Effects of protein kinase C activators on phorbol ester-sensitive and -resistant EL4 thymoma cells

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Phorbol ester-sensitive EL4 murine thymoma cells respond to phorbol 12-myristate 13-acetate with activation of ERK mitogen-activated protein kinases, synthesis of interleukin-2, and death, whereas phorbol ester-resistant variants of this cell line do not exhibit these responses. Additional aspects of the resistant phenotype were examined, using a newly-established resistant cell line. Phorbol ester induced morphological changes, ERK activation, calcium-dependent activation of the c-Jun N-terminal kinase (JNK), interleukin-2 synthesis, and growth inhibition in sensitive but not resistant cells. A series of protein kinase C activators caused membrane translocation of protein kinase C’s (PKCs) α, η, and θ in both cell lines. While PKCγ was expressed at higher levels in sensitive than in resistant cells, overexpression of PKCδ did not restore phorbol ester-induced ERK activation to resistant cells. In sensitive cells, PKC activators had similar effects on cell viability and ERK activation, but differed in their abilities to induce JNK activation and interleukin-2 synthesis. PD 098059, an inhibitor of the mitogen activated protein (MAP)/ERK kinase MEK, partially inhibited ERK activation and completely blocked phorbol ester-induced cell death in sensitive cells. Thus MEK and/or ERK activation, but not JNK activation or interleukin-2 synthesis, appears to be required for phorbol ester-induced toxicity. Alterations in phorbol ester response pathways, rather than altered expression of PKC isoforms, appear to confer phorbol ester resistance to EL4 cells.

Introduction

The EL4 murine thymoma cell line exists in phorbol ester-sensitive and insensitive forms. Sensitive, or ‘wild-type’ (WT), cells respond to phorbol 12-myristate 13-acetate (PMA*) with induction of interleukin-2 (IL-2) synthesis, while insensitive, or ‘variant’ cells, do not exhibit this response (1). Variant cells, originally selected for survival in the presence of chronic incubation with PMA, retain their resistant phenotype when cultured in the absence of PMA (2). The critical differences between wild-type and variant cells have not yet been established. Activation of the ERK mitogen-activated protein kinase (MAPK) phosphorylation cascade is elicited by various mitogenic agonists by PMA, an activator of protein kinase C (PKC) isoforms (3). ERKs are activated by PMA in wild-type but not in variant EL4 cells (4). In variant cells, PMA also fails to activate pp90 ribosomal S6 kinase (RSK), which is activated by ERKs, and ERK kinase (MEK), which activates ERK (5). All of these kinases are expressed and can be activated in both cell types in response to okadaic acid (OA) (4,5), a protein phosphatase inhibitor with tumor promoter activity (6). The mechanism by which phorbol esters induce ERK activation has not been established, but may involve phosphorylation of proteins upstream of Raf-1 (7).

Members of the MAPK family, are involved in regulating IL-2 synthesis in T-cells (8). The IL-2 promoter is regulated by multiple transcription factors, including AP-1, NFAT, NFκB, CD28RE and NFIL2 (15). c-Fos and c-Jun are components of the AP-1 complex. Wild-type, but not variant, EL4 cells induce c-jun transcription in response to phorbol ester (9). Activated ERKs phosphorylate and activate Elk, a factor responsible for induction of c-Fos [16]. c-Jun N-terminal kinases (JNKs) phosphorylate and activate c-Jun. Thus, activations of ERKs and JNKs can co-operate to induce IL-2 transcription. JNKs are activated by ‘stress-related’ stimuli, such as UV light (10), as well as by growth factors (11), cytokines (12) and (in T-cells) via co-stimulation of protein kinase C (PKC)- and calcium-dependent pathways (8). JNK activation can be mediated via the small GTP-binding protein rac (13,14). Prolonged JNK activation is correlated with induction of apoptosis in some cell types (10). TCR-mediated activation of the ERK and JNK cascades is deficient in anergic T-cells, underscoring the importance of MAPKs in T-cell activation (17,18). However, since ERKs appear to be required for T-cell differentiation but not for proliferation (19), the roles of MAPKs in T-cell signaling require further clarification.

PMA is the prototypical tumor promoter, as classically defined for skin carcinogenesis (20). In this model, incomplete tumor promoters (e.g. thymeleatoxin) act as partial agonists, while complete promoters (e.g. PMA) are full agonists. PMA activates all diacylglycerol-dependent PKC isoforms, while incomplete tumor promoters can selectively activate particular isoforms in vitro (21). Down-regulation of PKCs likely plays a role in tumor promotion. Bryostatins activate PKC, but antagonize promotion by preventing PKC down-regulation (22).

In this study, a series of PKC activators was used to examine the roles of PKCs, ERKs, and JNKs in PMA responsiveness. The results indicate that activation of the ERK pathway, but not the JNK pathway, is required for PMA toxicity.

Materials and methods

Cell culture

EL4 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, and penicillin/streptomycin. The wild-type EL4 cell line was kindly provided by Dr David Morris (University of Washington). The new variant (NV) cell line was developed by culturing wild-type cells in medium containing 100 nM PMA for 7 days, with a change of medium every 2 days. At the conclusion of this

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period, the cells were transferred to medium lacking PMA. The cells were first tested for ERK activation in response to PMA after ~1 month. Wild-type (WT) and NV cells were regularly evaluated for phenotype by measuring ERK activation in response to PMA (see below). In all of the experiments shown, NV cells showed no PMA responsiveness by this assay.

**Cell proliferation and viability assays**

EL4 cells were cultured in complete culture medium to which various agents were added. PMA, 4t-PMA, thymeleatoxin, mezerein, indolactam V, prostratin (12-deoxyphorbol-13-acetate), phorbol 12-tigliate-13-decanoate and 4-O-methyl PMA were from LC Services. Bryostatin 5 was provided by Dr Khapil Bhalla (MUSC). PD 098059 was obtained from Calbiochem. At various times after treatment, cell density and viability (trypan blue exclusion) were determined using a hemacytometer. Cells were photographed using a Nikon Diaphot microscope with Hoffman interference optics (×20 objective).

**Interleukin-2 assays**

IL-2 secretion was measured either by bioassay or using an ELISA method. For the bioassay, culture supernatant harvested from cells treated with various agents for 20–24 h was added to CTLL, an IL-2 dependent T-cell line. After 48 h, [3H]thymidine was added. Thymidine incorporation was assessed 24 h later, as previously described (23). A standard curve was generated by adding recombinant IL-2 to CTLL cells in the same assay. ELISA assays for murine IL-2 in the culture supernatant were carried out using reagents obtained from Biosource International.

**Protein kinase assays**

ERK and RSK activities were measured in cytosolic extracts as previously described (5,24), using myelin basic protein (MBP) and 56 peptide (RRLSSLRA; S6P) as substrates for ERK and RSK, respectively. JNK assays used were able to elicit maximal ERK activation (Fig. 1 B). The effects of PMA on growth of the WT and NV cells The phorbol ester-resistant EL4 cells characterized previously were examined. Cells were incubated overnight with PMA (100 nM), prostratin (100 nM), and phorbol 12-tigliate-13-decanoate (100 nM). PMA is a strong tumor promoter, 4tPMA is an inactive isomer of PMA, and the remaining compounds are weak or incomplete tumor promoters. The PKC activators used were able to elicit maximal ERK activation in WT cells at the concentrations used (see below). Results are shown only for selected compounds. PMA and thymeleatoxin were toxic to WT cells while 4tPMA was not (Fig. 1A). Viability of NV cells was not reduced by any of the compounds tested. In Figure 1B, cell density (cells/ml) represents that attained by proliferation during the 20-h incubation. Proliferation was inhibited in WT cells incubated with PMA or thymeleatoxin, as compared with the control of 4tPMA-incubated cells. This result is consistent with PMA-induced cell cycle arrest noted previously in these cells (25,26). Proliferation of NV cells was not affected by any of the compounds used. In the same experiments, the effects of the following agents on viability and proliferation were similar to those of PMA (data not shown): mezerein, indolactam V, bryostatin 5, prostratin, 4-O-methyl PMA, and phorbol 12-tigliate-13-decanoate. Thus, a variety of PKC activators are toxic to PMA-sensitive EL4 cells, but not to resistant cells.

**Effects of PKC activators on cell morphology**

Cells were photographed following a 24-h incubation with various agents (Fig. 2). Untreated NV cells are more adherent than untreated WT cells, as was the case for the PMA-resistant line used previously in our laboratory. PMA, mezerein, and thymeleatoxin caused morphology changes (blebbing and swelling) in WT cells. The morphology of NV cells was unaffected by these compounds. Inhibition of proliferation is apparent from the decreased density of WT cells treated with PKC activators. All PKC activators tested affected cell morphology in a similar manner to PMA, with effects seen for WT but not NV cells (data not shown).

**Activation of ERK and RSK**

The phorbol ester-resistant EL4 cells characterized previously in our laboratory were unable to activate ERKs in response to PMA (4,5). As shown in Figure 3, ERKs were activated in response to PMA in WT but not NV cells. The inactive phorbol ester, 4tPMA, had no effect on ERK activity. All other PKC activators tested caused ERK activation, with no consistent differences in time course or magnitude of response in a 60-min incubation. The concentrations of the activators used...
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Fig. 4. Effects of PD 098059 on ERK activation. Whole-cell extracts were prepared from WT and NV EL4 cells that had been incubated for 15 min in the absence (‘□’) or presence (‘△’) of 100 nM PMA and/or the indicated concentrations of PD 098059. When present, PD 098059 was added 90 min prior to PMA. ERKs were immunoblotted using antibodies to ERK-1 (A and C) and active-ERK (B and D).

throughout this study were chosen on the basis of their ability to elicit maximal ERK activation in WT cells in dose–response experiments (data not shown). None of the compounds tested activated ERKs in NV cells (Figure 3).

In previous studies, we have shown that PMA-resistant EL4 cells can activate MEK and ERK in response to okadaic acid (OA), a protein phosphatase inhibitor that does not activate PKCs (4,5). Incubation with 10 µM OA for 10 min resulted in activation of ERK and RSK in both WT and NV cells, as detected using in vitro assays for kinase activity (ERK: 450% of control in WT, 206% of control in NV). The NV cells appeared less sensitive to OA than the original variant cells. This point is reinforced in Figure 3. When cells were treated for various times with a low dose of OA (1 µM), a gradual activation of ERKs was seen only in WT and not in NV cells.

An alternative method for examining activation of ERKs is to examine their phosphorylation state. When whole-cell extracts of control and PMA-treated WT and NV cells were immunoblotted using an antibody that recognizes ERK-1/ERK-2, both ERK1 and ERK2 showed shifts in electrophoretic mobility on SDS–PAGE in extracts from PMA-treated WT cells (24). Such shifts are correlated with activating phosphorylations of ERKs (27). Shifts in ERK mobility were not observed in NV cells treated with PMA (Figure 4A). Immunoblots using an anti-active-ERK (dually phosphorylated ERK) antibody confirmed that ERKs were activated by PMA in WT, but not NV cells (Figure 4A). ERK mobility shifts were seen in cytosol from WT cells treated with PMA, mezerein or bryostatin (but not 4α-PMA), and were not seen in cytosol from NV cells treated with PMA, mezerein, bryostatin 5 or 4α-PMA (data not shown).

PD 098059 interferes with the activation of MEK by Raf-1, resulting in inhibition of ERK activation (29). PD 098059, at 20 µM, partially inhibited PMA-induced phosphorylation of ERKs in WT cells (Figure 4A). Results of a dose–response
Expression and translocation of PKC isoforms in WT and NV EL4 cells. (A) Cytosolic extracts were prepared from untreated WT and NV cells. Equal amounts of cytosolic protein were separated on SDS–PAGE and immunoblotted using antibodies to the indicated isoforms of PKC. All samples were obtained in the same experiment and analyzed in parallel. (B) WT and NV EL4 cells were incubated for 10 min in the absence (‘C’) and presence (‘P’) of 100 nM PMA. Cytosolic and membrane fractions were prepared. Equal amounts of protein were separated on SDS–PAGE and immunoblotted for PKCs α and η. All samples were obtained in the same experiment and analyzed in parallel. (C) WT EL4 cells were incubated for the indicated times with 100 nM 4αPMA, 100 nM bryostatin 5 or 1 µM mezerein. Cytosolic and membrane fractions were prepared. Equal amounts of protein were separated by SDS–PAGE and immunoblotted for PKCη.

study are shown in Figure 4B. While a dose-dependent inhibition of ERK phosphorylation was observed, complete inhibition was not observed at the highest dose of PD 098059 used (50 µM). It was not possible to use higher doses, because of the limited solubility of the drug at 50 µM (29). Quantitative results, obtained by measuring MBP kinase activity in cytosolic extracts, showed that 20 µM PD 098059 inhibited PMA-induced ERK activation by 41%. This incomplete inhibition is consistent with a report that PD 098059 does not completely inhibit ERK activation in response to PMA or high doses of growth factors (29).

Expression and translocation of PKC isoforms in WT and NV cells

The mechanisms underlying PMA responsiveness were further explored. Since PMA exerts its effects via binding to PKC isoforms, immunoblotting was utilized to examine the expression and localization of these target proteins. Expression of PKCs α, ε, η, θ and ζ was detected in both WT and NV cells. Results are shown in Figure 5A for α, ε, η and θ. In this experiment, levels of η and α were significantly reduced in NV cells, while levels of θ were similar between the two cell lines. PKCe was detected at such low levels that comparison between cell lines was compromised. As shown in Figure 5B, 100 nM PMA caused rapid translocation of PKCη from cytosol to membrane in both WT and NV cells. This response is most readily seen by changes in cytosolic PKCη. Similar results were seen for PKCα (Figure 5B) and PKCθ (data not shown). Bryostatin 5 and mezerein, but not 4αPMA, likewise caused translocation of PKCη from cytosol to membranes in WT cells within 5 min (Figure 5C). Similar results were seen for all other PKC activators used (data not shown), suggesting that multiple PKC activators can activate PKCη in EL4 cells.

Compounds that activate PKC can induce down-regulation of some PKC isoforms. PKCα was severely depleted from both cytosolic and membrane fractions following a 20-h incubation with PMA (Figure 6). For PKCs η and θ, cytosolic and total levels remained depressed after 20 h. Thus, PMA treatment causes down-regulation of three different PKC isoforms in WT EL4 cells.

Overexpression of PKCη in EL4 cells

The results described above suggested that expression levels of PKCs η or α might be important in modulating PMA sensitivity. Underexpression of PKCη in NV cells was a consistent finding, while relative levels of expression of PKCα were sometimes similar between WT and NV cells (data not shown). We examined whether enhanced expression of PKCη
might restore PMA sensitivity. NV cells were transiently transfected with an expression vector for PKC\(\eta\). As shown in Figure 7A, transfected cells showed an increase in expression of PKC\(\eta\) that was correlated with the amount of DNA transfected. However, the transfected cells did not activate ERKs in response to PMA (Figure 7B). PMA-induced activation of RSK was likewise not observed in PKC\(\eta\)-transfected NV cells (Figure 7C). Similar negative results were obtained with PKC\(\alpha\) transfections, either with or without co-transfection of PKC\(\eta\) (data not shown). To test whether the number of transfected cells was sufficient, immunolocalization of PKC\(\eta\) was performed (data not shown). The results confirmed that expression of PKC\(\eta\) was lower in NV than in WT cells. Following transfection of NV cells with DNA encoding PKC\(\eta\), at least 15% of the cells expressed high levels of PKC\(\eta\). Any restoration of PMA-induced ERK activation should have been detectable, since ERK activation was detected when a mixture of 85% NV/15% WT cells was incubated with PMA (data not shown). Thus, enhanced expression of PKC\(\eta\) is not sufficient to restore PMA responsiveness.

Effects of PKC activators on JNK activity
JNKs have been implicated as an important mediator of IL-2 expression in T-cells, and are activated in other cell types by agents that induce programmed cell death (10). JNKs can be weakly activated by PMA alone, but are activated in a synergistic manner by a combination of PMA and calcium ionophore (8). JNK activity was assessed following affinity purification of the kinases on a solid-phase substrate. JNK activity was slightly increased in response to PMA alone in WT cells (Figure 8A), as previously reported (24), and was synergistically activated with co-stimulation by PMA plus ionomycin. In NV cells, JNKs were slightly activated in response to ionomycin, but not to PMA; a combination of PMA and ionomycin produced no further increase. Activation by PMA and ionomycin in WT cells was maximal by 20 min, and activity declined to basal levels by 90 min (Figure 8B).

The effects of other PKC activators were examined. Bryostatin activated JNK in a calcium-dependent manner at 10 min, but to a lesser extent than PMA (Figure 8C). Thymeleatoxin induced little or no JNK activation at 10 min. Some stimuli induce delayed and/or prolonged JNK activation (30). Activation of JNKs was not observed in response to PMA, mezerein or thymeleatoxin alone, or to mezerein or thymeleatoxin with ionomycin, at up to 120 min (data not shown).

**Effects of PKC activators on IL-2 synthesis**

PMA-resistant EL4 cell lines have previously been shown to be deficient in production of IL-2 in response to PMA (1). IL-2 secretion was examined using a bioassay for IL-2 (Figure 9A). WT cells secreted IL-2 in response to PMA, while NV cells did not. OA did not increase IL-2 synthesis in either cell
line, indicating that ERK activation is not sufficient to induce IL-2 synthesis.

The IL-2 studies were extended to other PKC activators, using an immunoassay for murine IL-2 (Figure 9B). WT cells secreted IL-2 in response to PMA. Ionomycin did not enhance the response to PMA. NV cells did not produce IL-2 in response to PMA, with or without ionomycin (data not shown). Phorbol 12-tiglate 13-decanoate, indolactam V, mezerein and thymeleatoxin induced IL-2 synthesis when added alone, with levels of IL-2 similar to those seen in response to PMA. Responses to mezerein and thymeleatoxin were slightly enhanced by ionomycin. Prostratin, bryostatin 5, and 4-O-methyl-PMA alone did not induce IL-2 secretion, but did induce IL-2 when added with ionomycin. No response was observed with 4αPMA or OA, with or without ionomycin (data not shown). These data differentiate the abilities of PKC activators to induce ERK activation and cell death from their abilities to induce JNK activation and IL-2 synthesis.

**Effects of PD 098059 on PMA-induced toxicity**

The results presented above suggested that neither JNK activation nor IL-2 expression was required for PMA-induced toxicity in EL4 cells. The role of ERK activation was therefore examined. WT cells were incubated in complete culture medium in the absence and presence of 100 nM PMA (or without PMA) for 20 h. Ionomycin (100 nM) completely blocked the ability of PMA to inhibit both proliferation and viability. PD 098059 completely blocked the ability of PMA to inhibit proliferation and viability. PD 098059 also inhibited the
effects of PMA on cell morphology (Figure 10C). Activation of JNKs in response to co-stimulation of WT EL4 cells with PMA and ionomycin was not inhibited by 50 μM PD 098059 (data not shown). Since PD 098059 is apparently a specific inhibitor of MEK (29), these results suggest that ERK activation is required for PMA-induced toxicity.

Discussion

In this study, we have examined the effects of a variety of PKC activators on phorbol ester-sensitive and -resistant EL4 thymoma cells. In the sensitive (WT) cell line, treatment with PMA activates the ERK phosphorylation cascade. This response is present in most mammalian cells, including primary T-cells and T-cell lines. Resistant EL4 cells not only fail to activate ERKs in response to phorbol ester, but are resistant to its toxic effects. A new variant (NV) EL4 cell line, with a phenotype similar to that of previously-derived variant lines, was selected by incubation of WT cells with phorbol ester. ERK activation is induced by a variety of toxic PKC activators in WT but not NV cells. Failure to activate ERKs in response to PKC activation is thus a hallmark of the PMA-resistant phenotype in EL4 cells.

Our data are consistent with a minor role for ERKs in the induction of IL-2 transcription, in that activation of ERKs is not sufficient to induce IL-2 synthesis. Sensitive, but not resistant, EL4 cells activate JNKs in response to co-stimulation with PMA and ionomycin. Although this result is consistent with the failure of PMA-resistant EL4 cells to transcribe c-Jun (10) and IL-2 in response to PMA, regulation of the IL-2 promoter involves multiple factors in addition to c-Jun. Also, WT EL4 cells synthesize IL-2, but only slightly activate JNKs, in response to PMA alone. Since the PKC activators tested all activate ERKs, but vary in their abilities to induce JNK activation and IL-2 transcription in WT cells, activation of both ERKs and JNKs is not sufficient to induce IL-2 transcription. Thymeleatoxin, which activated ERK but not JNK, nonetheless induced IL-2 synthesis. These data indicate that pathways in addition to ERK and JNK are required for IL-2 synthesis, and that PKC activators can differ in their abilities to stimulate these pathways.

Our results indicate that various PKC activators do not discriminate between calcium-dependent and -independent PKC isoforms in EL4 cells. Similar conclusions have been reached by other investigators for some of these compounds (31–34). All of the compounds tested caused similar responses in terms of cell toxicity, ERK activation and translocation of PKCη, a calcium-independent isoform. The abilities of PKC activators to show isoform selectivity in some cell types, but not in others, likely reflects aspects of PKC regulation that have not yet been fully defined (e.g. phosphorylation of PKC, interaction of PKC with docking proteins). The responses observed in WT EL4 cells were independent of the abilities of the compounds to act as complete or incomplete tumor promoters in mouse skin. For example, 4-O-methyl-PMA, which causes proliferation but not tumor promotion in mouse skin (35), activated PKCs and ERKs in EL4 cells with a potency similar to that of PMA. This compound was previously shown to be much less potent than PMA as a proliferative agent in mouse skin (35). These results emphasize that the actions of PKC activators should be established for each cell type in which they are utilized.

The major effects of PMA appear to be mediated via PKC isoforms. Decreased expression of particular PKC isoforms could theoretically confer resistance to phorbol ester. Our data suggest that this is not the case in NV EL4 cells. The cause and effect(s) of the reduced level of expression of PKCs α and η in NV cells are not yet clear. While PMA-resistant EL4 cells underexpress PKCη in comparison with sensitive cells, PKCη responds to PKC activators in both cell types. The role of PKCη in T-lymphocyte signaling has not been established. PKCη, a major isoform in mouse skin (36), is down-regulated in mouse epidermis in response to application of PMA (37). In a leukemia cell line with the multidrug resistance phenotype, originally selected for resistance to okadaic acid, PMA toxicity is retained despite underexpression of PKCε (38). In the case of resistant EL4 cells, underexpression of PKCs likewise does not appear to confer the phorbol ester-resistant phenotype.

We have shown that a variety of PKC activators have similar effects on EL4 cell viability, morphology, translocation of PKCη, and ERK activation. JNK activation does not appear to be required for PMA-induced cell death. The finding that MEK/ERK activity is apparently required for PMA-induced toxicity in EL4 cells was unexpected, in view of the known roles of ERKs in mitogenesis. It is now clear that the roles of ERKs and JNKs are not entirely segregated. The balance between ERK and JNK activities can regulate apoptosis in some cell types, with sustained activation of JNKs being associated with apoptosis (38). However, JNKs are not significantly activated by PMA alone in EL4 cells. In T-cells, it is possible that JNKs play a predominant role in proliferation, with ERKs being more critical for other responses. Further work will be required to establish the mechanism linking ERK activation to growth arrest and cell death in EL4 cells. PD 098059 completely inhibited the effects of PMA on cell growth at concentrations that did not completely block acute ERK activation. It is possible that the magnitude and duration of ERK activation are critical factors in determining the role of ERKs in EL4 cells, as is the case for PC12 cells (39). Alternatively, the possibility that MEK is involved in ERK-independent signaling pathways has recently been raised (40).

In a recently-published study of the mechanism of PMA-induced cell death in EL4 cells, Desrivieres and co-workers concluded that PMA induced cell cycle arrest was not mediated by MEK/ERKs (26). These investigators showed that PD 098059 partially inhibited PMA-induced ERK activation, but did not inhibit the reduction in [3H]thymidine incorporation observed after a 6-h incubation with PMA. The differences in the conclusions drawn between this study and our own may arise from differences in the protocols used to assess cell proliferation and viability. In our study, the ability of PD 098059 to block PMA-induced cytotoxicity was most apparent after 20 h of incubation. It is thus possible that the late phases of the response to PMA are most dependent on MEK/ERK activation.

The molecular mechanism responsible for coupling PKC activation to ERK or JNK activation has not been completely established in any cell type. While differences in signal transduction between PMA-sensitive and resistant EL4 cells remain to be explored, our observations point to potential roles for alterations in ERK signaling pathways in mediating phorbol ester toxicity in T-lymphocytes.

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References


5. In this article, the activation of MAPKs (and other kinases) is often referred to as the mitogen-activated protein kinase (MAPK) signal transduction pathway. The pathway involves multiple levels of phosphatidylinositol 3-kinase (PI3K) and the RAS-MAPK pathway.


