Evidence for Protein Kinase C-Dependent and -Independent Activation of Mitogen-Activated Protein Kinase in T Cells: Potential Role of Additional Diacylglycerol Binding Proteins

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Evidence for Protein Kinase C-Dependent and -Independent Activation of Mitogen-Activated Protein Kinase in T Cells: Potential Role of Additional Diacylglycerol Binding Proteins

Lawrence G. Puente,* James C. Stone,† and Hanne L. Ostergaard2*

Activation of mitogen-activated protein kinases (MAPK) is a critical signal transduction event for CTL activation, but the signaling mechanisms responsible are not fully characterized. Protein kinase C (PKC) is thought to contribute to MAPK activation following TCR stimulation. We have found that dependence on PKC varies with the method used to stimulate the T cells. Extracellular signal-regulated kinase (ERK) activation in CTL stimulated with soluble cross-linked anti-CD3 is completely inhibited by the PKC inhibitor bisindolylmaleimide (BIM). In contrast, only the later time points in the course of ERK activation are sensitive to BIM when CTL are stimulated with immobilized anti-CD3, a condition that stimulates CTL degranulation. Surprisingly, MAPK activation in response to immobilized anti-CD3 is strongly inhibited at all time points by the diacylglycerol (DAG)-binding domain inhibitor calphostin C implicating the contribution of a DAG-dependent but PKC-independent pathway in the activation of ERK in CTL clones. Chronic exposure to phorbol ester down-regulates the expression of DAG-responsive PKC isoforms; however, this treatment of CTL clones does not inhibit anti-CD3-induced activation of MAPK. Phorbol ester-treated cells have reduced expression of several isoforms of PKC but still express the recently described DAG-binding Ras guanyl-nucleotide-releasing protein. These results indicate that the late phase of MAPK activation in CTL clones in response to immobilized anti-CD3 stimulation requires PKC while the early phase requires a DAG-dependent, BIM-resistant component. The Journal of Immunology, 2000, 165: 6865–6871.

Mitogen-activated protein kinases (MAPK)3 are a family of serine-threonine kinases involved in numerous eukaryotic signal transduction processes including those controlling cell proliferation, cell differentiation, yeast pheromone responses, and cytokine biosynthesis (1). MAPK function as the distal element of three-step kinase cascades. The best studied MAPK are the extracellular signal-regulated kinases ERK1 and ERK2. These are activated by the tyrosine-threonine kinases MEK1 and MEK2, which are themselves activated by the serine-threonine kinase Raf (2–4). Raf activity is thought to be regulated principally by Ras-mediated recruitment of Raf to the cell membrane (5). Ras molecules cycle between an inactive GDP-bound state and an active GTP-bound state. The ratio of GTP-bound Ras to GDP-bound Ras is modulated by GTPase-activating proteins (GAPs) and guanylnucleotide exchange factors (GEFs) (6). GAPs decrease Ras effector function by enhancing the intrinsic GTPase activity of Ras, while GEFs accelerate the release of GDP from Ras, allowing GTP to bind.

The best characterized Ras–MAPK activation pathway is that stimulated through the receptor tyrosine kinase family of growth factor receptors (7). When ligated, these receptors become autophosphorylated, allowing for the binding of the adaptor molecule Grb2. Grb2 associates with Sos, a RasGEF, and the recruitment of Grb2-Sos complexes to the membrane activates the membrane-associated Ras. In T lymphocytes, ligation of the TCR results in activation of Ras, Raf, and ERK (8). This pathway is required for T cell function, including IL-2 production (8, 9) and CTL degranulation (10). The mechanism linking TCR stimulation to activation of the Ras-Raf-MEK-ERK pathway in T cells is unclear although several mechanisms have been proposed.

Both TCR stimulation and addition of PMA induce accumulation of GTP-bound Ras in T cells (11). Because both treatments activate protein kinase C (PKC), it was originally thought that TCR stimulation might be linked to the Ras-MAPK pathway by PKC (11). Early work suggested that PKC could activate Ras by inhibiting RasGAPs (11, 12). However, studies in which PKC was inhibited by a pseudosubstrate peptide indicated that PKC-independent pathways for TCR-mediated activation of Ras also exist (12, 13). Other studies indicate that PKC can act downstream of Ras at the level of Raf. TCR-mediated activation of Raf-1 has been reported to be PKC dependent (14), and at least one isoform of PKC is capable of activating Raf-1 by direct phosphorylation (15), although mutation of the PKC phosphorylation site does not block activation of Raf by PMA (16). A second model for activation of the Ras-MAPK pathway in T cells is that, analogous to the mechanism of Ras activation by growth factor receptor protein tyrosine kinases, Ras activation occurs when the Ras-GEF Sos is recruited to the cell membrane by protein-protein interactions (17). In T cells, this is thought to be
mediated by the adapter protein, linker for the activation of T cells (LAT). LAT is a transmembrane protein containing multiple Grb2-binding sites that become phosphorylated in response to TCR stimulation and, therefore, could recruit Grb2-Sos complexes to the membrane (18, 19). However, it is not known whether Sos is the physiologically relevant RasGEF in T cells (17).

Ras guanyl-nucleotide-releasing protein (RasGRP), also known as CalDag-GEFII, is a novel RasGEF expressed in neuronal cells and lymphoid cells (20–22). RasGRP contains a diacylglycerol (DAG) binding domain and can mediate Ras activation in response to PMA (20, 21). In Jurkat T cells, TCR stimulation induces RasGRP association with the membrane and RasGRP overexpression enhances Ras-ERK activation in response to TCR stimulation (23). Furthermore, RasGRP has been shown to be essential for T cell development (24). Therefore, DAG production downstream of TCR stimulation likely mediates effects through RasGRPs as well as through PKC. Additional mechanisms of MAPK regulation in T cells have been suggested, including direct recruitment of Shc-Grb2-Sos complexes to phosphorylated CD3ζ (17, 25), interactions between Lck and MAPK (26), modulation by NO (27), and regulation of phosphatases (28).

In the present study, we evaluate the role of PKC in the regulation of ERK1 and ERK2 in Ag and IL-2-dependent CTL clones. We demonstrate that dependence on PKC for ERK1/2 activation varies with stimulation conditions and varies over the time course of stimulation. Our results indicate that there are PKC-independent but DAG-dependent mechanisms, in addition to a PKC-dependent pathway, regulating MAPK in T cells.

Materials and Methods

Cell lines

Murine CD8+ CTL clones, clone 11 and clone AB.1 (29) were maintained by weekly stimulation with irradiated splenocytes from C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) and IL-2, as described previously (29), and used 4–7 days later.

Antibodies

The hybridoma producing 145-2C11 (anti-CD3ε) was obtained from the American Type Culture Collection (Manassas, VA). The hybridoma producing PV-72 (anti-phosphotyrosine) was obtained from Dr. B. Selton (The Salk Institute, La Jolla, CA). Anti-MAPK (ERK1 plus ERK2) mAb was purchased from Zymed (San Francisco, CA). Phospho-p44/42 MAPK (Thr202/Tyr204) Abs 9101S and 9105S were purchased from New England Biolabs (Beverly, MA). Abs to PKC-α (H-7), PKC-β (C-16), PKC-δ (C-17), PKC-ε (E-5), and anti-PKC-θ (C-18) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An additional PKC-ε Ab was purchased from BD Biosciences (Mississauga, Ontario, Canada). RAS10 Ab was obtained from Upstate Biotechnology, was used to add Ras and ERK1/2 protein and the remainder of the lysate was used for Raf-1 affinity precipitation. A total of 15 μl Raf-1 binding domain fusion protein bound to glutathioneagarose, obtained from Upstate Biotechnology, was added to each sample rotated at 4°C. After 30 min the beads were washed three times with magnesium-containing lysis buffer and resuspended in Laemmli sample buffer. The entire sample was loaded onto a 12% SDS-PAGE gel and probed with RAS10 Ab (Upstate Biotechnology).

Degranulation assay

Degranulation assays were performed as previously described (10). All samples were analyzed in triplicate and the SD is shown.

PKC assay

Cells that had been previously treated for 18 h with PMA or with carrier were lysed at 107/ml in extraction buffer containing 25 mM Tris (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM 2-ME, 1 mM PMSF. Postnuclear lysates were assayed for PKC activity using the Sigma PKC assay system from Promega (Madison, WI) as described in the product bulletin. This system uses the biotinylated peptide neurogenin as a substrate. The ratio of 32P cpm incorporation into the peptide from extract assayed in the presence of phosphatidylinerine and DAG to the incorporation of extract assayed in the absence of activators was determined. In all experiments, the cpm incorporated into the peptide also decreased with increasing concentration of PMA in the overnight pretreatment.

Results

TCR stimulation results in the activation of multiple signaling proteins including phospholipase C-γ1 (PLC-γ1) (30). PLC-γ1 converts phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and DAG. Inositol 1,4,5-trisphosphate activates calcium-dependent pathways while DAG activates PKC. PKC has been reported to promote MAPK activation in a number of cell types including T cells. As expected, treatment of CTL clone AB.1 with the DAG analogue PMA resulted in a rapid increase in the
phosphorylation of ERK1 and ERK2 and this response was completely inhibited by the PKC inhibitor BIM (Fig. 1A).

To examine whether ERK activation in response to TCR/CD3 stimulation was also PKC dependent, we stimulated CTL clones either in suspension with anti-CD3 mAb and cross-linking Ab or in anti-CD3-coated assay plates and in the presence or absence of BIM. Immobilized anti-CD3 stimulation of these CTL clones, but not cross-linked anti-CD3 stimulation, results in a degranulation response (10). CTL clones stimulated by soluble, cross-linked anti-CD3 mAb exhibited a transient activation of ERK1 and ERK2 that was maximal at 5 min and was inhibited by BIM, indicating that ERK activation under these conditions is PKC dependent (Fig. 1B).

In dramatic contrast to the results obtained with cross-linked anti-CD3 Ab, ERK phosphorylation resulting from immobilized anti-CD3 stimulation comprised an initial phase from 15 to 25 min after plating that was resistant to BIM, followed by a phase that was BIM-sensitive (Fig. 2, B–D). The drug was effective in these cells as BIM treatment inhibited the degranulation response in a dose-dependent manner (data not shown). The delay in inhibition of ERK activation was not due to slow uptake of BIM because this drug completely inhibited the much higher level of ERK phosphorylation induced by PMA after only 10 min (Fig. 2A). The decline in ERK activation was not due to cell death because BIM-treated cells exhibited no significant decrease in viability as measured by trypan blue exclusion after 120 min of exposure, and remained viable when replated in growth media and cultured overnight. Similar results were observed using CTL clone 11 and with Con A blasts generated using C57BL/6 spleen cells (data not shown). The PKC inhibitors Gö-6983 or Ro-31-8220 exerted modest or no inhibition, respectively (data not shown), of the ERK phosphorylation induced by immobilized anti-CD3 mAb. Because the PKC isoform selectivities of BIM, Gö-6983, and Ro-31-8220 differ, this result suggests that only particular PKC isoforms are involved in ERK activation.

**FIGURE 1.** ERK activation is BIM-sensitive when CTL are stimulated in suspension. CTL clone AB.1 was stimulated by PMA (A) or soluble, cross-linked 2C11 (B), in the presence of the indicated concentrations of BIM or DMSO carrier control. Activated ERK1 (p44) and ERK2 (p42) were detected by Western blotting (WB) with mAb 9105S, which is specific for the Thr202/Tyr204 phosphorylated form of these MAPK (right panels). Equal loading of ERK1 and ERK2 was confirmed by reblotting with anti-MAPK (ERK1 plus ERK2) mAb (left panels). Similar results were obtained with CTL clone 11 (data not shown).

**FIGURE 2.** ERK activation in response to immobilized anti-CD3 stimulation comprises BIM-resistant and -sensitive phases. A, Effectiveness of BIM treatment was verified by inhibition of ERK activation in response to acute PMA stimulation. Activated ERK1 (pp44) and ERK2 (pp42) were distinguished from their inactive forms by mobility shift assay as described in Materials and Methods. B, Clone AB.1 cells were added to wells coated with anti-CD3 mAb 145-2C11 and lysed after the indicated times. Active and inactive ERK1 and ERK2 were visualized as in A. C, The ratio of activated to inactive ERK2 was quantitated from B, using NIH Image, and plotted as the percent of ERK2 in the active form over time. D, The identification of the mobility-shifted bands shown in B as activated ERK1 and ERK2 was confirmed by reprobing the same membrane with phospho-p44/42 MAPK (Thr202/Tyr204)-specific mAb 9105S. This is representative of at least four different experiments.
To determine whether BIM exerted its effect upstream of MAPK, at the level of Ras, we determined the degree of Ras activation after stimulation with immobilized anti-CD3 in the presence or absence of BIM. As shown in Fig. 3A, Ras activation, as measured by the ability to bind a Raf-1 fusion protein, is observed in the presence of BIM, particularly at the 15-min time point. By 30 min there is a significant decrease in the level of Ras activity in the 145-2C11-stimulated cells in the presence of BIM compared with control cells. The BIM was effective in this experiment as PMA stimulation of cells from the same pool of cells used for the Ras assay was inhibited in the BIM-treated cells but not in the control cells (Fig. 3B). These results suggest that there is BIM-sensitive and BIM-resistant activation of Ras that mirrors the results observed in Fig. 2 with ERK1/2 activation.

BIM inhibits PKC activity by interacting with the PKC kinase domain. We next examined the effect of the inhibitor calphostin C, which interacts with the DAG binding domain found in DAG-responsive PKC isoforms and in RasGRP (31). Phosphorylation of ERK1 and ERK2 in response to immobilized anti-CD3 stimulation was drastically reduced in the presence of calphostin C (Fig. 4). In striking contrast with the effects of BIM, inhibition was evident at all time points. This result suggests that all phases of ERK activation following immobilized anti-CD3 stimulation require one or more DAG binding proteins even though the early phase is BIM-resistant.

To confirm that the inhibition of ERK phosphorylation observed with calphostin C was due to a requirement for DAG, we used the PLC-γ1 inhibitor U73122. U73122, but not its inert analogue U73343, completely inhibited ERK1 and ERK2 phosphorylation at all time points examined (Fig. 5). Taken together, the effects of these inhibitors suggested the existence of an activity upstream of MAPK ERK1 and ERK2 that was PLC-γ1 dependent and DAG dependent, but independent of BIM-inhibitable PKC activity.

As an alternate method to examine the role of PKC in ERK activation, PKC expression was down-regulated by overnight exposure of cells to PMA. This treatment is known to down-regulate the DAG-responsive PKC isoforms. Consistent with the BIM results (Fig. 1), the ERK phosphorylation normally induced by acute PMA treatment did not occur when cells were pretreated overnight with 100 ng/ml PMA (Fig. 6A). Also consistent with the BIM inhibition data, ERK phosphorylation in response to immobilized anti-CD3 stimulation was not significantly inhibited by this treatment (Fig. 6A). When PMA-pretreated cells were stimulated with immobilized anti-CD3 mAb, degranulation was partially inhibited at 10 and 50 ng/ml and substantially inhibited at 100 ng/ml PMA in the overnight treatment (Fig. 6B). Abs to CD45, which do not trigger degranulation were used as a control to show that the overnight PMA treatment did not cause the cells to nonspecifically degranulate. The incomplete inhibition in cells treated with the higher concentrations of PMA is likely due to the re-expression of PKC during the 4–5 h of incubation required for the degranulation assay. These results also confirm that PKC activity is required for optimal degranulation as was observed with the PKC inhibitor studies.

To confirm that overnight treatment of PMA did down-regulate PKC activity, the activity in total extracts from control and PMA-treated cells was measured. The ratio of activity measured in the extracts assayed in the presence of PKC activators to the activity measured in the absence of activators was determined. A ratio of one would be predicted in cells that had no DAG-stimulated PKC.
activity. The results in Fig. 7A demonstrate that total PKC activity was diminished in the presence of PMA pretreatment, particularly at the 50 and 100 ng/ml concentrations. To further confirm that DAG-responsive PKC isozymes were down-regulated after overnight PMA exhaustion, lysates from the PMA-pretreated and control cells were probed with Abs to various PKC isozymes. As shown in Fig. 7A, PKC-α, PKC-βI, PKC-δ, PKC-ε, and PKC-θ were all significantly down-regulated in response to PMA. We could not detect PKC-βII or PKC-γ in these cells (data not shown). Very little of each isozyme remained in the cells after overnight treatment with 50 or 100 ng/ml PMA. Taken together with the lack of PMA-stimulated ERK1/2 activity, the decreased degranulation, and the decreased enzymatic activity, our results strongly suggest that overnight PMA treatment substantially depletes the cells of DAG-responsive PKC isozymes. Despite the significant decrease in PKC activity, there is still substantial anti-CD3-stimulated ERK1/2 activation (Fig. 6A).

FIGURE 6. PKC down-regulation by chronic PMA treatment inhibits acute PMA-stimulated ERK activation but does not inhibit anti-CD3 stimulated ERK activation. Clone AB.1 cells were treated with 10, 50, or 100 ng/ml PMA or an equivalent volume of ethanol carrier control for 18 h. A. Cells were stimulated for the indicated time with immobilized anti-CD3 or with PMA for 15 min. MAPK (ERK1 and ERK2) activation was assayed by gel mobility shift assay as described in Materials and Methods. B. PMA-pretreated or control AB.1 cells were stimulated with anti-CD3 (145-2C11) or with anti-CD45 (I3/2) as a control. After a 5-h incubation, degranulation was measured by assaying culture supernatant for serine esterase activity. Both the ERK1/2 blot and the degranulation assay were performed on samples from the same pool of cells.

The surprising activation of ERK in response to anti-CD3 in these CTL clones treated overnight with PMA suggested that a factor activated by anti-CD3, but not down-regulated in response to chronic PMA stimulation, was acting upstream of ERK phosphorylation. The novel RasGEF, RasGRP is expressed in CTL clones (our unpublished observations), and is regulated by DAG (20, 21, 31). Therefore, we examined the expression of RasGRP in cells that had been chronically treated with PMA. The PKC-α blot shown in Fig. 7A was stripped and reprobed with a mAb to RasGRP. All experiments in this figure and in Fig. 6 were performed on samples from the same pool of cells. The experiments in Figs. 6 and 7 have been repeated three times with identical results.

FIGURE 7. Chronic PMA treatment down-regulates PKC activity and expression but not RasGRP expression. Clone AB.1 cells were preincubated with 10, 50, or 100 ng/ml PMA or an equivalent volume of ethanol carrier control, for 18 h. A. Cells were assayed for PKC activity as described in Materials and Methods. B. Cell lysates were assayed for PKC expression by immunoblotting for the indicated PKC isozyme. C. The blot used for PKC-α analysis in A was stripped and reprobed with a mAb to RasGRP. All experiments in this figure and in Fig. 6 were performed on samples from the same pool of cells. The experiments in Figs. 6 and 7 have been repeated three times with identical results.

Discussion

Several mechanisms have been proposed for the regulation of MAPK in T lymphocytes. Activation of the MAPK ERK1 and ERK2 in response to TCR stimulation is generally thought to be mediated either by PKC (12) or by recruitment of Grb2-Sos complexes to phosphorylated LAT (19). In our present study, when T cells were stimulated in suspension by PMA or by cross-linked anti-CD3 mAb, ERK activation was completely blocked by the PKC inhibitor BIM. In contrast, when cells were stimulated by
immobilized anti-CD3 Ab, we identified BIM-sensitive and BIM-resistant phases of Ras and ERK activation.

CTL that are stimulated in suspension do not degranulate, whereas CTL degranulation is effectively stimulated by immobilized anti-CD3. These disparate outcomes correlate with the duration of signaling through various pathways including ERKs (10). The difference in duration of signaling between the two conditions might reflect quantitative differences in the duration of TCR stimulation, or qualitative differences in the pathways activated. Here we have found that each condition activates pathways upstream of ERK1 and ERK2 that have differential dependence on PKC, as indicated by the difference in BIM sensitivity. Our observation that BIM inhibits degranulation suggests that PKC is required either for sustained ERK1/2 activation or for some other aspect of degranulation.

An earlier study reported that PKC was not important for TCR-mediated ERK2 activation based on the ineffectiveness of the PKC inhibitor Ro-31-8425 (32). Indeed, when we used the similar drug Ro-31-8220, little effect on ERK activation was seen. This suggests that the Ro-sensitive PKC isoforms α, β1, γ, and ε are not important for ERK activation. BIM is known to inhibit PKC isoforms α, β, δ, ε, and ζ with varying efficiencies. Therefore, it is highly likely that only certain PKC isoforms such as δ or ζ are involved in the TCR-mediated regulation of ERK. PKC-θ is unlikely to play a major role because T cells deficient in PKC-θ have normal ERK responses (33), although later time points after stimulation were not specifically examined in this study. It is important to note that PMA-stimulated ERK activation is highly sensitive to all three inhibitors, indicating that this mode of activation is not equivalent to the PKC-dependent phase of ERK activation induced by immobilized anti-CD3.

The initial increase in ERK activity in response to immobilized anti-CD3 stimulation was relatively insensitive to the presence of BIM, whereas the sustained phase was inhibited by the drug. This pattern of regulation for the Raf-MEK-ERK pathway has also been found in other cell types. Howe and Juliano (34) found that only the late phase of integrin-mediated Raf activation is PKC dependent in fibroblasts, whereas the initial phase is dependent on Raf membrane localization, induced either artificially or by Ras-Raf interaction.

Ras activation in T cells has been proposed to be mediated by the recruitment of Grb2-Sos complexes to the cell membrane via LAT (18, 19). This process is solely dependent on protein-protein interactions involving inducible protein tyrosine phosphorylation. However, ERK activation is strongly inhibited by the PLC-γ1 inhibitor U73122 in Jurkat T cells (23) and CTL clones (Fig. 5). One of the functions of PLC-γ1 is the production of DAG and we found that ERK activation in CTL is sensitive to the DAG binding domain inhibitor calphostin C (Fig. 4). LAT is involved in the recruitment and activation of PLC-γ1 following TCR stimulation (18, 19, 35). Interestingly, a mutant of LAT that is unable to bind PLC-γ1 but is still capable of binding Grb2 is unable to support MAPK activation suggesting that a PLC-γ1-dependent pathway is important for the regulation of MAPK activity in Jurkat cells (36). In Slp-76-deficient T cells, which recruit Grb2 to LAT, but do not activate PLC-γ1, Ras, and ERK2, are not activated (35). Taken together, these data suggest that the major function of LAT with respect to ERK activation is to activate PLC-γ1, and that PLC-γ1 is essential for TCR-mediated ERK activation, most likely due to a requirement for DAG. Our results do not necessarily rule out a role for Grb2-Sos in TCR-induced signaling but suggest that the bulk of MAPK activity is regulated by both DAG-dependent but PKC-independent and PKC-dependent mechanisms.

Our data indicate that although DAG is essential for ERK activation in T cells, the major DAG receptor, PKC, is not required for the initial period of ERK activation as indicated by insensitivity to BIM and Ro-31-8220. These results suggest the existence of a DAG-responsive, but non-PKC, component upstream of ERK activation in T cells. The novel Ras-GEF, RasGRP contains a DAG binding domain and can mediate Ras and ERK activation in response to PMA (20, 21). We found that long-term PMA exposure did not down-regulate RasGRP expression, suggesting that it could be responsible for the CD3-stimulated ERK activation observed following chronic PMA treatment. RasGRP can mediate Ras activation in response to TCR cross-linking in Jurkat T cells and RasGRP over-expression enhances TCR-mediated activation of ERK (23). Therefore, RasGRP is likely to be one of the mediators of DAG-dependent, PKC-independent activation of ERK in T cells.

In summary, the extended period of ERK activation required for CTL degranulation consists of an early PKC-independent phase and a late PKC-dependent phase. The early phase, while PKC-independent, is PLC-γ1-dependent and DAG-dependent and may involve non-PKC DAG-responsive signaling elements such as RasGRP. Clearly, regulation of the Ras-ERK pathway in T cells is complex. Further studies will be required to identify all the relevant pathways and to distinguish their relative roles in T cells.

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References


