

Cross-talk with Ca^{2+} Influx Does Not Underlie the Role of Extracellular Signal-regulated Kinases in Cytotoxic T Lymphocyte Lytic Granule Exocytosis*

Received for publication, January 12, 2004, and in revised form, March 18, 2004
Published, JBC Papers in Press, April 1, 2004, DOI 10.1074/jbc.M400296200

Allan F. Fierro, Georjeana A. Wurth, and Adam Zweifach‡

From the Department of Physiology and Biophysics, University of Colorado Health Sciences Center, Denver, Colorado 80262

One important mechanism cytotoxic T lymphocytes use to kill target cells is exocytosis of lytic granules that contain cytotoxic agents such as perforin and granzyme. Ca^{2+} influx and activation of protein kinase C have been known for many years to be key signals for granule exocytosis. Recent work has suggested that activation of extracellular signal-regulated kinases (ERK), members of the mitogen-activated protein kinase (MAP kinase) family, may be a third required signal. We surmised that the involvement of ERK in lytic granule exocytosis could be mediated through cross-talk with Ca^{2+} influx, rather than constituting an independent signal. We tested this idea using TALL-104 human leukemic CTLs as a model system and discovered the following. 1) ERK inhibition caused a modest decrease in the amplitude of increases in intracellular Ca^{2+} concentration, but this effect cannot account for the profound inhibition of granule exocytosis. 2) Ca^{2+} influx can activate ERK in TALL-104 cells, but this effect does not contribute to ERK activation stimulated by solid phase anti-CD3 monoclonal antibodies. We conclude that cross-talk between ERK signaling and Ca^{2+} does not mediate the role of ERK in CTL lytic granule exocytosis.

Cytotoxic T lymphocytes (CTLs)¹ kill virus-infected cells, tumor cells, and transplanted tissues and organs (1, 2). One important mechanism they use is exocytosis of lytic granules containing cytotoxic agents such as perforin and granzymes. Despite the fundamental importance of this mechanism to immune function, relatively little is known about the intracellular signals that are required for granule exocytosis. Physiologically, stimulation of T lymphocytes occurs via the T cell receptor (TCR) and the associated signaling molecule CD3 (3). Two

kinds of evidence support the idea that activation of protein kinase C (PKC) and elevation of intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), both triggered by TCR cross-linking, are critical events in lytic granule exocytosis. First, stimulation of cells with ionomycin, which elevates $[\text{Ca}^{2+}]_i$, and phorbol esters, which activate PKC, can bypass TCR stimulation (4, 5). Second, TCR-stimulated granule exocytosis is inhibited when activation of PKC is impaired (4, 6, 7) or Ca^{2+} influx is blocked (8–13).

Recent evidence indicates that there may be a third intracellular signaling pathway that is important for granule exocytosis: activation of extracellular signal-regulated kinases (ERKs). ERKs are members of the mitogen-activated protein kinase (MAP kinase) family that also includes the p38 MAP kinases and c-Jun kinases (JNKs) (14, 15). There are two isoforms of ERK, ERK1 and ERK2 (also referred to as p44 ERK and p42 ERK). They are activated upon dual phosphorylation of critical threonine and tyrosine residues by the upstream kinases MEK1 and MEK2. Whereas most work on biological roles of ERK has focused on cell growth, differentiation, and death, recent work has shown that stimuli that trigger CTL exocytosis, such as TCR cross-linking by solid phase anti-CD3 monoclonal antibodies or contact with target cells, activate ERK, and pharmacological inhibitors of ERK block granule exocytosis (16, 17). Similar findings have been reported in natural killer (NK) cells, which also use lytic granule exocytosis to kill targets (18–21). Reorientation of lytic granules and the microtubule-organizing center (MTOC) toward the site of contact with the target cell has been reported to be an ERK-dependent step in target cell killing by both CTLs (16) and NK cells (19).

We suspected that the role of ERK in lytic granule exocytosis, rather than constituting a third independent signaling pathway, could rather be related to the well established requirement for Ca^{2+} influx. We envisioned two possible mechanisms based on published data. First, ERK inhibition has been reported to inhibit capacitative calcium entry (CCE) in platelets (22, 23). As CCE is likely responsible for Ca^{2+} influx in CTLs (24), and exocytosis is dependent on Ca^{2+} influx (8–13), a reduction in CCE due to ERK inhibition would be expected to block granule exocytosis. Second, evidence from a number of cell types (25–27) including T lymphocytes (28, 29) indicates that increases in $[\text{Ca}^{2+}]_i$ due to influx can activate ERK, raising the possibility that activation of ERK could constitute a critical Ca^{2+} -dependent step in granule exocytosis. At present, the identity of molecule(s) that confer Ca^{2+} dependence on lytic granule exocytosis is unclear, although published results have implicated calmodulin (12) and the Ca^{2+} -dependent protein phosphatase calcineurin (30). The idea that the involvement of ERK in lytic granule exocytosis might be mediated by cross-

* This work was supported by National Institutes of Health Grant AI054839. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Physiology and Biophysics, University of Colorado Health Sciences Center, 4200 E. 9th Ave., Denver, CO 80262. Tel.: 303-315-5007; Fax: 303-315-8110; E-mail: adam.zweifach@UCHSC.edu.

¹ The abbreviations used are: CTL, cytotoxic T lymphocyte; TCR, T cell receptor; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; Ca^{2+}_o , extracellular Ca^{2+} ; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; CCE, capacitative calcium entry; MTOC, microtubule-organizing center; PMA, phorbol myristate acetate; mAb, monoclonal antibody; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; 7-AAD, 7-amino-actinomycin D; TG, thapsigargin; BLT, $N\alpha$ -benzyloxycarbonyl-L-lysine thiobenzyl ester; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; NK, natural killer cells.

talk with Ca^{2+} influx has, to our knowledge, never been tested. It is a simpler hypothesis than the idea that there are three independent signaling pathways and could conveniently explain, for example, reports that granule/MTOC reorientation is dependent both on ERK (16, 19) and on Ca^{2+} influx (31, 32).

In the present study, we used TALL-104 human leukemic CTLs (13, 33) to investigate whether the role of ERK in lytic granule exocytosis is mediated by cross-talk with Ca^{2+} influx. Our results indicate that ERK inhibition modestly reduces the magnitude of TCR-stimulated $[\text{Ca}^{2+}]_i$ signals. However, this effect cannot account for the profound inhibition of TCR-stimulated granule exocytosis. Furthermore, while Ca^{2+} influx can activate ERK in TALL-104 cells, this effect does not contribute significantly to ERK activation stimulated via TCR cross-linking. We conclude that cross-talk between Ca^{2+} influx and ERK activation does not account for the role of ERK in lytic granule exocytosis.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Salts for physiological solutions and poly-L-lysine were from Sigma-Aldrich. Fura-2/AM, calcein-AM, and 7-amino-actinomycin D (7-AAD) were from Molecular Probes (Eugene, OR). Thapsigargin was purchased from Alexis Biochemicals (San Diego, CA). Fetal calf serum was from Summit Biotechnology (Ft. Collins, CO). Anti-CD3-coated beads (Dynabeads M-450 Pan T) were purchased from DYNAL A.S. (Oslo, Norway). Rabbit polyclonal antibodies against total ERK and p90^{rsk} were from Cell Signaling (Beverly, MA). Rabbit polyclonal antibodies against dually phosphorylated ERK and PD98059 were from Promega (Madison, WI). Phosphatase inhibitors (set II) and protease inhibitors (set V, no EDTA) were from Calbiochem (San Diego, CA).

Cells and Solutions—TALL-104 cells were obtained from the American Type Culture Collection and grown in Iscove's medium supplemented with 10% fetal calf serum, glutamine, antibiotics, and 100 international units of interleukin-2. Raji human B lymphoma cells were obtained from the American Type Culture Collection and grown in RPMI 1640 supplemented with 10% fetal calf serum, glutamine, and antibiotics. Both cell types were maintained in a humidified incubator at 37 °C in 10% CO_2 . Ringer's solution contained (in mM): 145 NaCl, 4.5 KCl, 1 MgCl_2 , 2 CaCl_2 , 5 HEPES, and 10 glucose (pH 7.4 with NaOH). Zero Ca^{2+} (or Ca^{2+} -free) Ringer's was identical, except CaCl_2 was replaced with MgCl_2 , and 1 mM EGTA was added. For experiments with the MEK inhibitor PD98059 (34), TALL-104 cells were pretreated with the drug at the indicated concentration for 1 h at 37 °C. PD98059 was dissolved in Me_2SO . In all cases, the total Me_2SO concentration was maintained constant at 0.2%, and control cells were treated for 1 h with the same Me_2SO concentration. PD98059 was also included in the experimental solution.

$[\text{Ca}^{2+}]_i$ Imaging—TALL-104 cells were loaded with Fura-2 by incubating them with 1 μM Fura-2/AM in cell culture medium for 30 min at room temperature. Imaging experiments were performed at room temperature with an imaging system described previously (13). For analysis of calcium data, background-subtracted images were thresholded on F340, and ratios were computed pixel by pixel. Ratios were converted to $[\text{Ca}^{2+}]_i$ according to the method of Grynkiewicz *et al.* (35) using calibration values obtained *in vitro*. Data were analyzed further using Igor Pro software (Wavemetrics, Lake Oswego, OR).

Granzyme Release Assays—Granzyme released from TALL-104 cells was assayed by measuring hydrolysis of $N\alpha$ -benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) essentially as described previously (36). TALL-104 cells were suspended at a density of 5×10^7 cells/ml of cell culture medium. 5 μl of the cell suspension (2.5×10^5 cells) was then pipetted into wells of a microtiter plate. 200 μl of appropriate experimental solution was added to each well. Cells were stimulated by adding anti-CD3-coated beads, and then incubated for 50 min at room temperature, followed by centrifugation at $200 \times g$ for 0.5 min. 50 μl of supernatant was transferred to another 96-well plate, and 150 μl of BLT solution (0.2 mM BLT and 0.22 mM 5,5'-dithiobis(2-nitrobenzoic acid) in phosphate-buffered saline (pH 7.2) was added to the supernatant of each condition and allowed to react for 40 min at room temperature. After removal of the remaining supernatant, 200 μl of 0.1% Triton X-100 in phosphate-buffered saline was added to the pellet of each experimental well, and 50 μl of this solution was treated as described above to determine the residual cellular granzyme activity. Absorbance measurements were made either with a Dynatech II plate



FIG. 1. Stimulation of TALL-104 cells by TCR cross-linking activates ERK. A, Western blot of TALL-104 whole cell lysates prepared from unstimulated cells, cells stimulated with 50 nM PMA, and cells stimulated with anti-CD3 beads. The blot was probed with antibodies against total ERK1/2 and p90^{rsk}. B, Western blot of the same lysates as in A probed with antibodies against dually phosphorylated (active) ERK1/2 and p90^{rsk}. Stimulation with PMA or anti-CD3 beads increased active ERK, but did not change total ERK levels.

reader or a Bio-Tek Synergy HT-I plate reader (Bio-Tek Instruments, Winooski, VT) read at 410 nm after subtraction of an appropriate blank. The percent of BLT esterase activity released spontaneously was measured from unstimulated CTLs that were treated identically to stimulated cells, and incubated in normal Ringer's. Release was calculated as $\text{absorbance}_{\text{supernatant}} / \text{absorbance}_{\text{pellet}} + \text{absorbance}_{\text{supernatant}}$. Spontaneous release was subtracted from percent release obtained upon stimulation to obtain percent-specific stimulated release.

CARE-LASS Assay—The CARE-LASS method (37) was used to monitor hitting of target cells. 5×10^4 Raji cells per condition were loaded with 1 μM calcein-AM for 15 min in Normal Ringer's, and 30 min after washing were mixed with TALL-104 cells at CTL: target ratios of 1:1, 2.5:1, 5:1, and 10:1 in 50 μl of Normal Ringer's for 50 min at room temperature in 96-well v-bottom microtiter plates. At the end of the incubation period, the plate was centrifuged, and 50 μl of the supernatant were transferred to a flat-bottomed 96-well plate. The remainder of the supernatant was discarded, and the pellets were lysed by resuspending them in 200 μl of Normal Ringer's containing 1% Triton X-100. 50 μl of this solution was then transferred to the flat-bottomed 96-well plate for analysis. Fluorescence of the supernatant and pellet samples were measured using a Bio-Tek Fx800 fluorescence plate reader equipped with fluorescein excitation and emission filters (Bio-Tek Instruments, Winooski, VT). Specific calcein release was calculated after subtraction of spontaneous release, which was determined for Raji cells incubated without TALL-104 cells for 50 min. Experiments were performed in triplicate.

Measurements of Cellular Viability with 7-AAD—Cells were incubated in phosphate-buffered saline supplemented with 50 $\mu\text{g}/\text{ml}$ 7-AAD for 15 min at room temperature, then washed twice, and kept on ice until they were analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA). As a positive control, cells were electroporated with an Amaxa Nucleofector (Amaxa, Inc., Gaithersburg, MD), using program T-20 and nucleofection solution V.

Western Blotting for Detection of ERK Activation—Whole cell lysates were prepared from $1\text{--}2.5 \times 10^6$ TALL-104 cells per experimental condition by incubating them for >30 min in ice-cold Tris-buffered sample buffer containing 10% CHAPS, protease inhibitor mixture, phosphatase inhibitor mixture, 1 mM EGTA, and 1 mM EDTA. Lysates were centrifuged at $16,000 \times g$ for 10 min at 4 °C. The resulting supernatants were mixed 1:1 with Laemmli sample buffer containing β -mercaptoethanol then boiled for 5 min. Samples were run on Bio-Rad pre-made 10% acrylamide Ready gels and transferred to 0.2 μm nitrocellulose membranes using a wet-transfer apparatus. Blots were blocked with 5% bovine serum albumin in TBS + 0.1% Tween (TBST), then incubated with primary antibodies diluted in 5% bovine serum albumin in TBST overnight at 4 °C. After washing with TBST, blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) for 1.5 h at room temperature. Blots were visualized using enhanced chemiluminescence (Supersignal West Pico reagent, Pierce Biotechnology) and a Kodak 440CF Image Station (Eastman Kodak, New Haven, CT). Blots were quantitated using NIH Image. Mean intensity for identically sized regions of interest was calculated for bands corresponding to total or phosphorylated ERK, for p90^{rsk}, and for appropriate background regions. After background subtraction, ERK intensity in each lane was normalized to p90^{rsk} levels.

Statistics—Data are presented as mean \pm S.E. Statistical significance was assessed using free on-line Student's *t* tests available at www.graphpad.com. *p* values less than 0.05 were taken to indicate statistically significant differences.

RESULTS

TCR Cross-linking Activates ERK in TALL-104 Cells—We used Western blotting to investigate whether stimulating

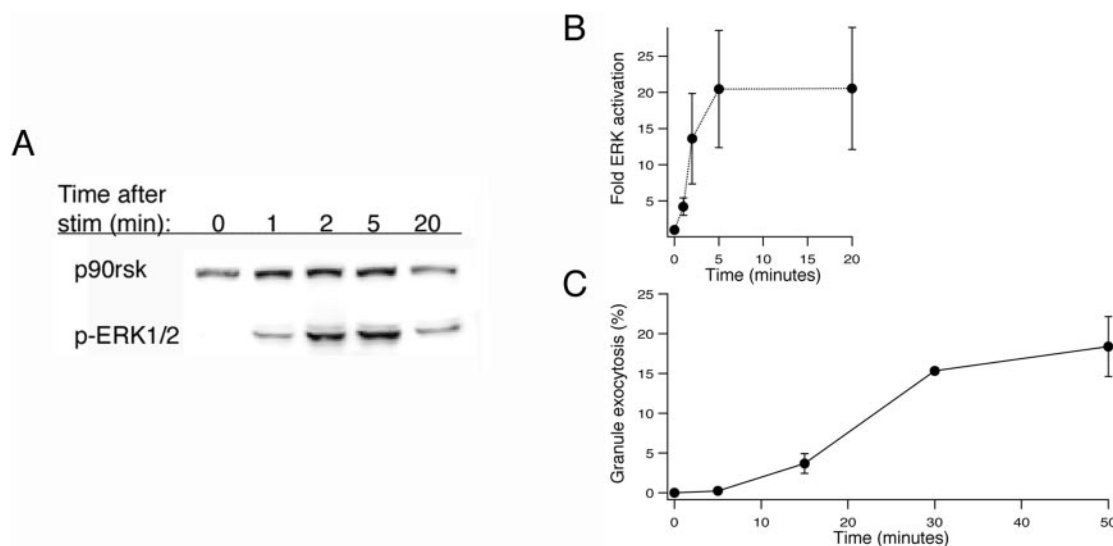


FIG. 2. **Activation of ERK by TCR cross-linking precedes granule exocytosis.** *A*, Western blot of TALL-104 whole cell lysates prepared at the indicated times following stimulation with anti-CD3 beads probed with rabbit polyclonal antibodies to dually phosphorylated ERK1/2 and to p90^{rsk}. Levels of dually phosphorylated (active) ERK increased following stimulation. *B*, average time course of the increase in phospho-ERK from three experiments. Data are normalized to the prestimulation (zero) time point. *C*, time course of granule exocytosis measured using BLT-esterase assays. Comparison of *B* and *C* indicates that ERK activation precedes granule exocytosis.

TALL-104 leukemic CTLs with solid phase anti-CD3 antibodies activates ERK1/2 (Fig. 1). Solid phase anti-CD3 antibodies activate CTLs via TCR cross-linking, and are therefore a physiologically relevant stimulus. Cells were washed and resuspended in Normal Ringer's and then stimulated with 50 nM phorbol myristate acetate (PMA); beads were coated with an anti-CD3 monoclonal antibody (mAb) or not stimulated. Whole cell lysates were prepared 20 min after stimulation and run on a gel in duplicate. After transfer to nitrocellulose, the blot was cut in half and probed either with rabbit polyclonal antibodies against total ERK or against dually phosphorylated (*i.e.* active) ERK. In both cases, the blot was also probed with a rabbit polyclonal antibody specific for p90^{rsk} to serve as a loading control (28, 29). Bands with molecular masses of ~44 and 42 kDa were detected in the blot probed with the antibody recognizing total ERK (Fig. 1A). The intensity of the lower molecular mass band (p42 ERK or ERK2) was always greater than that of the higher molecular mass form, which may suggest a relatively greater abundance of ERK2 in TALL-104 cells, although this could also reflect the affinity of the antibody for the two proteins. Fig. 1B shows that stimulation with PMA or anti-CD3 beads resulted in an increase in levels of active ERK. As was the case with the antibody against total ERK, the intensity of the band corresponding to active ERK2 was higher than that corresponding to active ERK1. Consistent with previous reports (17), we found that stimulating cells with 10 μ g/ml soluble anti-CD3 monoclonal antibody (UCHT1) stimulated neither ERK activation nor granule exocytosis, although it did trigger a modest increase in $[Ca^{2+}]_i$ (data not shown).

To investigate the time course of ERK activation in response to stimulation with solid phase anti-CD3 mAb, cell lysates were prepared at various times following stimulation with beads, and probed for active ERK as described above. Fig. 2A is a representative experiment that shows that contact with beads resulted in a time-dependent increase in the amount of dually phosphorylated ERK. Fig. 2B presents results pooled from three experiments similar to (and including) the one shown in A. ERK activation reached a maximum ~5 min after stimulation with anti-CD3 beads, and remained at this elevated level for at least 20 min.

To compare the temporal relationship between ERK activation and lytic granule exocytosis, we measured the time course

of exocytosis using BLT-esterase assays (36), which monitor the release of granzymes from populations of cells (Fig. 2C). Granule exocytosis had a much slower time course than ERK activation. At 5 min, when ERK was essentially maximally activated, granule exocytosis was barely detectable. That ERK activation precedes granule exocytosis is consistent with the idea that ERK activation is required for granule exocytosis.

Both ERK Activation and Granule Exocytosis Stimulated by TCR Cross-linking Are Blocked by the MEK Inhibitor PD98059—To confirm that the activation of ERK detected above is important for granule exocytosis, we examined the effects on granule exocytosis triggered by anti-CD3 beads of treating cells with PD98059. This compound blocks the upstream kinases MEK1 and MEK2 that phosphorylate and thus activate ERK1/2 (34). Fig. 3A shows that PD98059 inhibited granule exocytosis in a dose-dependent manner. When cells were treated with 50–100 μ M PD98059, granule exocytosis was inhibited by ~75%.

PD98059 treatment also inhibited target cell killing assessed using the CARE-LASS assay (37). This method measures release of the fluorescent dye calcein from target cells into the supernatant as a result of lytic granule exocytosis. Target cells in these experiments were Raji B lymphoma cells that were treated with an anti-CD3-containing bispecific antibody (38). The CARE-LASS assay provides a convenient means of measuring target cell killing: consistent with our previous results (13), killing of Raji cells was not detected using this method in the absence of Ca^{2+}_o or if Raji cells were not pretreated with bispecific antibody (data not shown). As expected based on the inhibition of granule exocytosis detected using BLT-esterase assays (Fig. 3A), we found that treatment of cells with 100 μ M PD98059 inhibited calcein release at all CTL:target ratios tested by ~75% (Fig. 3B).

We next confirmed that PD98059 in fact inhibits ERK activation in response to anti-CD3 beads. Fig. 3C shows that pretreatment of cells with 100 μ M PD98059 for 1-h inhibited bead-stimulated ERK activation. In two such experiments, treatment of cells with 100 μ M PD98059 reduced ERK activation by 80 and 96%.

To ensure that the effects of PD98059 described above do not result from drug-induced cellular toxicity, we used 7-AAD (39) to assess viability (Fig. 4). This fluorescent drug binds to DNA.

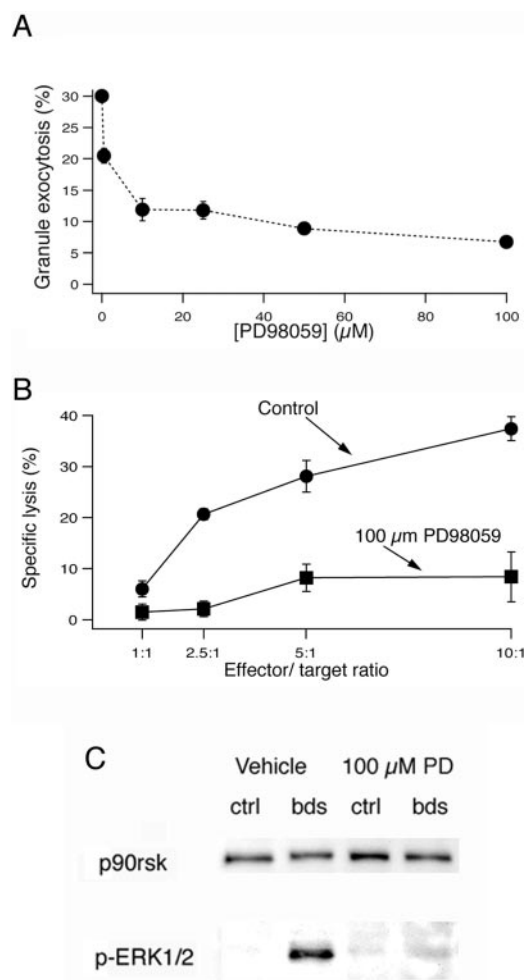


FIG. 3. PD98059 inhibits both ERK phosphorylation and granule exocytosis. *A*, effects of treating cells with various concentrations of PD98059 on granule exocytosis measured using BLT-esterase assays. Cells were stimulated with anti-CD3 beads, and exocytosis was measured 50 min after stimulation. PD98059 caused a dose-dependent inhibition in granule exocytosis. Data are from four experiments. *B*, effects on target cell killing measured using the CARE-LASS assay of treating cells with 100 μM PD98059. Data are from three experiments performed in triplicate. *C*, Western blot of TALL-104 whole cell lysates prepared 20 min after stimulation with anti-CD3 beads probed with rabbit polyclonal antibodies to dually phosphorylated ERK1/2 and to p90^{rsk}. Cells were treated either with vehicle (Me₂SO) or 100 μM PD98059. PD98059 treatment reduces ERK activation.

It is excluded by viable cells, but apoptotic cells are moderately permeable to it, and necrotic cells with compromised plasma membrane integrity are still more permeable. 7-AAD fluorescence therefore displays a three-part distribution. Live cells do not stain with 7-AAD, whereas apoptotic cells show a low level of staining, and necrotic cells show a higher level of staining. Using this probe, we found that treatment with 100 μM PD98059 did not induce significant cell death compared with untreated or vehicle-treated control cells (Fig. 4 shows data that are representative of three similar experiments). In all three cases, >85% of cells were viable. In contrast, when we examined 7-AAD staining 24 h after electroporating cells, we found that ~33% of cells were viable, ~33% were apoptotic, and ~33% were necrotic, confirming that 7-AAD staining can be used to assess viability in TALL-104 cells. Note that the results of [Ca²⁺]_i imaging experiments presented below (Fig. 5) also suggest that cells treated with 100 μM PD98059 are viable, because resting [Ca²⁺]_i was not elevated in drug-treated cells, and significant bead-stimulated [Ca²⁺]_i responses were observed. We might expect non-viable cells to have elevated basal

[Ca²⁺]_i and to fail to respond to beads.

PD98059 Treatment Inhibits [Ca²⁺]_i Signals, but This Effect Does Not Account for the Inhibition of Granule Exocytosis—ERK inhibition has been reported to inhibit CCE in platelets (22, 23). If a similar effect occurred in TALL-104 cells, it would likely inhibit granule exocytosis, because exocytosis in CTLs absolutely requires Ca²⁺ influx (8–13) through channels that are likely CCE channels (24). To examine whether inhibition of [Ca²⁺]_i increases contributes to the effects of PD98059 on granule exocytosis, we measured [Ca²⁺]_i signals stimulated by anti-CD3-coated beads in Fura-2-loaded TALL-104 cells using digital imaging (Fig. 5A). In three sets of experiments in which a total of 355 control cells and 400 cells treated with 100 μM PD98059 were imaged, there was a significant decrease in the peak [Ca²⁺]_i levels in cells treated with 100 μM PD98059 compared with vehicle-treated controls (337 ± 8 nM versus 514 ± 15 nM) (Fig. 5A). Importantly, [Ca²⁺]_i levels at relatively long times (~1200 s after stimulation) were reduced in PD98059-treated cells as compared with vehicle-treated control cells (170 ± 2 nM versus 191 ± 4 nM).

To determine whether the decrease in bead-stimulated [Ca²⁺]_i signals accounts for the inhibition of granule exocytosis caused by PD98059 treatment, we examined the effects on granule exocytosis of decreasing the extracellular Ca²⁺ concentration (Fig. 5B). We measured [Ca²⁺]_i signals in response to bead stimulation at a number of different Ca²⁺_o and found that reducing extracellular Ca²⁺ from 2 to 0.1 mM caused a reduction in both peak and plateau [Ca²⁺]_i levels that was greater than or comparable to the reduction caused by PD98059 treatment. In 398 cells imaged in three experiments in the presence of 0.1 mM Ca²⁺_o, peak [Ca²⁺]_i was reduced more when Ca²⁺_o was lowered to 0.1 mM than when cells were treated with PD98059. Plateau [Ca²⁺]_i ~1200 s after stimulation was comparable: 173 ± 2.5 nM in the presence of 0.1 mM Ca²⁺_o compared with 170 ± 2 nM in PD98059-treated cells. However, the reduction in [Ca²⁺]_i elevation caused by reducing Ca²⁺_o to 0.1 mM did not decrease granule exocytosis (Fig. 5B). We therefore conclude that reduction in [Ca²⁺]_i signals cannot account for the effects of PD98059 on granule exocytosis.

To examine whether inhibition of CCE contributes to the reduction in bead-stimulated [Ca²⁺]_i elevations, we assessed the effects on thapsigargin (TG)-stimulated [Ca²⁺]_i increases of treating cells with 100 μM PD98059 (Fig. 5C). TG activates CCE by inhibiting SERCA (Sarco- and endo-plasmic reticulum Ca²⁺ ATPase) ATPases, causing store depletion (40). We used a protocol commonly employed to assess the magnitude of CCE. Cells were stimulated with TG in Ca²⁺-free extracellular solution, and then Ca²⁺_o was added. Treatment with PD98059 did not affect the release of Ca²⁺ from intracellular stores (*inset i*). In three sets of experiments in which a total of 426 control cells and 296 PD98059-treated cells were imaged, there was a significant decrease in the peak and plateau of the Ca²⁺ rise following Ca²⁺_o addition, but the slope of the [Ca²⁺]_i rise upon Ca²⁺_o addition (*inset ii*) was not lower in PD98059-treated cells compared with vehicle-treated controls (34.4 ± 9 nM/s versus 31.4 ± 3 nM/s).

Ca²⁺ Influx Can Activate ERK, Although This Effect Is Not Important for ERK Activation by TCR Cross-linking—Previous work in primary human T lymphocytes and the Jurkat human leukemic T lymphocyte line indicates that elevation of [Ca²⁺]_i due to influx can activate ERK (28, 29). To confirm whether this is also true in TALL-104 cells, we investigated the effects of applying TG on ERK activation. Fig. 6A shows that stimulating cells with TG in Normal Ringer's increased levels of dually phosphorylated ERK in a time-dependent manner. To confirm that TG stimulation of ERK requires Ca²⁺ influx, cells

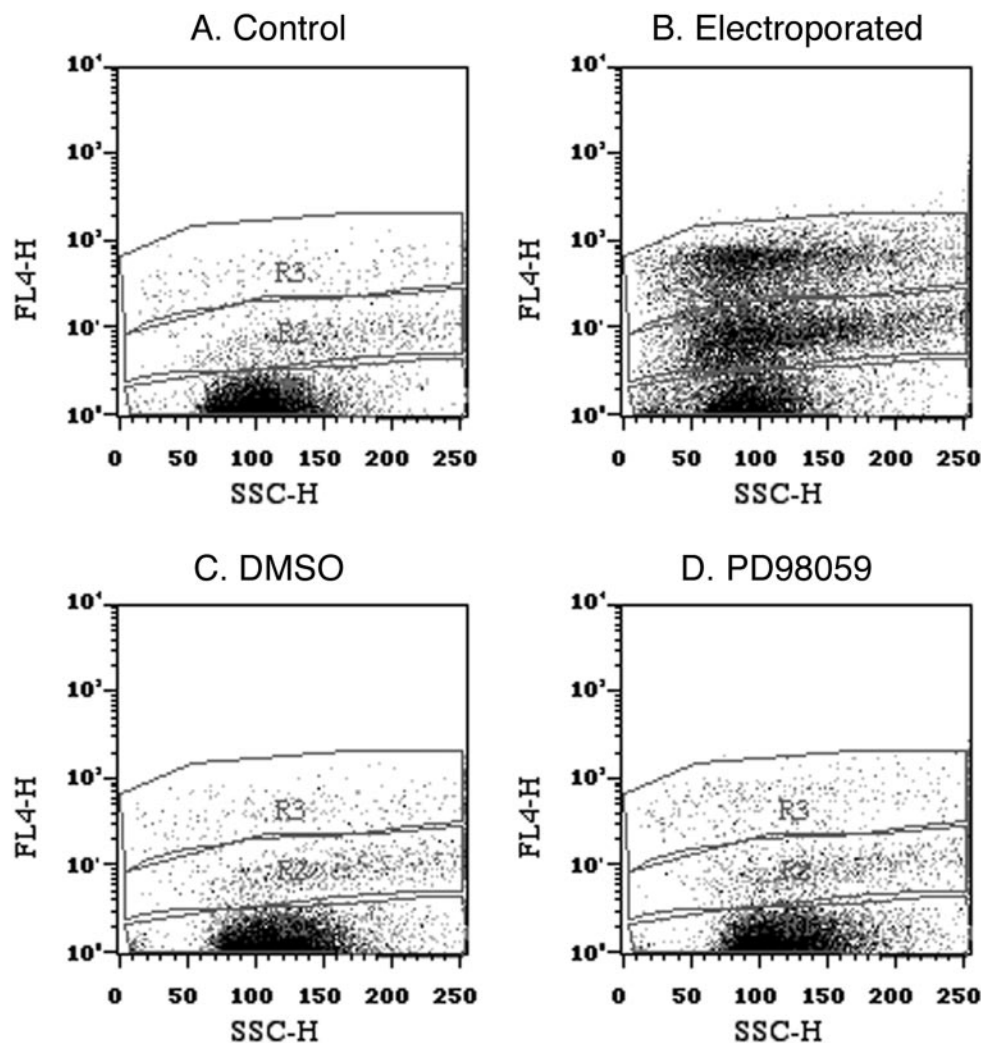


FIG. 4. **Toxicity cannot account for the inhibitory effects of PD98059 on lytic granule exocytosis.** 7-AAD was used to assess viability of untreated control cells (A), vehicle-treated control cells (C), and cells treated with 100 μ M PD98059 (D). Fluorescence was assessed using flow cytometry. Data are plots of 7-AAD fluorescence (FL4-H) versus side scatter (SSC-H). As a positive control, electroporated cells were treated similarly (B). On each plot, three regions are indicated. R1 (the lowest) corresponds to viable cells, R2 (intermediate) to apoptotic cells, and R3 (highest) to necrotic cells. Treatment of cells with Me₂SO (DMSO) or 100 μ M PD98059 did not decrease the percent of viable cells, although electroporation did.

were stimulated with TG in the absence of Ca²⁺_o (Fig. 6B). At 20 min after stimulation with TG, ERK phosphorylation was reduced in the absence of Ca²⁺_o to $18.7 \pm 7\%$ ($n = 3$) of its levels in the presence of Ca²⁺_o. Taken together, the results of Fig. 6 indicate that Ca²⁺ influx can stimulate ERK activation in TALL-104 cells.

To test whether the ability of Ca²⁺ influx to stimulate ERK contributes to bead-stimulated ERK activation, we compared ERK activation in response to anti-CD3 beads in the presence and absence of Ca²⁺_o (Fig. 7). In five such experiments, there was no significant difference in ERK activation in the presence or absence of Ca²⁺_o, indicating that Ca²⁺ influx is not required for full ERK activation in response to TCR stimuli.

DISCUSSION

We have investigated the idea that cross-talk with Ca²⁺ influx underlies the role of ERK in CTL granule exocytosis. Our results indicate that it does not. The reduction in [Ca²⁺]_i signals caused by ERK inhibition cannot account for the reduction in granule exocytosis (Fig. 5), and, although Ca²⁺ influx can activate ERK (Fig. 6), this effect is not important for ERK activation stimulated by solid phase anti-CD3 mAbs (Fig. 7). Our previous work (13, 41) indicates that granule exocytosis in TALL-104 cells exhibits many of the properties characteristic

of CTL granule exocytosis, including dependence on Ca²⁺ influx and PKC, and polarized target-directed exocytosis of lytic granules. The results of this communication demonstrate that granule exocytosis is ERK-dependent. We therefore expect that our results will be applicable to other CTL systems.

Previous work in platelets demonstrated that maximal ERK inhibition reduced the magnitude of CCE by ~50% (22, 23). We found that the peak and plateau of the [Ca²⁺]_i rise upon Ca²⁺_o addition in TG-stimulated cells were reduced by treatment with TG, but the slope of the [Ca²⁺]_i increase was not altered (Fig. 5). The slope of the [Ca²⁺]_i increase is likely the most direct measure of the magnitude of Ca²⁺ influx, although the peak [Ca²⁺]_i has been found in one study to correlate closely (42). The most likely explanation for the discrepancy between our results and the previous work is that the identity of the CCE pathways is different in CTLs and platelets. There are likely multiple types of CCE pathway (43). Influx in CTLs is mediated by a CCE pathway that has the same selectivity for Ca²⁺ over Ba²⁺ and Sr²⁺ as I_{CRAC} in Jurkat T cells, suggesting that I_{CRAC} mediates influx in CTLs (24). The identity of the CCE pathway involved in platelets is not clear. If CCE is not inhibited in TALL-104 cells by treatment with PD98059, what might account for the reduction in the amplitude of the [Ca²⁺]_i

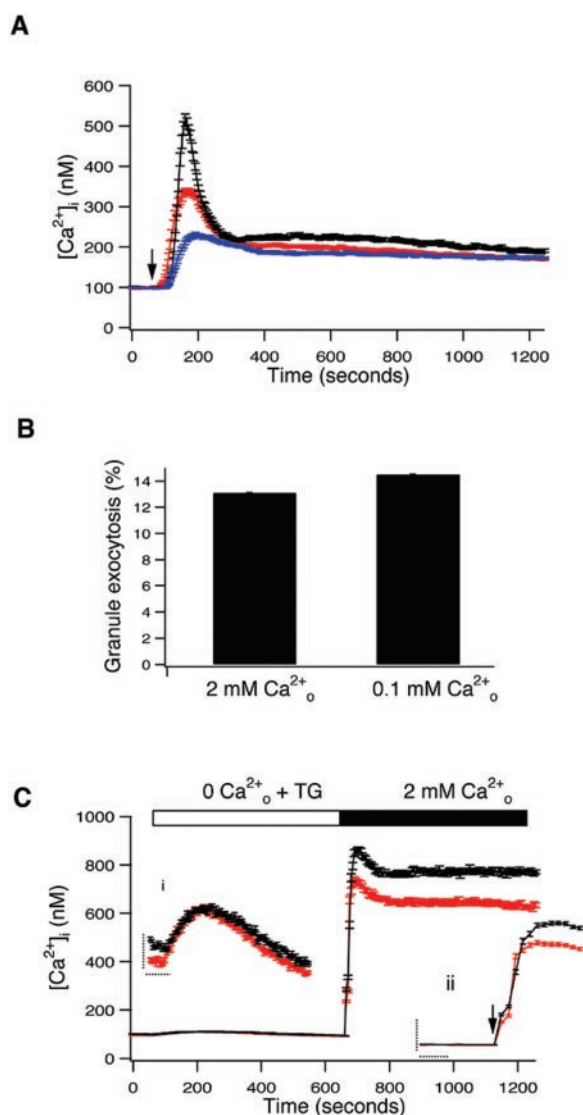


FIG. 5. Inhibition of $[Ca^{2+}]_i$ increases does not account for PD98059 inhibition of granule exocytosis. *A*, $[Ca^{2+}]_i$ increases measured in vehicle-treated cells (black trace), cells treated with 100 μ M PD98059 (red trace), and cells treated with vehicle in the presence of reduced (0.1 mM) Ca^{2+}_o (blue trace). PD98059 treatment reduced the magnitude of $[Ca^{2+}]_i$ increases, but reduction of Ca^{2+}_o to 0.1 mM had a more extreme effect. *B*, inhibition of $[Ca^{2+}]_i$ increases cannot account for decreases in granule exocytosis caused by PD98059 treatment. BLT-esterase assays were used to monitor granule exocytosis from vehicle-treated cells in the presence of 2 or 0.1 mM Ca^{2+}_o . Data are from six experiments. Reducing Ca^{2+}_o did not significantly affect granule exocytosis, indicating that PD98059-induced reduction in $[Ca^{2+}]_i$ increases does not account for inhibition of exocytosis. *C*, TG-stimulated $[Ca^{2+}]_i$ increases in vehicle-treated cells (black traces) and cells treated with 100 μ M PD98059 (red traces). *Inset i* shows the release of Ca^{2+} from stores following treatment with TG. Scale bars indicate 10 nM and 100 s. *Inset ii* shows the rise in $[Ca^{2+}]_i$ upon Ca^{2+}_o addition (arrow). Scale bars indicate 200 nM and 20 s. Neither the release of Ca^{2+} from stores or the slope of the $[Ca^{2+}]_i$ increase upon Ca^{2+}_o addition were affected by treatment with PD98059, but the peak and plateau $[Ca^{2+}]_i$ values after Ca^{2+}_o addition were reduced.

increase following Ca^{2+}_o addition in TG-stimulated cells? One possibility is an alteration in the function of plasma membrane Ca^{2+} -ATPases, which clear Ca^{2+} from the cytoplasm and thus shape $[Ca^{2+}]_i$ signals in T cells (44).

Results obtained from a variety of different cell types, including PC12 cells (27), cardiac fibroblasts (45), astrocytes (26), and T lymphocytes (28, 29) indicate that $[Ca^{2+}]_i$ increases due to influx can activate ERK. Our results are consistent with these

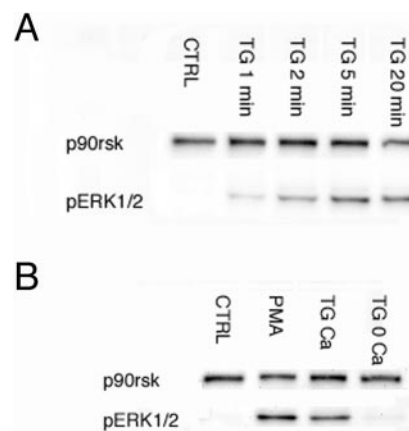


FIG. 6. Ca^{2+} influx activates ERK. *A*, Western blot of TALL-104 whole cell lysates prepared at the indicated times following stimulation with 1 μ M TG in the presence of 2 mM Ca^{2+}_o probed with rabbit polyclonal antibodies to dually phosphorylated ERK1/2 and to p90^{rsk}. Stimulation with TG increased the levels of dually phosphorylated (active) ERK. *B*, Western blot of TALL-104 lysates prepared 20 min after the indicated stimulation. The increase in ERK phosphorylation stimulated by TG was markedly attenuated in the absence of Ca^{2+}_o , indicating that Ca^{2+} influx is required.

previous reports, as we found that treating cells with TG activated ERK in a Ca^{2+}_o -dependent manner (Fig. 6). These results differ from results obtained in platelets (22, 23), in which treatment of cells with TG activated ERK even when changes in $[Ca^{2+}]_i$ were prevented by loading cells with the high affinity fast Ca^{2+} chelator dimethyl BAPTA. Again, the difference in cell types provides the most likely explanation for the difference between platelets and the other cells in which activation of ERK requires Ca^{2+} increases due to influx.

Despite the fact that $[Ca^{2+}]_i$ elevation can activate ERK (Fig. 6), this effect is unlikely to contribute significantly to ERK activation stimulated by TCR cross-linking, as ERK activation stimulated by beads was not significantly different in the presence and absence of Ca^{2+}_o (Fig. 7). These results can readily be understood in light of recent progress in delineating the pathway(s) that activate ERK in T cells. RasGRP (46), a recently identified Ras guanyl nucleotide exchange factor that contains Ca^{2+} -binding EF-hand domains and a diacylglycerol binding domain, is believed to link TCR signaling to Ras activation and thus to ERK activation via the tyrosine kinase-dependent activation of PLC- γ 1 (47). Previous work in T lymphocytes suggests that Ca^{2+} -dependent ERK activation may be mediated via activation of calmodulin-dependent protein kinase, which phosphorylates and activates the tyrosine kinase p56 Lck (29). Lck activation is one of the earliest signaling events stimulated by TCR cross-linking (48), and is likely to play an important role in the activation of PLC- γ 1. TCR stimulation and $[Ca^{2+}]_i$ increases both thus likely activate Lck, which in turn activates PLC- γ 1, thus initiating ERK activation via RasGRP. If this scheme is correct, then it explains why Ca^{2+} influx is not required for bead-stimulated ERK activation: TCR cross-linking could maximally activate the same pathway that influx-dependent $[Ca^{2+}]_i$ increases can activate in the absence of TCR cross-linking. Note that activation of PKC may also play a role in activating ERK in T cells. We found that treating cells with the PKC inhibitor bisindolylmaleimide I reduced bead-stimulated ERK activation 20 min after stimulation by $59 \pm 3\%$ ($n = 6$, data not shown). Both PKC-dependent and PKC-independent activation of ERK have been reported previously in CTLs (49). In that study, analysis of the time course of ERK activation revealed that early ERK activation (times less than ~ 30 min) was PKC-independent, requiring instead participation of a non-PKC diacylglycerol binding protein, likely RasGRP. We

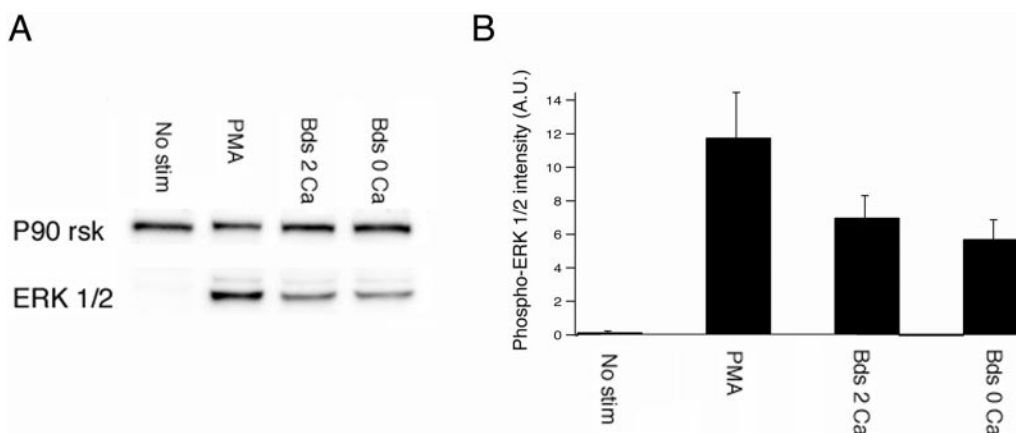


FIG. 7. Ca^{2+} influx is not required for ERK activation stimulated by TCR cross-linking. **A**, Western blot of TALL-104 whole cell lysates prepared from unstimulated (control) cells, cells stimulated with 50 nM PMA, cells stimulated with anti-CD3 beads in the presence of extracellular Ca^{2+} , and cells stimulated with anti-CD3 beads in the absence of extracellular Ca^{2+} . The blot was probed with rabbit polyclonal antibodies to dually phosphorylated ERK1/2 and to p90^{rsk}. **B**, quantitation of results from five experiments like that shown in **A**. Bead-stimulated ERK activation was not significantly different in the presence or absence of Ca^{2+} , indicating that Ca^{2+} influx is not required for ERK activation stimulated by solid phase anti-CD3.

were examining ERK activation at 20 min after stimulation, when, based on the previous results described above, it might be expected to be PKC-independent. However, differences in cell types may account for this discrepancy, as TALL-104 cells are extremely efficient and rapid killers. That PKC likely plays a role in activating ERK raises questions about the possibility of cross-talk between PKC and ERK. Future work will be required to determine whether PKC and ERK play independent roles in lytic granule exocytosis, or whether the sole role of PKC is ERK activation.

Reports from CTLs (16) and NK cells (19) are consistent with the idea that reorientation of the MTOC and lytic granules toward the site of contact with the target are inhibited when ERK activation is blocked. However, granule/MTOC reorientation may not be the only process that is dependent on ERK activation. Our observations indicate that treating cells with 100 μM PD98059 inhibits granule exocytosis elicited by stimulating cells with 1 μM TG and 50 nM PMA.² PD98059-treated cells released $29.1 \pm 2.9\%$ of their total BLT esterase activity, compared with release of $44.5 \pm 1.3\%$ of BLT esterase activity in vehicle-treated controls, a difference that is statistically significant ($p = 0.001$, $n = 6$). If, as seems likely to us, exocytosis stimulated by TG and PMA occurs without granule/MTOC reorientation, these results may also be consistent with the possibility that the actual fusion of lytic granules with the plasma membrane is also ERK-dependent.

Acknowledgments—We thank Drs. Bruce G. Wallace and S.R. Levinson for their patient help in instructing us in Western blotting techniques.

REFERENCES

- Berke, G. (1994) *Annu. Rev. Immunol.* **12**, 736–753
- Berke, G. (1995) *Cell* **81**, 9–12
- Lewis, R. S. (2001) *Annu. Rev. Immunol.* **19**, 497–521
- Langki, D. W., Weiss, A., and Fitch, F. W. (1987) *J. Immunol.* **138**, 3646–3653
- Nishimura, T., Burakoff, S. J., and Herrmann, S. H. (1987) *J. Immunol.* **139**, 2888–2891
- Langlet, C., and Schmitt-Verhulst, A. M. (1992) *J. Immunol. Methods* **151**, 107–115
- Juszczak, R. J., and Russell, J. H. (1989) *J. Biol. Chem.* **264**, 810–815
- Haverstick, D. M., Engelhard, V. H., and Gray, I. S. (1991) *J. Immunol.* **146**, 3306–3313
- Esser, M. T., Haverstick, D. M., Fuller, C. L., Gullo, C. A., and Bracale, V. L. (1998) *J. Exp. Med.* **187**, 1057–1067
- Fortier, A. H., Nacy, C. A., and Sitkovsky, M. V. (1989) *Cell. Immunol.* **124**, 64–76
- Sitkovsky, M. V. (1988) *Immunol. Rev.* **103**, 127–160
- Takayama, H., and Sitkovsky, M. (1987) *J. Exp. Med.* **166**, 725–743
- Lyubchenko, T. A., Wurth, G. A., and Zweifach, A. (2001) *Immunity* **15**, 847–859
- Platanias, L. C. (2003) *Blood* **101**, 4667–4679
- Johnson, G. L., and Lapadat, R. (2002) *Science* **298**, 1911–1912
- Lilic, M., Kulig, K., Messaoudi, I., Remus, K., Jankovic, M., Nikolic-Zugic, J., and Vukmanovic, S. (1999) *Eur. J. Immunol.* **29**, 3971–3977
- Berg, N. N., Puente, L. G., Dawicki, W., and Ostergaard, H. L. (1998) *J. Immunol.* **161**, 2919–2924
- Djeu, J. Y., Jiang, K., and Wei, S. (2002) *Clin. Cancer Res.* **8**, 636–640
- Jiang, K., Zhong, B., Gilvary, D. L., Corliss, B. C., Hong-Geller, E., Wei, S., and Djeu, J. Y. (2000) *Nat. Immunol.* **1**, 419–425
- Trotta, R., Puorro, K. A., Paroli, M., Azzoni, L., Abebe, B., Eisenlohr, L. C., and Perussia, B. (1998) *J. Immunol.* **161**, 6648–6656
- Milella, M., Gismondi, A., Roncioli, P., Bisogno, L., Palmieri, G., Frati, L., Cifone, M. G., and Santoni, A. (1997) *J. Immunol.* **158**, 3148–3154
- Rosado, J. A., and Sage, S. O. (2001) *J. Biol. Chem.* **276**, 15659–15665
- Rosado, J. A., and Sage, S. O. (2002) *Trends Cardiovasc. Med.* **12**, 229–234
- Zweifach, A. (2000) *J. Cell Biol.* **148**, 603–614
- Elzi, D. J., Bjornsen, A. J., MacKenzie, T., Wyman, T. H., and Silliman, C. C. (2001) *Am. J. Physiol. Cell Physiol.* **281**, C350–360
- Schliess, F., Sinning, R., Fischer, R., Schmalenbach, C., and Haussinger, D. (1996) *Biochem. J.* **320**, 167–171
- Egea, J., Espinet, C., and Comella, J. X. (1999) *J. Biol. Chem.* **274**, 75–85
- Atherfold, P. A., Norris, M. S., Robinson, P. J., Gelfand, E. W., and Franklin, R. A. (1999) *Mol. Immunol.* **36**, 543–549
- Franklin, R. A., Atherfold, P. A., and McCubrey, J. A. (2000) *Mol. Immunol.* **37**, 675–683
- Dutz, J. P., Fruman, D. A., Burakoff, S. J., and Bierer, B. E. (1993) *J. Immunol.* **150**, 2591–2598
- Kupfer, A., Dennert, G., and Singer, S. J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 7224–7228
- Kupfer, A., and Dennert, G. (1984) *J. Immunol.* **133**, 2762–2766
- Cesano, A., and Santoli, D. (1992) *In Vitro Cell. Dev. Biol.* **28A**, 648–656
- Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saitel, A. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7686–7689
- Gryniewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Takayama, H., Trenn, G., and Sitkovsky, M. V. (1987) *J. Immunol. Methods* **104**, 183–190
- Lichtenfels, R., Biddison, W. E., Schulz, H., Vogt, A. B., and Martin, R. (1994) *J. Immunol. Methods* **172**, 227–239
- Haagen, I. A., van de Griend, R., Clark, M., Geerars, A., Bast, B., and de Gast, B. (1992) *Clin. Exp. Immunol.* **90**, 368–375
- Vezina, J., Grossmuller, F., and Muller, K. (2001) *J. Immunol. Methods* **252**, 163–169
- Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R., and Dawson, A. P. (1990) *77*, 2466–2470
- Lyubchenko, T. A., Wurth, G. A., and Zweifach, A. (2003) *J. Physiol.* **547**, 835–847
- Fanger, C. M., Hoth, M., Crabtree, G. R., and Lewis, R. S. (1995) *J. Cell Biol.* **131**, 655–667
- Parekh, A. B., and Penner, R. (1997) *Physiol. Rev.* **77**, 901–930
- Bautista, D. M., Hoth, M., and Lewis, R. S. (2002) *J. Physiol.* **541**, 877–894
- Murray, A. W. (1992) *Nature* **359**, 599–604
- Ebinu, J. O., Bottorff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J., and Stone, J. C. (1998) *Science* **280**, 1082–1086
- Ebinu, J. O., Stang, S. L., Teixeira, C., Bottorff, D. A., Hooton, J., Blumberg, P. M., Barry, M., Bleakley, R. C., Ostergaard, H. L., and Stone, J. C. (2000) *Blood* **95**, 3199–3203
- Zamoyaska, R., Basson, A., Filby, A., Legname, G., Lovatt, M., and Seddon, B. (2003) *Immunol. Rev.* **191**, 107–118
- Puente, L. G., Stone, J. C., and Ostergaard, H. L. (2000) *J. Immunol.* **165**, 6865–6871

² A. Zweifach and G. A. Wurth, unpublished observations.

**Cross-talk with Ca²⁺ Influx Does Not Underlie the Role of Extracellular
Signal-regulated Kinases in Cytotoxic T Lymphocyte Lytic Granule Exocytosis**

Allan F. Fierro, Georjeana A. Wurth and Adam Zweifach

J. Biol. Chem. 2004, 279:25646-25652.

doi: 10.1074/jbc.M400296200 originally published online April 1, 2004

Access the most updated version of this article at doi: [10.1074/jbc.M400296200](https://doi.org/10.1074/jbc.M400296200)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 47 references, 24 of which can be accessed free at
<http://www.jbc.org/content/279/24/25646.full.html#ref-list-1>