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ERK activation is only one role of PKC in TCR-independent cytotoxic T cell granule exocytosis

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Abstract

Cytotoxic T cells (CTLs) kill target cells by releasing lytic agents via regulated exocytosis. Three signals are known to be required for exocytosis: an increase in intracellular Ca^{2+} , activation of protein kinase C (PKC) and activation of extracellular signal regulated signal kinase (ERK). ERK activation required for exocytosis depends on activity of PKC. The simplest possibility is that the sole effect of PKC required for exocytosis is ERK activation. Testing this requires dissociating ERK and PKC activation. We did this using TCR-independent stimulation of TALL-104 human leukemic CTLs. When cells are stimulated with thapsigargin and PMA, agents that increase intracellular Ca^{2+} and activate PKC, respectively, PKC-dependent ERK activation is required for lytic granule exocytosis. Expressing a constitutively-active mutant MAP kinase kinase activates ERK independent of PKC. However, activating ERK without PKC does not support lytic granule exocytosis, indicating that there are multiple effects of PKC required for granule exocytosis.

Keywords

Signal transduction; PKC; ERK; flow cytometry; cytotoxicity; lytic granules; granule exocytosis; lymphocyte

INTRODUCTION

One mechanism cytotoxic T cells (CTLs) use to kill virus infected, tumor or transplanted target cells is regulated exocytosis of molecules such as perforin and granzymes from specialized preformed lytic granules (reviewed in [1;2;3]). Killing is initiated by T cell receptor (TCR) activation by antigen-MHC upon contact with an appropriate target cell, which leads to a complex set of signaling steps that ultimately trigger three signals required for the lytic interaction: protein kinase C (PKC) activation [4;5;6], activation of extracellular signal regulated kinases (p42/p44 ERKs, members of the mitogen activated protein kinase (MAPK) family [6;7;8]) and elevation of intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) [9;10].

Drugs that increase $[\text{Ca}^{2+}]_i$ and activate PKC can promote granule exocytosis in the absence of TCR engagement [11;12]. These stimuli, while not physiological, provide a simplified

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system for probing the signals regulating granule exocytosis. The involvement of PKC in both TCR-dependent and TCR-independent lytic granule exocytosis has been investigated [4;5;12;13], revealing that the novel PKC isoform PKC θ does not play the kind of unique role in lytic granule exocytosis [4;6] that it does in important helper T cell functions such IL-2 gene expression, Fas ligand expression and c-jun N terminal kinase activation (reviewed in [14]). However, knowledge of signaling events in CTLs downstream of PKC activation remains rudimentary, and PKC is likely to be involved in multiple steps of the lytic interaction, including regulating granule reorientation [15;16] as well as granule exocytosis.

ERK activation, which, as described above is critical for granule exocytosis, appears to be a downstream target of PKC [6;7;17]. It is possible that the effects of PKC are mediated solely through ERK activation. If this were the case, then our view of lytic granule exocytosis would be greatly simplified. Importantly, it would mean that there would be no need to try to identify additional currently unknown PKC substrates required for the fusion of lytic granules with the plasma membrane. To date, the only evidence against the idea that the sole role of PKC is ERK activation is pharmacological. Puente et al. showed that Go-6976, used as a conventional PKC inhibitor, blocked granule exocytosis stimulated by solid-phase anti-CD3, but did not block ERK activation [18]. However, the complexity of responses stimulated by cross-linked anti-CD3 makes it difficult to conclude from these data that it is the exocytic step that is affected. Also, the possibility of off-target effects of drugs is always a concern.

To test directly whether the sole role of PKC in promoting exocytosis is ERK activation requires finding a way to activate ERK without activating PKC. We used a constitutively-active mutant human MAP Kinase Kinase (hMKK) [19] which phosphorylates and activates ERK independently of PKC in TALL-104 human leukemic CTLs, a cell-line we have used as a model for dissecting signaling events downstream of TCR engagement [4;8;9;20;21;22]. Our results indicate that PKC has multiple roles required for TCR-independent lytic granule exocytosis.

MATERIALS AND METHODS

cDNA constructs and transfections

The constitutively active Δ N-S218E-S222D MAPKK Mutant (hMKK) [19] was given to us by Dr. Lynn Heasley (UCHSC, CO). Standard PCR methods were used to subclone it into pEGFPN1 (Clontech) using engineered KpnI and BamHI restriction sites. 2.5×10^6 TALLs were transfected using an Amaxa Nucleofector (Amaxa Biosystems, Gaithersburg, MD) using program T-20 and solution V. Experiments were performed 6–7 hrs post-transfection. PKC mutant constructs have been previously described [4].

Chemicals and reagents

Salts and PD 98059 were purchased from Sigma-Aldrich (St. Louis, MO). Fetal calf serum was from Hyclone (South Logan, UT). Thapsigargin and PMA were from Alexis Biochemicals (San Diego, CA). ERK Inhibitor II (FR 180204) and Ro31-8220 were from EMD Biosciences (San Diego, CA). Anti-CD107a (clone H4A3) was purchased from BD Biosciences (San Diego, CA), and was conjugated to Alexafluor 647 using a kit from Molecular Probes/Invitrogen (Eugene, OR). Alexafluor 647-conjugated anti-phospho p42/44 was purchased from Cell Signaling (Danvers, MA). Recombinant human IL-2 was provided by the National Cancer Institute.

Cells and solutions

TALL-104 cells were from American Type Culture Collection (Rockville, Maryland) and cultured in Modified Dulbecco's Iscoves medium with 10% FCS, 100 IU IL-2, 2mM L-

glutamine and penn-strep antibiotic. Cells were grown in a humidifier incubator at 37°C in 10% CO₂. Ringer's solution contained (in mM): 145 NaCl, 4.5 KCl, 1 MgCl₂, 2 CaCl₂, 5 HEPES and 10 glucose (pH 7.4 with NaOH).

Immunostaining and flow cytometry

LAMP externalization was monitored as described previously [4;21;22]. The anti-LAMP-1 antibody was either conjugated to Alexafluor 647, or purchased conjugated to phycoerythrin (PE) from BD Biosciences. In both the cases conjugated Anti-LAMP-1 antibody was used at 0.09 micrograms per test. For phospho-staining experiments, cells were fixed and permeabilized using CALTAG's Fix/Perm kit (CALTAG laboratories, Burlingame, California) with methanol modification. Flow cytometry was performed on a FACSCalibur at the University of Connecticut at Storrs Flow Cytometry and Confocal Microscopy Facility. FlowJo software (TreeStar, Ashland, Oregon) was used to analyze data offline. For multi-color experiments, data were acquired without hardware compensation. Unstained and single-color controls were run, and, when needed, data were compensated off-line using FlowJo.

Statistics

Statistical significance was assessed using repeated measures ANOVA (Instat, Graphpad Software, San Diego, CA). Statistically significant data ($p < 0.05$) have been indicated with an asterisk (*).

RESULTS

TCR-independent lytic granule exocytosis requires PKC-dependent ERK activation

We have previously shown that TCR-dependent lytic granule exocytosis in TALL-104 cells is ERK dependent [8]. We tested whether TCR-independent lytic granule exocytosis is also ERK dependent by treating cells with two different inhibitors of ERK signaling, PD 98059 (100 μ M) and FR 180204 (100 μ M), and measuring their effects on exocytosis by measuring LAMP-1 externalization. PD 98059 blocks the MAPKKs MEK 1/2 that phosphorylate and activate ERK [23], while FR 180204 blocks substrate phosphorylation by ERK by occluding its ATP-binding site [24]. Cells were pretreated with drugs or vehicle for one hour and then stimulated with thapsigargin (TG) in combination with phorbol myristate acetate (PMA) for 50 minutes in the continued presence of drug or vehicle. TG activates Ca²⁺ influx, while PMA activates PKC. Representative flow histograms are shown in Figure 1Ai, and quantification of results from three such experiments are shown in Figure 1Aii. Both drugs significantly reduced TCR-independent granule exocytosis. FR180204 treatment had a more pronounced effect (~85%) than PD 98059 treatment (~35%), likely reflecting the fact that FR 180204 blocks ERK-dependent phosphorylation, while PD 98059 only partially blocks ERK activation, leaving some residual ERK activity.

PKC is involved in both TCR-dependent and TCR-independent lytic granule exocytosis. We tested the role of PKC in ERK activation (Figure 1B) by measuring ERK phosphorylation levels. We used flow cytometry to quantify ERK phosphorylation levels [25; 26; 27] in our experiments for the ease of multi-parameter assessment at a single cell level, particularly in transfected cells (see below). The anti-phospho ERK antibody we used specifically detected two bands of appropriate molecular weight in Western blots (data not shown). We measured ERK phosphorylation levels upon treatment with TG+PMA in control cells or cells treated with inhibitors of either MAPKK or PKC. Representative flow histograms of anti phospho-ERK staining are shown in Figure 1Bi, and quantitation is shown in Figure 1Bii. We found that the MAPKK inhibitor PD 98059 applied at 100 μ M reduced ERK phosphorylation levels by ~60%. Consistent with a role for PKC, the general PKC inhibitor Ro31-8200 (4 μ M, a concentration that similar to what others have used to assess the role of PKCs in granule

exocytosis [18; 28] and which we have previously shown blocks TCR-independent lytic granule exocytosis [4]) almost completely blocked ERK activation. This indicates that ERK activation during TCR-independent stimulation occurs primarily via PKC-dependent pathways. At least part of this effect is likely due to MAPKK activation, but MAPKK-independent ERK activation may also be involved [29]. Further consistent with the involvement of PKC in ERK activation, we found that constitutively-active mutants of two PKC isoforms, PKC α (a conventional PKC isoform) and PKC θ (a novel PKC isoform), which can bypass the requirement for PMA and stimulate granule exocytosis when cells are also treated with TG [4], also significantly activate ERK compared to unstimulated controls or compared to GFP negative cells in the same sample, although the PKC θ mutant did so more robustly than the PKC α mutant and neither reached the levels observed when cells were treated PMA treatment (data not shown).

Overexpressing a constitutively-active mutant human MAPKK activates ERK

A constitutively active human MAPKK (hMKK) mutant with ~400-fold higher activity than wild type has been previously characterized by others [19]. We fused this mutant to green fluorescent protein (GFP). To confirm that the mutant activates ERK at the expression levels we can attain, we transfected cells with either EGFP (vector alone) or hMKK-EGFP, and measured ERK phosphorylation levels 6–7 hours post transfection. At no level of expression of GFP alone was ERK phosphorylated (Figure 2A, left). However, a clear dose-dependent ERK phosphorylation was caused by transfection with the hMKK mutant (Figure 2A, middle). Consistent with the expectation for a constitutively-active hMKK, ERK activation was PD 98059 sensitive (Figure 2A, left). Representative flow histograms of ERK phosphorylation levels for untransfected (NEG) and highly transfected (POS) populations of vector and mutant-transfected cells are shown in Figure 2B, along with quantification from three similar experiments. At the highest expression levels of the hMKK mutant, ERK phosphorylation was comparable to levels seen with PMA treatment (Figure 2B).

ERK activation in the absence of PKC activations is not sufficient to support exocytosis

Because the hMKK mutant activates ERK robustly, it provides a tool allowing us to determine whether ERK activation is the sole effect of PKC that is required for TCR-independent exocytosis; if it is, the hMKK mutant should render exocytosis independent of the need to activate PKC with PMA. Just as in a previous study PKC mutants replaced the requirement for PMA, we transfected cells with either EGFP (vector alone) or hMKK-EGFP then 6–7 hrs post transfection stimulated them with TG or with TG and PMA and measured granule exocytosis using the LAMP externalization assay (Figure 3). Representative flow histograms of LAMP staining levels for untransfected (NEG) and highly transfected (POS) populations of vector and mutant transfected cells are shown in Figure 3A, and quantifications are shown in Figure 3B. Even at expression levels of the hMKK mutant that phosphorylated ERK to levels comparable to those attained with PMA treatment (Figure 2B) there was no significant increase in granule exocytosis when compared with GFP-transfected cells at any level of expression, or compared to the untransfected cells in the hMKK1 samples (NEG). Transfected cells stimulated with both TG and PMA responded normally, indicating that hMKK expression does not inhibit exocytosis.

DISCUSSION

The results presented here demonstrate two main points. First, PKC-dependent ERK activation is required for TCR-independent lytic granule exocytosis. Second, activating ERK cannot be the sole role of PKC in promoting exocytosis. While some might view results obtained with TCR-independent stimuli as being non-physiological, we do not see any feasible way in which ERK activation could be dissociated from PKC activation using TCR stimulation. Furthermore,

as noted above, TCR-independent stimulation likely bypasses TCR-dependent events such as MTOC/ granule reorientation, allowing isolation of effects on exocytosis from processes upstream.

PKC regulation of ERK activation appears to be complex. When stimulated through plate bound anti-CD3, novel PKC isoform(s) act upstream of Ras to activate ERK [6]. PI3 kinase may be involved in this pathway [30], and it appears that when nPKCs such as PKC- θ are knocked out, other isoforms can participate [6]. When stimulated via soluble anti-CD3, conventional PKC isoform(s) activate ERK downstream or independent of Ras [6], although exocytosis is not triggered, even after cross-linking of the CD3 with secondary antibodies [17]. Additionally, there may be MAPKK-independent routes of PKC-dependent ERK activation, including direct phosphorylation of ERK by PKC [29]. This might account for the fact that we found that PD98059 only partially inhibited ERK phosphorylation, while the PKC inhibitor Ro31-8200 blocked completely (Figure 1B). In addition, non PKC-dependent mechanisms of ERK activation have been reported in lymphocytes, including a calmodulin-dependent pathway [31]. We have previously shown that raising intracellular calcium levels activates ERK using Western blotting with a different anti-phospho-ERK antibody, although this effect did not contribute to TCR-dependent ERK activation [8].

Although the results presented here were obtained with TALL-104 human leukemic CTLs, we expect that they will be representative of events in other CTL systems. We have extensively used TALL-104 cells as a model system to study signaling events downstream of TCR engagement [4;8;9;20;21;22] and to our knowledge all known key features of CTL exocytosis are preserved in TALL -104 cells.

The substrate(s) of ERK that participate in exocytosis are unknown. The only candidate is paxillin, a cytoskeletal adaptor protein that has been suggested to be involved in TCR-dependent exocytosis based on its localization to the microtubule organizing center (MTOC) and its translocation to the point of target contact upon TCR activation [30]. A thorough examination of ERK substrates will be needed to understand the role of this kinase in lytic granule exocytosis.

Apart from its participation in activating ERK, very little is known about potential substrates of PKC involved in granule exocytosis. PKC modulates exocytosis in many cell types that undergo regulated secretion, including chromaffin cells, mast cells, and some neurons (reviewed in [32]). Of identified PKC substrates, SNAP-25 and Munc 18 play a role in regulated exocytosis [32]. Munc13-4 plays a role in lytic granule exocytosis [33], but there are no known Munc 18 isoforms in CTLs. SNAP-25, found primarily in excitable cells, is not expressed in TALL-104 cells (A.P.F and A.Z., unpublished observations). However, the ubiquitously expressed SNAP-25 isoform SNAP-23 is expressed in TALL-104 cells, and at least some of it is plasma membrane resident (A.P.F. and A.Z., unpublished observations). PKC-dependent phosphorylation of SNAP-23 is important in exocytosis in platelets [34] and mast cells [35]. SNAP-23 may thus be a PKC substrate in CTLs. Also, PKC likely also participates in upstream steps of the lytic interaction such as granule reorientation [15;16]. Thus, there may be different PKC substrates that are involved in different steps of the lytic interaction. Future experiments will be needed to identify PKC substrates and to identify the step(s) at which they participate.

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Figure 1. PKC-dependent ERK activation is required for TCR-independent lytic granule exocytosis
 A) LAMP-1 externalization is inhibited by the ERK inhibitors PD 98059 and FR180204. (i) Histograms of PE-Anti-LAMP fluorescence for one representative experiment for (from back to front) untreated cells, cells stimulated with TG and PMA (STIM), cells treated with and stimulated in the presence of 100 μ M PD 98059, and cells treated with and stimulated in the presence of 100 μ M FR 180204. (ii) Quantification for three such experiments. Data are normalized to the levels measured in stimulated cells. B) Effect of PKC and ERK inhibitors on ERK phosphorylation. Cells were pretreated with the inhibitors for one hour and then stimulated with TG+PMA in the presence of the inhibitors for 30 minutes. (i) Representative flow histograms of Alexafluor 647-anti-phospho ERK fluorescence levels for (from back to front) untreated cells, cells stimulated with TG+PMA, stimulated cells treated with PD 98059, and stimulated cells treated with Ro31-8220. (ii) Quantification for three such experiments. Asterisks denote values that are significantly different from the stimulated condition. # denote values that are significantly different from the unstimulated condition.

Figure 2. Overexpressing a constitutively-active MAPKK mutant activates ERK

A) Dot plots of GFP or hMCK-GFP expression (x-axis) vs. phospho-ERK levels (y-axis) for GFP transfected cells, hMCK-GFP transfected cells and hMCK-GFP transfected cells treated with 100 μ M PD 98059 for one hour. Superimposed are dot plots of phospho-ERK levels for PMA treated cells (shown in grey). B) (i) Histogram of hMCK-GFP expression. Two gating regions are indicated: NEG corresponding to untransfected cells and POS corresponding to highly transfected cells. (ii) Representative histograms of Alexafluor 647 anti-phospho ERK staining for (from back to front) untreated cells, NEG-gate cells, POS-gate cells and PMA treated cells. (iii) Quantification of 4 such experiments. Data were normalized to the levels measured in PMA treated cells. Asterisks denote values that are significantly different from the untreated condition.

Figure 3. Activating ERK without PKC is not sufficient to support exocytosis in TG-treated cells
 A) (i) Representative histograms of GFP and hMCK-GFP fluorescence intensity. Two gating regions are indicated: NEG, corresponding to untransfected cells, and POS, corresponding to highly expressing cells. (ii) Representative Alexafluor 647 anti-LAMP1 fluorescence histograms from the experiment shown in A for TG treated or TG+PMA-treated (STIM) cells for NEG gate cells and POS gate cells expressing GFP (left) or hMCK-GFP (right). B) Quantification of 3 such experiments. Black bars indicate cells from the NEG gate and white bars indicate cells from the POS gate. Data are normalized to the levels measured in stimulated cells of GFP NEG population.