Calcineurin Activation Is Only One Calcium-dependent Step in Cytotoxic T Lymphocyte Granule Exocytosis*

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We have tested the idea that calcineurin, a calcium-dependent phosphatase that is critical for activating cytokine gene expression in helper T cells, plays a role in lytic granule exocytosis in cytotoxic T lymphocytes (CTLs). We used TALL-104 human leukemic CTLs as a model. Our results confirm an earlier report (Dutz, J. P., Fruman, D. A., Burakoff, S. J., and Bierer, B. E. (1993) J. Immunol. 150, 2591–2598) that immunosuppressive drugs inhibit exocytosis in CTLs stimulated either via the T cell receptor (TCR) or via TCR-independent soluble agents. Of the two recently reported alternate targets of immunosuppressive drugs (Matsuda, S., Shibasaki, F., Takehana, K., Mori, H., Nishida, E., and Koyasu, S. (2000) EMBO Rep. 1, 428–434 and Matsuda, S., and Koyasu, S. (2000) Immunopharmacology 47, 119–125), JNK is not required for lytic granule exocytosis, but we were not able to exclude a role for P38. Exocytosis could be inhibited by expressing GFP fused to a C-terminal fragment of CAIN (cabin 1), but not by expressing VIVIT-GFP. Finally, expressing either full-length or truncated constitutively active mutant calcineurin A enhanced lytic granule exocytosis. However, the mutant calcineurin was unable to support exocytosis when cells were stimulated in the absence of Ca\(^{2+}\) influx. Taken together, our results support the idea that activation of calcineurin is required for lytic granule exocytosis but suggest that it is not the sole Ca\(^{2+}\)-dependent step.

Cytotoxic T lymphocytes (CTLs)² play critical roles in antiviral immunity and in the immune response to tumors and transplants (4–6). One of the key mechanisms they use to kill target cells is exocytosis of lytic granules, specialized preformed secretory lysosomes that contain cell-killing agents such as perforin and granzymes. Despite the importance of this mechanism to immune function, knowledge of the signals underlying granule exocytosis remains fairly rudimentary. A great deal of evidence indicates that increases in intracellular calcium ([Ca\(^{2+}\)]) are required, (7–9), but the molecule(s) that couple [Ca\(^{2+}\)] increases to exocytosis remain unidentified.

It has been known for over a decade that the immunosuppressive drugs FK506 and cyclosporin A (CsA) inhibit lytic granule exocytosis stimulated via the T cell receptor (TCR) or via soluble TCR-independent chemicals that increase [Ca\(^{2+}\)] and activate protein kinase C (1). FK506 and CsA, when bound to proteins called FKBP (FK 506 binding proteins) and cyclophilins, respectively, are known to act as potent inhibitors of the Ca\(^{2+}\)-dependent phosphatase calcineurin (formerly designated CAIN) (10, 11). The effects of immunosuppressive drugs on CTLs therefore suggest a role for calcineurin in granule exocytosis. Consistent with this idea, CTLs were shown to express calcineurin using Western blotting, and the effects of immunosuppressive drugs on exocytosis were found to occur at concentrations that inhibited calcineurin using in vitro phosphatase assays (1). Calcineurin is believed to play a key role in mediating the Ca\(^{2+}\) dependence of helper T cell activation (12). The effects of immunosuppressive drugs on lytic granule exocytosis therefore raise the intriguing possibility that calcineurin activation is a central calcium-dependent event in both helper T cell and CTL activation.

Recently, however, additional important alternate signaling targets of FK506 and CsA have been identified. C-Jun N-terminal kinase (JNK) and P38, which are members of the mitogen-activated protein kinase family that also includes the extracellular signal-regulated kinases, have been shown to be blocked by immunosuppressive drugs in a calcineurin-independent manner (2, 3). Activation of extracellular signal-regulated kinase, a third member of the mitogen-activated protein kinase family, has been shown recently to be required for CTL granule exocytosis (13, 14), but the requirement for activation of JNK or P38 is not known. Importantly, the identification of alternate pharmacological targets of immunosuppressive drugs means that the effects of immunosuppressive agents can no longer be unambiguously taken as evidence for a role for calcineurin in lytic granule exocytosis.

In the present work, we have tested the idea that calcineurin is involved in granule exocytosis, using TALL-104 human leukemic CTLs (15), having previously provided evidence that these cells recapitulate key features characteristic of lytic granule exocytosis (13, 16). Consistent with previous work, we find that immunosuppressive drugs inhibit exocytosis stimulated either via the TCR or by TCR-independent chemicals. We provide evidence that JNK activation is not required for exocytosis but were unable to completely exclude the participation of P38.

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* This work was supported by National Institutes of Health Grant RO1AI054839 (to A. Z.). 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.

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2 The abbreviations used are: CTL, cytotoxic T lymphocyte; TCR, T cell receptor; ([Ca\(^{2+}\)])\(\text{intracellular free}\); IL-2, interleukin-2; JNK, c-Jun N-terminal kinase; LAMP, lysosome-associated membrane protein; PMCA, phorbolester-activated; TG, thapsigargin; GFP, green fluorescent protein; YFP, yellow fluorescent protein; CnAmut-GFP, mutant constitutively active calcineurin A fused to GFP; CnA-YFP, wild-type calcineurin A fused to YFP; BLT, \(N^1\)-benzoyloxy carbonyl-L-lysine thiobenzyl ester; PE, phycoerythrin; CsA, cyclosporin A.
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To gather positive evidence supporting a role of calcineurin, we exploited a new assay of exocytosis that detects exposure of lysosome-associated membrane protein-1 (LAMP-1) to the extracellular solution as a consequence of exocytosis (17–21) to examine the effects of expressing calcineurin-inhibitory peptides and wild-type or mutant constitutively active Ca$^{2+}$-independent calcineurin A on exocytosis. Taken together, our results provide strong support for the idea that calcineurin activation is required for lytic granule exocytosis. However, results with mutant constitutively active Ca$^{2+}$-independent calcineurin A suggest that activation of calcineurin is unlikely to be the sole Ca$^{2+}$-dependent step.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Salts for physiological solutions were from Sigma-Aldrich. Fetal calf serum was from Atlas Biologicals (Ft. Collins, Colorado). Thapsigargin and PMA were from Alexis Biochemicals (San Diego, CA). Mouse IgG anti-CD107a (clone H4A3) was purchased from BD Biosciences. Dynabeads M450 anti-human pan T cell beads (Dynal Invitrogen, Brown Deer, WI) were used for solid-phase anti-CD3 stimulation.

Cells and Solutions—TALL-104 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Isco's medium supplemented with 10% fetal calf serum and 100 IU IL-2. They were grown 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). Ringer's solution with 0.5 mM Ca$^{2+}$ was identical, except that the total Ca$^{2+}$ added was 0.5 mM. Ca$^{2+}$-free Ringer's solution contained 145 NaCl, 4.5 KCl, 1 MgCl$_2$, 1 mM EGTA, 5 HEPES, and 10 glucose (pH 7.4 with NaOH).

N$^a$-Benzyloxy carbonyl-$\gamma$-lysine Thio benzyl ester (BLT) Esterase Assays—Granzyme B released from TALL-104 cells was assayed by measuring hydrolysis of BLT essentially as described previously (13, 20, 22). Absorbance measurements were made with a Bio-Tek Synergy HT-I plate reader (Bio-Tek Instruments, Winooski, VT) read at 410 nm after subtraction of an absorbance supernatant/absorbance pellet + absorbance supernatant. Spontaneous release was subtracted from percentage release obtained upon stimulation to obtain percentage specific stimulated release.

Immunoprecipitation Analysis of JNK Activity—To measure JNK activity, an immunoprecipitation kit from EMD/Calbiochem (San Diego, CA) was used according to the manufacturer's instructions. Following immunoprecipitation of JNK from cell lysates, the immunoprecipitate was incubated with c-JUN. The reaction product was then run on Bio-Rad premade 10% acrylamide Ready gels (Bio-Rad) and transferred to 0.2-$\mu$m nitrocellulose membranes using a wet-transfer apparatus. Blots were then probed with an anti-phospho-c-JUN antibody and detected with an horseradish peroxidase-conjugated secondary. Blots were visualized using enhanced chemiluminescence (Supersignal West Pico reagent; Pierce) and a Kodak 440CF Image Station (Eastman Kodak Co.).

Flow Cytometry—Flow cytometry was performed on two instruments: a Cytomics FC500 (Beckman Coulter, Miami, FL) at the University of Colorado Health Sciences Center and a FACSCalibur (BD Biosciences) at the University of Connecticut. For flow cytometry to detect LAMP externalization, cells were first washed with normal Ringer's and then stimulated as indicated in the conditions described. The anti-LAMP antibody was concentrated 20-fold from the stock provided by the manufacturer using Millipore Amicon columns with a 5000 molecular weight cutoff (Millipore, Bedford, MA), then diluted 1:50 before use. For experiments to measure IL-2 production, Jurkat human leukemic T cells were treated with monensin (GolgiStop; BD Biosciences) starting 1 h after stimulation to inhibit IL-2 secretion. They were fixed, permeabilized, and stained with PE-conjugated anti-IL-2 monoclonal antibody using Caltag’s Fix/Perm kit (Caltag Laboratories/Invitrogen) according to the manufacturer's protocol. For all flow cytometry experiments, cells were suspended in phosphate-buffered saline supplemented with 0.2–0.5% paraformaldehyde after processing. FlowJo software (TreeStar, Ashland, OR) was used to analyze data offline. For multicolor experiments, data were acquired without hardware compensation. Unstained and single-color controls were run, and data were compensated off-line using FlowJo.

cDNA Constructs—pEGFP was obtained from Clontech (Mountain View, CA). GFP-C-AIN (GFP upstream of bases 1881–2173 of CAIN (23)) was generously provided by Dr. Jianjie Ma (University of Medicine and Dentistry of New Jersey/Rutgers). VIVIT-GFP was obtained from Dr. Mark Dell’Acqua (University of Colorado Health Sciences Center at Fitzsimons) who obtained it from Dr. Patrick Hogan (The Center for Blood Research, Harvard University). This construct consists of the sequence MAGPHPVIVTGHEGPPVASM in pEGFP-N1. Mouse calcineurin A in pYFP-N2 (24) was also obtained from Dr. Dell’Acqua. Mutant constitutively active Ca$^{2+}$-independent calcineurin in PBj5 was obtained from Dr. Neil Clipstone (Loyola University/Stritch Medical School). This construct comprises the first 395 amino acids of murine calcineurin A α4. PCR was used to add Xhol and BamHI sites that were then used to insert the coding sequence into pEGFP-N1.

Transfections—TALL-104 cells were transfected using an Amaxa Nucleofector (Amaxa Biosystems; Gaithersburg, MD) using program T-20 and solution V. Cells were used 5–7 h post-transfection. Jurkat cells were transfected with Lipofectamine (Invitrogen). $1 \times 10^6$ cells were transfected by incubating them in a preformed mixture of 2 µg of DNA and 10 µl of Lipofetamine in 100 µl of Opti-MEM (Invitrogen) for 3–4 h, followed by addition of complete RPMI. Cells were used 14–16 h later.

Statistics—Statistical significance was assessed (for paired data with repeated measures analysis of variance; for unpaired data with single measure analysis of variance) using InStat (Graphpad Software, San Diego, CA).

RESULTS

Immunosuppressive Drugs Inhibit Both TCR-dependent and -independent Lytic Granule Exocytosis—We first confirmed that cyclosporin A (CsA) and FK506 inhibit TCR-stimulated...
lytic granule exocytosis in TALL-104 human leukemic CTLs, as has been reported for murine CTLs previously (1). For these experiments (Fig. 1A), cells were stimulated with polystyrene beads coated with anti-CD3 monoclonal antibodies. Exocytosis was assessed by measuring release of granzyme B using BLT-esterase assays. Both drugs inhibited bead-stimulated exocytosis. FK506 inhibited exocytosis at lower concentrations than CsA, consistent with the known affinity of drug-immunophilin complexes.

We also investigated whether immunosuppressive drugs inhibit exocytosis stimulated by TG and PMA, soluble drugs that bypass the TCR by activating Ca\(^{2+}\) influx and protein kinase C, respectively (Fig. 1, A and B). We used both BLT-esterase assays (Fig. 1A) and externalization of LAMP-1 (Fig. 1B) to test this. Results obtained with both methods support the idea that immunosuppressive drugs block TCR-independent exocytosis.

**JNK Activation Is Not Required for Lytic Granule Exocytosis, but a Role for P38 Is Not Excluded**—As an initial means of probing the involvement of JNK and P38 in lytic granule exocytosis, we used pharmacological agents that inhibit these kinases (Fig. 1, A and B). SP600125, a JNK inhibitor (25), blocked granule exocytosis. This result potentially supports a role for JNK in lytic granule exocytosis. To explore this further, we performed two kinds of experiment. First, we examined the effects of N\(^4\)-methyl-1,9-pyrazolanthrone (25), a compound that is chemically related to SP600125 but has been shown to have essentially no JNK inhibitory activity. We found that N\(^4\)-methyl-1,9-pyrazolanthrone completely blocked lytic granule exocytosis (Fig. 2A), a result that is inconsistent with the idea that the effects of SP600125 reflect a requirement for JNK activation in granule exocytosis. Second, we immunoprecipitated JNK from TALL-104 lysates treated in various ways and assessed its activation by monitoring its ability to phosphorylate recombinant c-JUN. An antibody raised against phosphorylated c-JUN was then used to assess phospho-cJUN levels (Fig. 2C). Stimulation with neither anti-CD3 beads or with TG and PMA caused robust activation of JNK compared with treatment of cells with anisomycin, a compound that is known to activate JNK.

As an initial means of investigating the role of P38, we used the compound SB203580, which blocks P38\(\alpha\) and P38\(\beta\) but not P38\(\gamma\) or P38\(\delta\). This compound had no effect on lytic granule exocytosis at either 100 or 500 nm, concentrations that have been shown to be effective in blocking interferon-\(\gamma\) production in helper T cells (26). However, we were unsuccessful in our attempts to assess P38 activation using immunoprecipitation assays. Considering the apparently nonspecific effects of SP600125 we observed, we decided not to pursue further pharmacological studies to probe the role of P38 in lytic granule exocytosis. We therefore cannot exclude a role of P38.

**Effects of Expressing GFP-C-CAIN or VIVIT-GFP on Lytic Granule Exocytosis**—JNK and P38 are the two currently known non-calcineurin targets of immunosuppressant drugs, but it is always possible that there are even more that have not yet been discovered. We therefore decided that, rather than trying to exclude molecules that could be alternate targets of immunosuppressive drugs, we would try to gather positive evidence for calcineurin involvement. As one means of doing this, we explored the effects of expressing VIVIT-GFP or the C-terminal of CAIN fused to GFP (GFP-C-CAIN). VIVIT is a higher affinity peptide analog of the PxxIIT docking motif found on NFAT to which calcineurin binds (27). VIVIT inhibits calcineurin binding to substrates via the PxxIIT motif but does not inhibit calcineurin phosphatase activity. CAIN (also known as CABIN) is an endogenous inhibitor of calcineurin. It binds to calcineurin via a PxxIIT motif and inhibits calcineurin phosphatase activity (28, 29). The C-terminus (C-CAIN) has been reported to be necessary and sufficient for this effect.

As a means of determining whether these peptides affect calcineurin activity at attainable levels of expression, we first
examined their effects on interleukin-2 synthesis in Jurkat human leukemic T cells (Fig. 3A). Fig. 3A shows histograms of PE-conjugated anti-IL-2 staining for unstimulated cells (dashed line) or for cells stimulated with TG + PMA (solid line). Monensin was added 1 h after stimulation to inhibit IL-2 secretion. After a further 5–7-h incubation, cells were fixed, permeabilized, and stained and then analyzed using flow cytometry. Typically, untransfected stimulated cells were 8–20% IL-2+ as assessed by gating on the unstimulated cell population.

We transfected Jurkat cells with cDNAs encoding GFP, VIVIT-GFP, or GFP-C-CAIN and then stimulated them and processed them as described above. Fig. 3B shows a plot of GFP fluorescence for cells transfected with GFP alone. Profiles for cells transfected with VIVIT-GFP or GFP-C-CAIN were similar. The numbered bars correspond to gating regions that were then used to analyze effects on IL-2 production. Fig. 3C shows histograms of PE-anti IL-2 fluorescence for cells from the gating regions shown in B. Non-expressers (GFP-negative cells) are shown at the back, and moving forward corresponds to progressively higher levels of expression. Fig. 3D shows pooled normalized results from three experiments comparing the effects of expressing GFP to expressing VIVIT-GFP or comparing expressing GFP to expressing GFP-C-CAIN. Expressing GFP alone had no effect on IL-2 production. In contrast, at all but the lowest level of expression analyzed, both VIVIT-GFP and GFP-C-CAIN significantly inhibited IL-2 production compared either to non-expressing cells in the same samples or to cells that expressed GFP alone. There was no statistical difference in efficacy between the two constructs.

To test the effects of these constructs on lytic granule exocytosis, we transiently transfected TALL-104 cells and measured exocytosis using LAMP externalization (Fig. 4). We have previously shown that this method is useful for assessing effects of expressing GFP fusion proteins on TCR-independent lytic granule exocytosis (21). It allows effects on viable transfected cells with given levels of expression to be monitored in a simple flow cytometry-based assay. Fig. 4A shows a representative experiment in which GFP was expressed, Fig. 4B shows a representative GFP-C-CAIN experiment, and Fig. 4C shows an experiment in which VIVIT-GFP was expressed. In all three panels, the top histogram plots GFP fluorescence intensity. The numbered bars are gating regions that were used to analyze PE-anti-LAMP-1 fluorescence. The bottom part of each panel shows histograms of PE anti-LAMP-1 fluorescence for the different gating regions. Histograms from GFP-negative cells (non-expressers, gating region 1) are at the back, while histograms from cells with increasing levels of expression are arranged toward the front. There was no effect apparent of expressing GFP alone or VIVIT-GFP at any level. However, expressing increasing amounts of GFP-C-CAIN resulted in a shift of the PE-anti-LAMP-1 fluorescence distribution toward lower levels. C presents pooled data from three paired experiments comparing GFP to GFP-C-CAIN. We compared cells with increasing levels of expression of either GFP or GFP-C-CAIN to non-expressing cells from the same samples (cells from region 1). We found that expressing GFP alone had no significant effect on exocytosis, while GFP-C-CAIN-expressing cells from gating regions 3 and 4 had reduced exocytosis compared with non-expressers. Furthermore, GFP-C-CAIN-expressing cells from gating regions 3 and 4 had significantly reduced exocytosis compared with cells expressing GFP at comparable levels.
We also conducted preliminary LAMP externalization experiments with a fusion of YFP to amino acids 321–360 of AKAP-79 (data not shown). AKAP-79 has been reported to act like C-CAIN, inhibiting calcineurin phosphatase activity, and the truncation has been reported to be more effective than the full-length protein (30). We found that the truncated AKAP construct had no inhibitory effect on lytic granule exocytosis. We did not test its effects on IL-2 production.

Effects of Expressing Mutant Ca\(^{2+}\)-independent Constitutively Active Calcineurin A or Wild-type Calcineurin A on Granule Exocytosis—We next investigated the effects of expressing calcineurin A on granule exocytosis (Fig. 5). We first expressed a truncated constitutively active form of calcineurin A fused to GFP (CnAmut-GFP) and measured exocytosis in cells stimulated with TG + PMA using LAMP externalization. We used reduced extracellular Ca\(^{2+}\) (0.5 mM) because our previous work indicates that exocytosis is maximal in the presence of higher Ca\(^{2+}\) (20); this would preclude detection of enhancement of exocytosis resulting from calcineurin expression. Fig. 5A shows histograms of GFP or CnAmut-GFP fluorescence for a representative experiment. The bars denote gating regions that were subsequently used to analyze anti-LAMP fluorescence. B and C show histograms of PE anti-LAMP fluorescence from the different gating regions for cells expressing GFP or CnAmut-GFP, respectively, from the experiment shown in A. In GFP-negative cells, there was a bimodal distribution of PE anti-LAMP fluorescence. There was a progressive shift of cells toward the right part of the bimodal distribution with increasing levels of expression in the CnAmut-GFP-expressing cells but not in cells expressing GFP alone. Fig. 5D shows pooled results from four similar experiments. Two tests of statistical significance were performed. First, we compared cells expressing increasing levels of GFP or CnAmut-GFP to non-expressing cells in the same sample populations. At no level of GFP expression were significant effects seen. In contrast, expressing CnAmut-GFP significantly enhanced exocytosis compared with the negative cells. We also compared CnAmut-GFP-expressing cells to cells expressing comparable levels of control GFP plasmid. Significant increases in exocytosis occurred at all levels of expression. There was no difference in the responses of non-expressing cells.

Next, we tested the effects of expressing this Ca\(^{2+}\)-independent form of calcineurin in cells stimulated with TG + PMA in the absence of extracellular Ca\(^{2+}\) (Fig. 5E). PE anti-LAMP fluorescence was assessed at different levels of CnAmut-GFP expression as in Fig. 5, A–D. At no level of expression was a shift of PE anti-LAMP fluorescence toward the right observed, indicating that expressing mutant calcineurin was insufficient to promote exocytosis. Identical results were obtained in two other experiments.

Finally, we examined whether expressing full-length calcineurin A fused to YFP (CnA-YFP) could also enhance lytic
granule exocytosis. The experimental design and analysis was identical to that described for Fig. 5, A–D. Fig. 5F displays pooled data from 6 experiments. At all levels of expression, cells expressing CnA-YFP had significantly higher responses than cells expressing YFP alone. As expected, there was no difference observed between the non-expressing populations. There were
no effects observed of expressing YFP or CnA-YFP on unstimulated cells (data not shown).

**DISCUSSION**

Taken together with previous work (1), our results provide strong support for the idea that calcineurin plays an important role in CTL lytic granule exocytosis. Consistent with that study, we found that immunosuppressive drugs block granule exocytosis stimulated either via the T cell receptor or via soluble TCR-independent drugs (Fig. 1). The results of Fig. 2 suggest that JNK does not play a role in lytic granule exocytosis. However, we were unable to exclude the possibility that P38 isoforms that are SB203580-insensitive are involved. Further work will be needed to resolve whether P38 activation is required. Such experiments might include the use of upstream regulators of P38 such as MEKK6 or dominant-negative P38 constructs (26).

However, strong support for the involvement of calcineurin in granule exocytosis comes from the observation that GFP-C-CAIN, a genetically encoded calcineurin inhibitory peptide, blocked exocytosis (Fig. 4). Finally, expressing catalytically competent calcineurin A enhanced lytic granule exocytosis (Fig. 5).

That TCR-independent granule exocytosis is affected by drugs, GFP-C-CAIN and calcineurin expression suggests that calcineurin may be involved in step(s) downstream of immunological synapse formation and/or granule/microtubule organizing center reorientation, as we think it unlikely that soluble chemical agents trigger these processes. Consistent with that idea, we have previously shown that protein kinase C isoforms display an essentially uniform plasma membrane distribution after stimulation with PMA (21), unlike their accumulation in the immunological synapse when in contact with target cells, and our unpublished observations indicate that TCR-
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independent lytic granule exocytosis in TALL-104 cells is not inhibited by disruption of microtubules.\(^3\) Note that participation of calcineurin in downstream steps does not preclude participation of calcineurin in upstream steps as well.

Our results are consistent with the idea that activation of calcineurin is likely to be an important step in granule exocytosis, contributing to the overall Ca\(^{2+}\) dependence of the process. However, expression of mutant calcium-independent calcineurin was not sufficient to promote exocytosis when cells were stimulated in the absence of extracellular Ca\(^{2+}\) (Fig. 5E). This suggests that there are likely multiple Ca\(^{2+}\)-dependent steps in lytic granule exocytosis triggered by soluble TCR-independent chemical stimuli. One possibility for an additional Ca\(^{2+}\)-dependent step is Ca\(^{2+}\) binding to a synaptotagmin isoform. Synaptotagmins (31) are C2 domain-containing proteins that are thought to trigger conformational changes in SNARE complexes (32, 33), leading to fusion of vesicles with the plasma membrane. Consistent with this idea, recent work indicates that target cell killing is reduced in mice in which synaptotagmin VII, an isoform associated with lysosomal exocytosis, has been knocked out (34). Interestingly, secretion of granzyme in response to TCR cross-linking was not reduced in the knockouts, an effect the authors attribute to the strength of stimulation.

The transcription factor NFAT is the best known substrate of calcineurin in T cells and is critical for mediating cytokine gene expression (see Regs. 10 and 35 for recent reviews). While NFAT is likely involved in the expression of at least some cytokines in CTLs, it seems unlikely to us that NFAT is involved in granule exocytosis. First, NFAT has not been reported to contain domains that would likely be involved in exocytosis. Second, NFAT translocates to the nucleus upon dephosphorylation by calcineurin (35). It seems unlikely that a protein residing in the nucleus would be involved in triggering fusion of granules with the plasma membrane. Finally, that VIVIT-GFP inhibited IL-2 expression in Jurkat cells but did not inhibit granule exocytosis in TALL-104 cells is expected if the substrate in CTLs is a molecule other than NFAT that does not use the PxIXIT motif for calcineurin docking.

As for potential calcineurin substrates other than NFAT, there are relatively few reports in the literature implicating calcineurin in exocytosis in mammalian cells to serve as a guide. In pancreatic acinar cells, release of amylase has been reported to be calcineurin-dependent (36), and a 24-kDa acidic phosphoprotein CRHSP-24 may be the substrate (37). In gastric chief cells, release of pepsinogen is calcineurin-dependent; a 55-kDa acidic cytoskeleton-associated protein may be the substrate (38). In pancreatic \(\beta\) cells, as in TALL-104 CTLs, expressing CAIN inhibits exocytosis; the kinesin heavy chain has been shown to be a calcineurin substrate and may mediate the effect (39). Motor proteins are believed to play important roles in CTLs, particularly in the reorientation of lytic granules to the site of contact with target cells. While granule reorientation is unlikely to occur in response to TCR-independent stimuli (see above), dephosphorylation of a motor protein by calcineurin could still be imagined to be important, perhaps by releasing granules from their association with the microtubule cytoskeleton allowing them to become capable of fusion with the membrane. Recent work suggests that plus end-directed motors are not involved in lytic granule exocytosis (40), arguing against participation of the kinesin heavy chain, which is a plus end-directed motor. However, the possibility that another motor protein is involved may be worth pursuing. To do so, it will be necessary to first identify the motors involved in granule exocytosis. Other reported substrates of calcineurin such as microtubule associated protein-2, tau factor, and tubulin (41) could also be important for granule exocytosis. Obviously, further work will be needed to address this issue. Interestingly, exocytosis in RBL mastocytoma cells is also blocked by immunosuppressive drugs (42), but as in CTLs the substrate is unknown. Thus, calcineurin dependence of secretory lysosome exocytosis may be a widespread phenomenon.

It is unlikely that effects on CTL lytic granule exocytosis make a significant contribution to the actions of immunosuppressive drugs in a clinical setting. CTLs require cytokine production by helper T cells to acquire effector function (5), and, as cytokine production is blocked by CsA and FK506, these agents will likely block the development of armed effector CTLs. However, natural killer cells also use exocytosis of lytic granule to kill their targets. If granule exocytosis in natural killer cells also involves calcineurin, this could contribute to the clinical efficacy of immunosuppressive drugs.

Compared with our knowledge of the signals triggering cytokine production in helper T cells, understanding of the signals mediating lytic granule exocytosis in CTLs remains rudimentary. While protein kinase C (21, 43–45), extracellular signal-regulated kinase (13, 46), and now calcineurin have been identified as key signaling molecules, in no case have their substrates been identified. Further work will be required to illuminate the next steps in the signal transduction cascade that leads to granule exocytosis.

Acknowledgments—Allan F. Fierro made important contributions to Fig. 2. We thank Dr. Karen Helm of the University of Colorado Center Flow Cytometry Core and Dr. Carol Norris of the University of Connecticut Microscopy and Flow Cytometry Facility for their help. We also thank Dr. David A. Knecht for helpful conversations.

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\(^3\) S. Gaur and A. Zweifach, unpublished observations.
