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

Arun T. Pores-Fernando, Surabhi Gaur, Michael J. Grybko, Adam Zweifach *

Department of Molecular and Cell Biology, University of Connecticut at Storrs, 91 N Eagleville Road, Unit 3125, Storrs, CT 06268-3125, USA

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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.

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 [1–3]). 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 [4–6], activation of extracellular signal regulated kinases (p42/p44 ERKs, members of the mitogen activated protein kinase (MAPK) family [6–8]) and elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) [9,10].

Drugs that increase $[Ca^{2+}]_i$ and activate PKC can promote granule exocytosis in the absence of TCR engagement [11,12]. These stimuli, while not physiological, provide a simplified 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 PKCθ 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 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 [6]. 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 off-target effects of drugs are 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) [18] 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

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* Corresponding author. Fax: +860 486 4331.
E-mail address: adam.zweifach@uconn.edu (A. Zweifach).

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events downstream of TCR engagement [4,8,9,19–21]. 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 ΔN3-S218E–S222D MAPKK Mutant (hMKK) [18] was given to us by Dr. Lynn Heasley (UCHSC, CO). Standard PCR methods were used to subclone it into pEGFPN1 (Clonetech) using engineered KpnI and BamH1 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 h 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 rom 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, MD) and cultured in Modified Dulbecco's Iscoves medium with 10% FCS, 100 IU IL-2, 2 mM 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 [4,20,21]. 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, CA) 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 μM) and FR 180204 (100 μM), and measuring their effects on exocytosis by measuring LAMP-1 externalization. PD 98059 blocks the MAPKKs MEK 1/2 that phosphorylate and activate ERK [22], while FR 180204 blocks substrate phosphorylation by ERK by occluding its ATP-binding site [23]. 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 min in the continued presence of drug or vehicle. TG activates Ca^{2+} influx, while PMA activates PKC. Representative flow histograms are shown in Fig. 1A-i, and quantification of results from three such experiments are shown in Fig. 1A-ii. 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 phosphor-

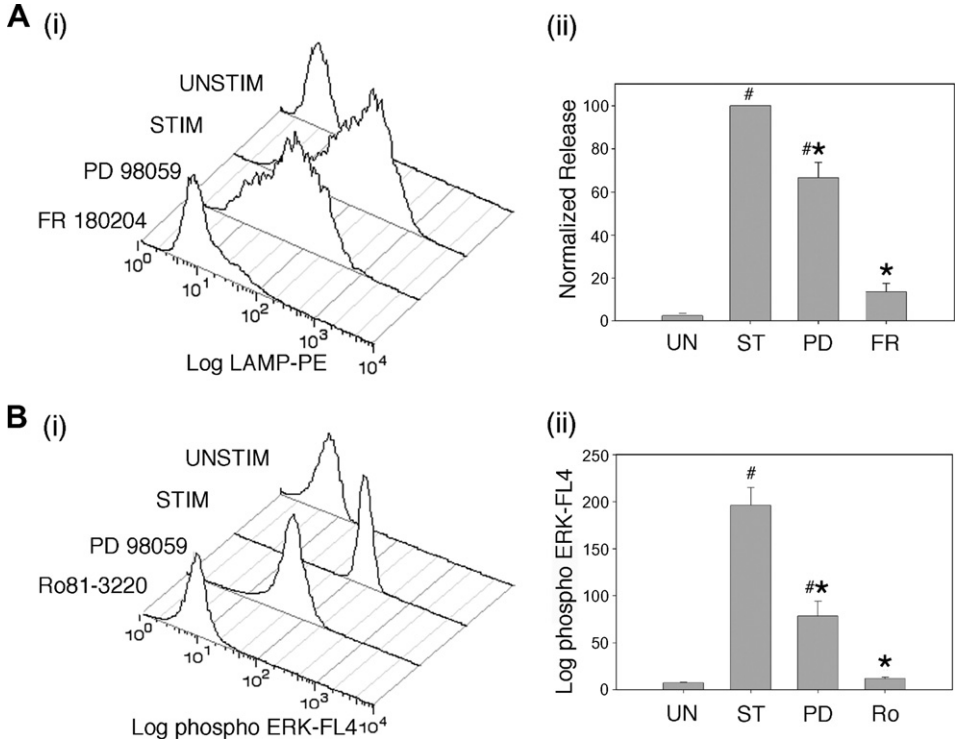

Fig. 1. PKC-dependent ERK activation is required for TCR-independent lytic granule exocytosis. (A) LAMP-1 externalization is inhibited by the ERK inhibitors PD 98059 and FR180204. (i) Histograms of PE-Anti-LAMP fluorescence for one representative experiment for (from back to front) untreated cells, cells stimulated with TG and PMA (STIM), cells treated with and stimulated in the presence of 100 μM PD 98059, and cells treated with and stimulated in the presence of 100 μM 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 min. (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.