6), chromatin condensation (6), and the activation of caspases (7). Although much is known about the physiological characteristics of apoptosis, the signal transduction events that regulate the induction of apoptosis are still enigmatic.

The mitogen-activated protein kinase (MAPK)[3] superfamily of serine/threonine kinases has emerged as an important component of cellular signal transduction. MAPK family members have been implicated in events such as proliferation, differentiation and apoptosis (8). Three MAPK families have been described: the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK) and the p38 kinases (for a review see (9)). The ERKs are activated by a cascade of kinases that originate with Raf1, a MAPKKK, that phosphorylates and activates a dual-specificity kinase, MEK1. MEK1, in turn, confers ERK activity by phosphorylating ERK on a specific tyrosine and threonine residue at the signature sequence T-E-Y (10). JNK/SAPK is activated by a similar cascade, MEKK1 → SEK1 → JNK/SAPK. However, SEK1 phosphorylates JNK/SAPK at the signature sequence T-P-Y (11).

Recent reports suggest that ERK and JNK/SAPK activity may be important to the cell's decision to undergo

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[3] Abbreviations used: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; MEK, MAPK/ERK kinase; SEK, SAPK/ERK kinase; MEKK, MEK kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; ara-C, 1-β-D-arabinofuranosylcytosine; BisM, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide; cal C, calphostin C; PD98059, 2′-amino-3′-methoxyflavone.

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apoptosis following exposure to stress (8). While it is apparent that mitogenic stimuli activate ERK (12,13) and environmental and physiological stresses stimulate JNK/SAPK activity (14-20), little is known about how these cascades communicate with each other to elicit a given response.

In this study, we investigated the role of ERK signaling in the regulation of apoptosis in HL-60 cells. Cells treated with the JNK/SAPK specific activator, anisomycin, underwent DNA fragmentation characteristic of apoptosis. TPA, a phorbol ester that stimulates ERK activity, was protective against anisomycin-induced apoptosis and this anti-apoptotic effect was reversible when TPA was used in conjunction with PKC inhibitors and the MEK inhibitor, PD98059. Therefore, we conclude that the anti-apoptotic effects of TPA in HL-60 cells are mediated in part through ERK activation.

MATERIALS AND METHODS

Materials. Anisomycin and TPA were purchased from Sigma (St. Louis, MO). PD98059 was from Biomol (Plymouth Meeting, PA). 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (or bisindolylmaleimide I (BisM)) and calphostin C (cal C) were obtained through Calbiochem (La Jolla, CA). Stocks of anisomycin, TPA, PD98059, BisM and cal C were prepared in dimethyl sulfoxide (DMSO). However, the amount of DMSO in the cell culture medium never exceeded 0.1% upon drug treatment. Agarose-conjugated rabbit polyclonal antibodies to JNK1 p46 (C-17) and ERK2 p42 (C-14) as well as GST-c-Jun (1-79) were from Santa Cruz Biotechnology (Santa Cruz, CA). The JNK1 antibody does not detect JNK2 p54 whereas the ERK2 antibody does cross-react to some extent with ERK1 p44. Myelin basic protein (MBP) was purchased from Life Technologies (Gaithersburg, MD). [γ-^{32}P] ATP (>4500 Ci/mmol) was obtained from ICN (Costa Mesa, CA). All other reagents were obtained through Sigma.

Cell culture and viability. The HL-60 cell line, a model of human promyelocytic leukemia, was grown in RPMI 1640 supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin G. Cell cultures were passaged thrice weekly to maintain logarithmic growth. Cell viability as determined by trypan blue exclusion was > 95%. Mycoplasma contamination was not detected by routine screening.

Internucleosomal DNA fragmentation assay. The procedure for analyzing internucleosomal DNA fragmentation was performed as reported previously with minor alterations (21). Briefly, HL-60 cells (2.5×10^6) were treated with drug or vehicle and incubated in a humidified incubator containing 95% air/5% CO_2. Drug incubations were terminated by centrifugation at 500 × g for 5 minutes at 4°C. The cells were washed and resuspended in lysis buffer (10 mM Tris, 20 mM EDTA, 0.1% NP-40, pH 7.4) for 20 minutes at 25°C. The lysates were subsequently incubated in the presence of RNase A (0.1 mg/ml) for 30 minutes at 37°C. Proteinase K (0.5 mg/ml) was then added to the lysates followed by a 16 hour incubation at 55°C. DNA was extracted by the addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the lysates followed by ethanol precipitation. Purified DNA was resuspended in 10 mM Tris: 1 mM EDTA (pH 8.0). DNA samples were then fractionated by agarose gel electrophoresis using a 2% agarose gel in Tris:Acetate:EDTA running buffer. Internucleosomal DNA fragmentation was visualized by ethidium bromide staining and UV illumination.

JNK/SAPK immunocomplex kinase assay. Following drug or vehicle treatment, cells (2.5×10^6) were washed twice in ice-cold PBS, pH 7.2, and then incubated in lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM Na_2EDTA, 0.5 mM 2-mercaptoethanol, 0.5% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM NaF, 20 mM β-glycerophosphate, 50 μM Na_3VO_4, and 10 mM ρ-nitrophenol phosphate) for thirty minutes at 4°C. Lysates were then centrifuged (12,000 × g, 10 minutes at 4°C) and the supernatants collected. To the supernatants, 5 μg of agarose-conjugated anti-JNK1 polyclonal antibody was added with 300 μl RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, and 0.1% SDS, 10 mM NaF, 20 mM β-glycerophosphate, 50 μM Na_3VO_4, and 10 mM ρ-nitrophenol phosphate) and incubated with rocking for two hours at 4°C. Following two washes in RIPA buffer and then two washes in kinase wash buffer (20 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 10 mM ρ-nitrophenol phosphate, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 50 μM Na_3VO_4), protein-antibody complexes were resuspended in 30 μl of kinase buffer (kinase wash buffer plus 40 μM [γ-^{32}P]-ATP (50 μCi/mmol) and 0.5 μg of GST-c-Jun(1-79) substrate). Kinase reactions were allowed to proceed at 30°C for 20 minutes. Reactions were terminated by the addition of 6X Laemmli sample buffer and boiled for 3 minutes. Samples were subsequently separated by 12% SDS-PAGE. Phosphorylation of the 39 kDa GST-c-Jun(1-79) peptide was determined by a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

ERK2 immunocomplex kinase assay. The procedure for assaying ERK2 kinase activity was basically the same as that performed for the JNK/SAPK activity assays with the following substitutions. ERK2 immunoprecipitations were carried out with 5 μg of agarose-conjugated ERK2 polyclonal antibody in 300 μl of RIPA buffer containing phosphatase inhibitors and rocked at 4°C for 2 hours. Following a wash and resuspension in 30 μl of kinase buffer, 10 μg of myelin basic protein (MBP) was added to the kinase reaction as a substrate for ERK2.

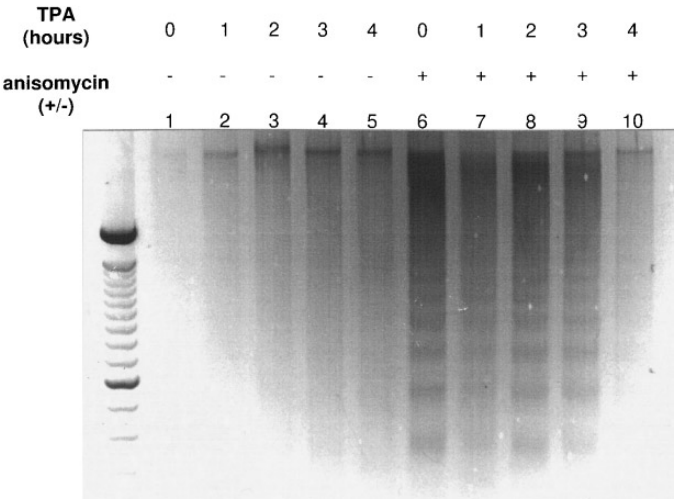

FIG. 1. TPA-mediated inhibition of anisomycin-induced apoptosis is time-dependent. HL-60 cells (2.5×10^6) were treated with 50 nM TPA from 0-4 hr with or without 25 ng/ml anisomycin for 4 hr at 37°C. Genomic DNA was extracted (see Materials and Methods) and then separated by agarose gel electrophoresis. DNA fragmentation was visualized by ethidium bromide staining and UV illumination.

RESULTS

HL-60 cells were treated with anisomycin and TPA and assayed for the presence of internucleosomal DNA fragmentation characteristic of apoptosis (Figure 1).

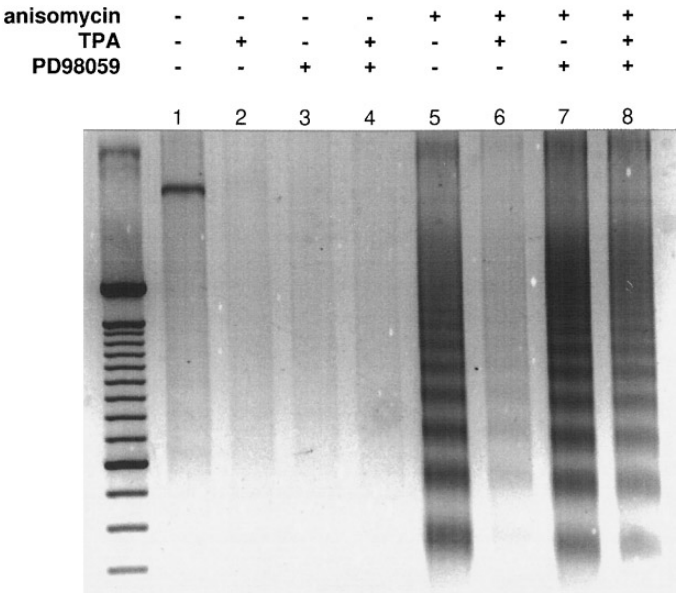

FIG. 2. PD98059 inhibits anti-apoptotic effects of TPA. HL-60 cells (2.5×10^6) were treated with drug vehicle or 25 μM PD98059 for 30 min followed by 25 ng/ml anisomycin and/or 50 nM TPA for 3 hr at 37°C. Genomic DNA was extracted (see Materials and Methods) and then separated by agarose gel electrophoresis. DNA fragmentation was visualized by ethidium bromide staining and UV illumination.

Preliminary experiments determined that 25 ng/ml (94 nM) anisomycin was the lowest concentration tested that induced apoptosis in HL-60 cells (data not shown). As shown in Figure 1, a 4 hour treatment with 25 ng/ml anisomycin produced internucleosomal DNA fragments or "ladders" (Figure 1, lane 6), whereas cells treated for 4 hours with 50 nM TPA did not (Figure 1, lane 2). Moreover, TPA was capable of inhibiting anisomycin-induced apoptosis in a time-dependent fashion (Figure 1, lanes 7-10). To this end, we found that the anti-apoptotic effect of TPA was maximal when TPA was treated concomitantly with anisomycin whereas a delay in TPA treatment corresponded to an attenuation in the inhibition of apoptosis.

Since TPA activates PKC and stimulates the Raf/MEK/ERK signal transduction pathway, we postulated that this pathway may be playing an important role in TPA's ability to inhibit apoptosis. Figure 2 illustrates the effects of the specific MEK inhibitor, PD98059, on TPA-mediated inhibition of apoptosis. PD98059 (25 μM) was incapable of inducing internucleosomal DNA fragmentation when used alone (Figure 2, lane 3). However, in cells treated with anisomycin and PD98059 it appeared that PD98059 slightly increased the amount of internucleosomal DNA fragments recovered (Figure 2, lane 7) when compared to anisomycin alone (Figure 2, lane 5). As seen in lane 6 of Figure 2, TPA inhibited the appearance of anisomycin-induced DNA fragmentation. This inhibition was reversed in cells that were pretreated with PD98059 (Figure 2, lane 8).

To investigate the signaling mechanisms responsible for PD98059's effects on apoptosis we used an immunocomplex kinase assay and myelin basic protein (MBP) as a substrate to measure the activity of ERK. TPA (50 nM) induced the sustained activation of ERK and this was inhibited by pretreating the cells with PD98059 (Figure 3A). Interestingly, cells co-stimulated with TPA and anisomycin resulted in a reduced level of ERK activity compared to TPA alone that was comparable to that achieved with PD98059 in combination with TPA (~ 50% inhibition). Additionally, when cells were treated with PD98059 followed by TPA and anisomycin, TPA-stimulated ERK activity was completely inhibited such that it resembled the untreated control (Figure 3A).

Since activation of the JNK/SAPK pathway has been associated with the induction of apoptosis (17), we measured JNK/SAPK activity by immunocomplex kinase assay using c-Jun as a substrate. As depicted in Figure 3B, anisomycin activated JNK/SAPK activity with this activity being potentiated by PD98059. Interestingly, we observed an increase in JNK/SAPK activity upon co-stimulation of cells with TPA and anisomycin. Thus, internucleosomal DNA fragmentation was detected in cells that displayed an induction of JNK/SAPK activity with little or no ERK activation (compare Figure 2 and Figure 3). Therefore, we conclude that in drug treatment regimens that elicited an increase in JNK/SAPK activity with a decrease or no change in ERK activity, internucleosomal DNA fragmentation occurred.

The PKC inhibitors, Bisindolylmaleimide I (BisM) and calphostin C (cal C), were then tested for their ability to inhibit the anti-apoptotic effects of TPA. Cells stimulated with anisomycin and TPA after a pretreatment with BisM (100 nM) displayed a level of DNA

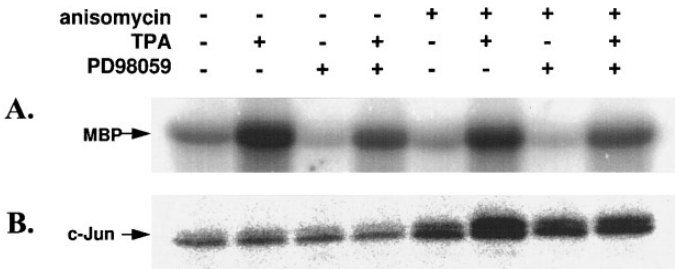

FIG. 3. Comparison of ERK and JNK activities following treatment with anisomycin, TPA, and/or PD98059. HL-60 cells (2.5×10^6) were treated with drug vehicle or 25 ng PD98059 for 30 minutes followed by 25 ng/ml anisomycin and/or 50 nM TPA for 3 hr at 37°C. (A) ERK2 and (B) JNK1 were immunoprecipitated with 5 μg of anti-ERK2-AC and anti-JNK1-AC, respectively, for 2 hr at 4°C. Immunocomplexes were washed and incubated in a reaction mixture containing 40 μM [γ-^{32}P] ATP (50 μCi/mmol) and substrate (ERK2 assay- 10 μg of MBP, JNK1 assay- 0.5 μg GST-c-Jun(79)) for 20 min at 30°C (see Materials and Methods). ERK2 and JNK1 activities were measured by resolving the substrates by SDS-PAGE and detecting the amount of phosphorylation by phosphorimaging.

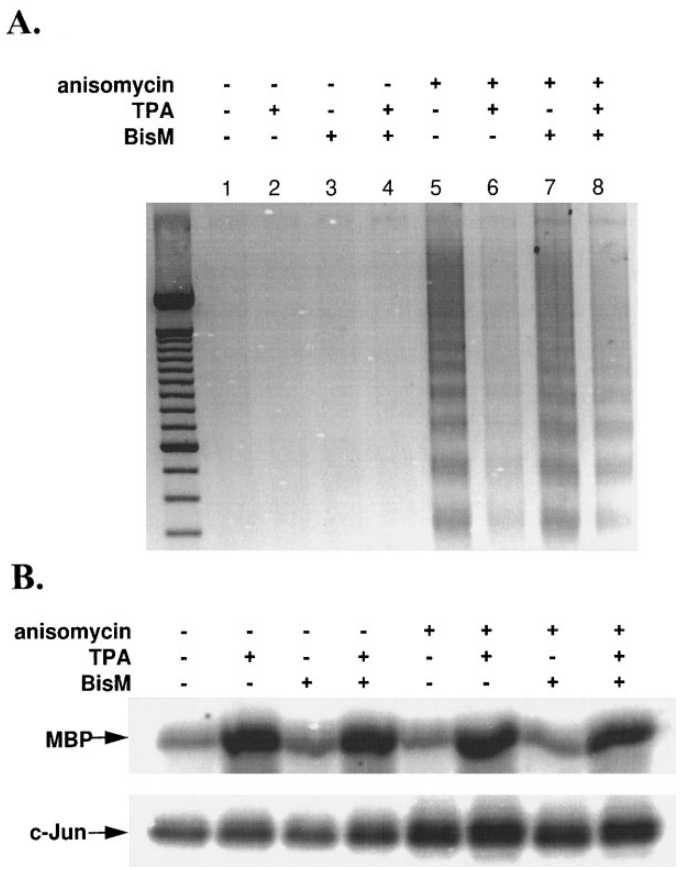

FIG. 4. BisM attenuates TPA mediated inhibition of apoptosis and ERK activity. Cal C attenuates TPA mediated inhibition of apoptosis and ERK activity. HL-60 cells (2.5×10^6) were treated with drug vehicle or 100 nM BisM for 30 min followed by 25 ng/ml anisomycin and/or 50 nM TPA for 3 hr at 37°C. (A) Genomic DNA was extracted (see Materials and Methods) and then separated by agarose gel electrophoresis. DNA fragmentation was visualized by ethidium bromide staining and UV illumination. (B) ERK2 and JNK1 were immunoprecipitated with 5 μg of anti-ERK2-AC and anti-JNK1-AC respectively for 2 hr at 4°C. Immunocomplexes were washed and incubated in a reaction mixture containing 40 μM [γ-^{32}P] ATP (50 μCi/mmol) and substrate (ERK2 assay- 10 μg of MBP, JNK1 assay- 0.5 μg GST-c-Jun(79)) for 20 min at 30°C (see Materials and Methods). ERK2 and JNK1 activities were measured by resolving the substrates by SDS-PAGE and detecting the amount of phosphorylation by phosphorimaging.

fragmentation that was only slightly more than that observed in cells treated with TPA and anisomycin (Figure 4A). ERK activity correlated well with the observed apoptotic effects in that BisM was only slightly effective in inhibiting TPA-induced ERK activity (~ 80%) (Figure 4B). However, the use of cal C (250 nM) was more effective than BisM at both blocking TPA-mediated ERK activity (~ 75%) and at inhibiting the anti-apoptotic effects of TPA (Figure 5).

DISCUSSION

TPA causes a number of effects in different cell types with proliferation being associated with acute TPA stimulation. Conversely, chronic TPA exposure has been implicated as playing a major role in the induction of differentiation (22) and apoptosis (23). In the present study, acute stimulation of cells with TPA induced a sustained increase in ERK activity that correlated with the inhibition of internucleosomal DNA fragmentation caused by anisomycin. Simultaneous treatment of TPA and anisomycin was necessary to observe inhibition of apoptosis since any delay in the application of TPA to anisomycin-treated cells resulted in the loss of DNA fragmentation inhibition (Figure 1). Therefore, TPA-mediated signaling events appear to act at an early point in apoptosis stimulated by anisomycin.

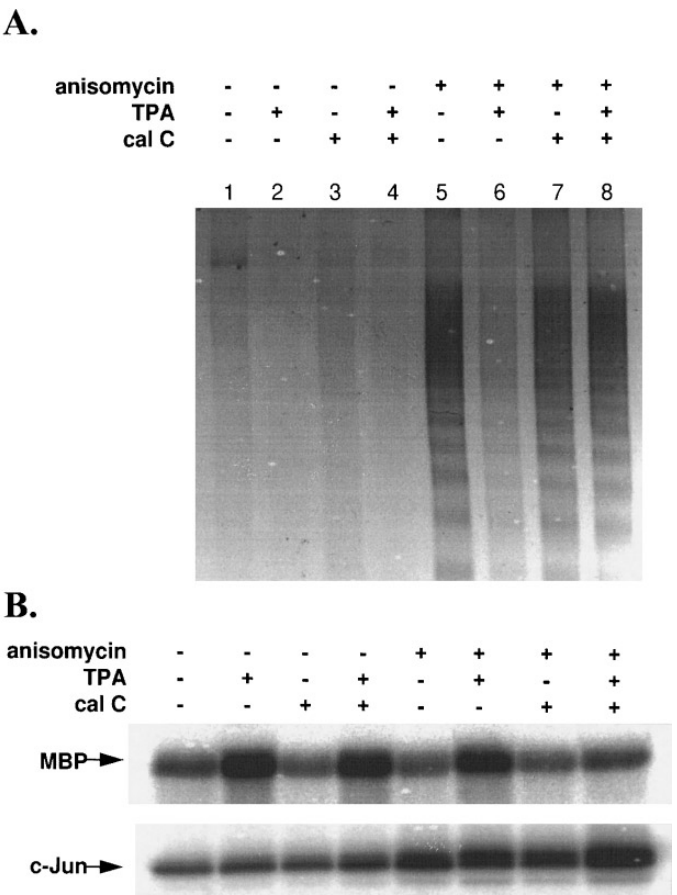

FIG. 5. Cal C attenuates TPA mediated inhibition of apoptosis and ERK activity. HL-60 cells (2.5×10^6) were treated with drug vehicle or 250 nM cal C for 30 min followed by 25 ng/ml anisomycin and/or 50 nM TPA for 3 hr at 37°C. (A) Genomic DNA was extracted (see Materials and Methods) and then separated by agarose gel electrophoresis. DNA fragmentation was visualized by ethidium bromide staining and UV illumination. (B) ERK2 and JNK1 were immunoprecipitated with 5 μg of anti-ERK2-AC and anti-JNK1-AC respectively for 2 hr at 4°C. Immunocomplexes were washed and incubated in a reaction mixture containing 40 μM [γ-^{32}P] ATP (50 μCi/mmol) and substrate (ERK2 assay- 10 μg of MBP, JNK1 assay- 0.5 μg GST-c-Jun(79)) for 20 min at 30°C (see Materials and Methods). ERK2 and JNK1 activities were measured by resolving the substrates by SDS-PAGE and detecting the amount of phosphorylation by phosphorimaging.

ERK and JNK/SAPK signaling cascades are activated by different factors which produce unique responses in the cell; ERK stimulation being associated with proliferative and differentiative processes and JNK/SAPK being implicated in apoptosis. Although these pathways are often considered as being mutually exclusive, these pathways more than likely exhibit crosstalk thereby regulating the activity of each other so as to fine tune a cell's response to a given stimulus. Although anisomycin had no affect on ERK activity in the absence of any other stimulus, this compound was able to inhibit TPA-induced ERK activity by 50% (Figure 3A). This effect may be a consequence of JNK/SAPK activation since anisomycin induces the potent and sustained activation of JNK/SAPK. However, this effect is not reciprocal because cells treated with TPA alone showed little, if any, affect on JNK/SAPK activity.

We observed in cells stimulated with anisomycin and TPA a degree of JNK/SAPK activation that was greater than anisomycin alone. We have no explanation for this observation other than the possibility that TPA may be negatively affecting the activity of a phosphatase that functions to regulate the activity of the JNK/SAPK cascade. Indeed, the kinetics of anisomycin-induced JNK/SAPK activation is characterized by a rapid increase in activity with a slow decrease in JNK/SAPK activity. Thus, the amount of JNK/SAPK activity measured at the 3 hour time point in this report is somewhat less than the peak activation which occurs at 30 minutes (data not shown). The fact that JNK/SAPK activity was greater in cells treated with anisomycin and TPA versus anisomycin alone may be due to the fact that the decay of anisomycin induced JNK/SAPK activity was abrogated by the TPA-mediated inactivation of a MAPK phosphatase or through SEK1, the upstream kinase responsible for JNK/SAPK activity. However, this latter possibility seems unlikely, because TPA alone was unable to stimulate the sustained activation of JNK/SAPK.

Since TPA and anisomycin together led to an inhibition of apoptosis, we conclude that elevated ERK activity may be responsible for the observed anti-apoptotic effects. This was supported by the use of the MEK1 inhibitor, PD98059. PD98059 binds to inactive MEK1 thus inhibiting ERK activation (24). We found that in the presence of 25 μM PD98059, TPA stimulated ERK activity was inhibited by $\sim$50% (Figure 3A). Incidentally, PD98059 given alone was unable to activate JNK/SAPK under the conditions used (Figure 3B). Therefore, we suggest that PD98059 did not induce apoptosis since JNK/SAPK activation was not increased. Moreover, when anisomycin was used in combination with PD98059, we observed a slight increase in apoptosis when compared to anisomycin alone. This increase in apoptosis correlated with a small increase in JNK/SAPK activity (Figure 2 and Figure 3B). The idea of JNK/SAPK involvement in apoptosis was further supported when PD98059 was used with combinations of anisomycin and TPA. In this treatment regimen apoptosis was observed along with an increase in JNK/SAPK activity coupled to an abrogation of ERK activity (Figure 3).

This notion of contextual MAPK signaling was investigated further by studies employing the PKC inhibitors, BisM and cal C. Both inhibitors were observed to have some activity in alleviating the anti-apoptotic effects of TPA with cal C being the more potent of the two. The weak antagonistic effects of BisM on the anti-apoptotic effects of TPA correlated well with its poor ability to inhibit ERK activity (Figure 4). BisM inhibits PKC by interacting with the ATP-binding site thus making it an attractive choice as a general inhibitor of PKC. We used BisM at concentrations of 100 nM and 1 μM and observed no difference in its ability to antagonize TPA-mediated inhibition of apoptosis (data not shown). Due to the fact that at higher concentrations BisM can inhibit other protein kinases in addition to PKC, we chose the 100 nM concentration for our ERK activity studies.

On the other hand, cal C was slightly more effective than BisM with respect to the antagonism of TPA-mediated effects. A probable explanation for these observations may be that cal C functions as an irreversible inhibitor of the phorbol ester binding site of PKC, the site where TPA binds and activates PKC. In any case, cal C was still less effective than PD98059 in inhibiting ERK activity induced by TPA. This suggests that TPA may be stimulating ERK activity through pathways that do not involve the recruitment and activation of Raf1. For instance, TPA has been reported to signal through phosphatidylinositol 3-kinase (PI 3-kinase) (25). This may represent a potential alternative mechanism by which TPA could stimulate ERK activity. Furthermore, BisM and cal C might be inhibiting ERK activity at earlier time points but after 3 hours, the inhibition may be attenuated due to unknown mechanisms. Experiments investigating these line of thought are currently underway in our laboratory.

How ERK activation may inhibit apoptosis is still largely unknown. ERK activity could inhibit apoptotic processes through the regulation of anti-apoptotic genes or by regulating existing proteins through phosphorylation. Evidence in support of these possibilities comes from reports investigating the anti-apoptotic gene product, Bcl-2. In one report, the levels of Bcl-2 protein in HL-60 cells were shown to increase in a time-dependent manner after stimulation with TPA (21). Furthermore, phosphorylation of Bcl-2 by MAP kinase has been reported to confer the anti-apoptotic effects of Bcl-2 (26).

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 TPA were abrogated by inhibiting PKC or MEK1, two enzymes necessary for TPA-induced ERK activation. However, we show that inhibition of ERK activity is not sufficient for the induction of apoptosis and instead, JNK/SAPK stimulation must be present for the apoptotic program to be initiated.

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