No Specific Subcellular Localization of Protein Kinase C Is Required for Cytotoxic T Cell Granule Exocytosis*

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Cytotoxic T cells kill virus-infected cells and tumor cells by releasing lytic granules that contain cell-killing contents. Exocytosis requires calcium influx and protein kinase C (PKC) activation. Here, we extend our previous finding regarding the lack of isoform specificity of PKCs in the granule release step, showing that mutant constitutively active PKCθ can substitute for phorbol esters and support exocytosis. PKCθ, a novel PKC isoform, was recently shown to play a role in lytic granule reorientation. Surprisingly, however, our results suggested that mutant PKCθ did not localize to the plasma membrane (PM). To test directly whether PKC has to be in the PM to drive exocytosis, we generated mutants of various PKC isoforms that were tethered either to the outer mitochondrial membrane or to the PM. Tethered mutant PKCθs were able to promote exocytosis as effectively as the untethered version. The substrates of PKCs involved in lytic granule exocytosis are currently unknown, but subcellular localization is believed to be a critical factor in determining PKC accessibility to substrates. That there is no requirement for specific PKC localization in lytic granule exocytosis may have important implications for the identity of PKC substrates.

One of the key mechanisms cytotoxic T lymphocytes (CTLs) use to kill target cells is release of specialized intracellular vesicles called lytic granules (reviewed in Ref. 1). These secretory lysosomes contain cytotoxic agents such as perforin (a pore-forming peptide) and granzymes (serine proteases), which, upon release from the CTL, damage target cell membranes and ultimately trigger apoptosis. CTLs are activated via T cell receptor (TCR) engagement upon contact with a target cell. Calcium influx and protein kinase C (PKC) activation are two primary signaling events that link TCR engagement to exocytosis (reviewed in Ref. 2). ERK MAP kinase activation is also required (3–5), and PI3-kinase activation is required for TCR-dependent but not TCR-independent granule exocytosis (6). PKC activation is important for activating ERK, but also plays other, as yet undefined, roles (7).

PKCs are serine/threonine protein kinases that are involved in many biologically important processes. There are 10 isoforms of PKC that are classified as classical, novel, or atypical based on their modulators (8). In helper T cells, the novel isoform PKCθ plays a preferential role in important cell functions (9). It synergizes with the calcium/calcmodulin-dependent phosphatase calcineurin to upregulate IL-2 gene transcription via activation of the nuclear factor of activated T-cells (NFAT) (10, 11), and also specifically localizes to the immunological synapse (12), a specialized membrane domain that forms during the interaction of T cells with antigen-presenting cells (12) or targets (13). In contrast, it appears at present that there is no isoform specificity for PKCs in the degranulation step of CTL lytic granule exocytosis, since when calcium is elevated with drugs, constitutively active mutants of multiple PKC isoforms can substitute for treatment with DAG analogs such as phorbol myristate acetate (PMA) (14, 15). However, there may be isoform specificity in other steps of the lytic interaction. Recently PKCθ, a novel isoform, was shown by Ma et al. (16) to play a specific role in controlling reorientation of lytic granules to the site of contact with target cells in murine CTLs. Further work from this group showed that the effects of PKCθ on target cell lysis required kinase activity, but that PKCθ localized to lytic granules in a manner that was independent of kinase activation (17). Although both reorientation of lytic granules and target cell killing were reduced in CTLs from PKCθ knock-out mice, it is important to note that block of granule reorientation could inhibit target cell killing without necessarily affecting the actual exocytosis of lytic granules, because reorientation precedes exocytosis.

In previous experiments using TALL-104 human leukemic CTLs, we did not detect expression of PKCθ and so did not determine whether a constitutively active mutant form of it, like PKCα and PKCθ, is capable of supporting exocytosis (14). However, there appear to be problems with the antibody we used. For example, it has been reported that it only detects an unphosphorylated form of PKCθ, not the phosphorylated form capable of catalytic activation (18). In light of the new information provided by Ma et al., we decided to revisit the role of PKCθ in lytic granule exocytosis. Here, we report that TALL-104 cells do express PKCθ, and that mutant constitutively active PKCθ can support exocytosis. Wild-type PKCθ displayed an apparent cytosolic localization, in contrast to what was observed in murine CTLs (17), and it translocated to the plasma membrane upon stimulation with PMA, similar to what we observed for PKCα and PKCθ (14). Strikingly, however, mutant PKCθ also displayed an apparent cytosolic localization in unstimulated cells, appearing in the PM only after stimulation with thapsi-

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2 The abbreviations used are: CTL, cytotoxic T lymphocyte; PKC, protein kinase C; TCR, T cell receptor; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; GFP, green fluorescent protein; PM, plasma membrane; TG, thapsigargin; PMA, phorbol myristate acetate.
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gargin (TG). Our previous work indicated that mutant PKCα and PKCθ displayed a predominantly PM localization, and we had suspected that this indicated that the PM was the site where PKC activity was required for granule exocytosis. Targeting of PKC isoforms to specific membranes is thought to be important for controlling their access to different substrates. This is thought to underlie the ability of isoforms to serve specific functions (reviewed in Ref. 19), because in general PKC isoforms display relatively little substrate specificity (20). As the emergent consensus appears to be that substrates of a given PKC isoform are likely to be associated with the membrane to which that isoform translocates upon stimulation, the idea that there might be no need for PKC to be localized to the PM to participate in exocytosis was quite surprising to us.

To specifically test whether there is a requirement for PM localization of PKC in granule exocytosis, we tethered constitutively active mutant PKCδ to the outer membrane of mitochondria using a targeting sequence derived from BCL-x (21). Mitochondrially tethered mutant PKCδ was able to support granule exocytosis essentially as effectively as the untethered version. A version of the mutant tethered to the PM by a myristoylation sequence was also able to promote exocytosis. These results indicate that no specific subcellular localization of protein kinase C is required for cytotoxic T cell granule exocytosis.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs and Transfections—Wild-type PKCδ and the constitutively active PKCδ mutant DR144/145A (22) were given to us by Dr. Mary Reyland (UCDHC, Aurora, CO). Standard PCR methods were used to subclone them into pEGFPN1 (Clontech). PKCα and PKCθ mutant constructs have been previously described (14). BCL-x in pEGFP-C3 (21) was kindly provided by Dr. Christoph Borner (Albert-Ludwigs-University Freiburg, Germany). Because we already had mutant PKCs in pEGFP-N1, to facilitate construction of mutant PKC-GFP-BCL-x chimeras, PCR methods were used to subclone the BCL-x sequence after the GFP coding sequence in pEGFP-N1 using the XbaI restriction enzyme site. This construct, which we refer to as GFP-BCL-x, was then used to make mitochondria using a targeting sequence derived from BCL-x (21). Mitochondrially tethered mutant PKCδ was able to support granule exocytosis essentially as effectively as the untethered version. A version of the mutant tethered to the PM by a myristoylation sequence was also able to promote exocytosis. These results indicate that no specific subcellular localization of protein kinase C is required for cytotoxic T cell granule exocytosis.

**RESULTS**

**TALL-104 Cells Express PKCδ, Which Translocates to the Plasma Membrane upon Stimulation with Soluble Chemical Agents**—We used Western blotting to probe for PKCδ expression in TALL-104 cells (Fig. 1A), and found that expression levels were comparable to those seen in Jurkat helper T cells and in a commercial human brain lysate. Next, we probed lysates from unstimulated cells and from cells stimulated with 50 nM PMA and 1 μM TG with an antibody against PKCδ phosphorylated on Thr-507 (this residue corresponds to Thr-505 in mouse, the species against which the antibody was raised) (Fig. 1B). Phosphorylation at this residue may be associated with PKCδ activation. Whereas there was no immunoreactivity in unstimulated cells, stimulation with PMA triggered recognition with the anti-phospho-Thr-507 PKCδ Ab. We conclude that PKCδ is not phosphorylated in unstimulated cells, and therefore have no ready explanation for our previous failure to detect PKCδ. However, that PKCδ is phosphorylated on Thr-507 after treatment with PMA may be consistent with the idea that PKC activates the native enzyme in TALL-104 cells. We next assessed the subcellular localization of PKCδ in unstimulated cells and in cells stimulated with TG and PMA (drugs that...
trigger calcium influx and activate PKC respectively) in two ways: heterologous expression of a PKCδ-GFP fusion protein (Fig. 1C) and immunocytochemical analysis with the same antibody used in Western blotting (Fig. 1D). These probes were then visualized using confocal microscopy. Results with the antibody were somewhat unsatisfying, because cells had to be permeabilized with methanol, which changed their morphology (see brightfield images in Fig. 1D). Nonetheless, both approaches suggest that PKCδ is found in the cytoplasm in unstimulated cells, and translocates to the PM upon stimulation, similar to what we observed previously with PKCα and PKCθ (14).

**A Constitutively Active Mutant PKCδ Can Substitute for PMA in Promoting Lytic Granule Exocytosis**—We next tested whether a constitutively active mutant PKCδ could promote exocytosis (Fig. 2). We overexpressed the constitutively active PKCδ mutant fused to GFP, and stimulated cells with TG. We assessed exocytosis by measuring externalization of LAMP-1 using flow cytometry, as described previously (14). Responses of untransfected cells to stimulation with TG or TG + PMA are shown in Fig. 2A, panel i. TG treatment alone triggered sub-maximal exocytosis in GFP-transfected cells (used as controls) at all levels of expression (Fig. 2A, panel iii). As we found previously was the case with constitutively active mutant PKCα and PKCθ (14), cells expressing high levels of constitutively active mutant PKCδ fused to GFP demonstrated significantly increased levels of anti-LAMP staining when compared with the non-expressing cells or cells expressing equivalent levels of GFP (Fig. 2, A, panel iv and B). Anti-LAMP fluorescence intensity increased with increasing levels of expression of mutant PKCδ. This indicates that PKCδ, like PKCα and PKCθ, can substitute for PMA in synergizing with calcium increases to promote TCR-independent lytic granule exocytosis. Interestingly, and in contrast to what we observed with PKCα and PKCθ mutants, ~10–15% of cells expressing high levels of PKCδ mutant were LAMP-positive in the absence of any overt stimulation (supplemental Fig. S1). This effect was at least partially calcium-independent, as adding 10 mM EGTA to cells 2 h after nucleofection (the earliest time at which GFP expression is detected) reduced the number of LAMP-positive cells, but did not fully prevent the response (supplemental Fig. S1).

**FIGURE 1. TALL 104 cells express PKCδ, which translocates to the plasma membrane on stimulation with TG + PMA.** A, Western blotting was used to probe for the expression of PKCδ in TALL-104 cells. Jurkat T helper cell and brain lysates were used as controls. 25 μg of total cell lysate was loaded per lane. B, PMA treatment stimulates phosphorylation of PKCδ on Thr-507. Equal amounts of lysate from unstimulated or PMA + TG-treated cells were probed with an antibody that recognizes phospho-Thr-505 PKCδ. C, confocal micrographs of unstimulated cells (unstim) and stimulated cells (stim) transfected with PKCδ fused to GFP. D, confocal images of unstimulated and stimulated cells immunostained with an anti-PKCδ antibody that was detected with a fluorescently labeled secondary antibody. Scale bars denote 5 μm.
Mutant PKCδ-GFP Is Not Associated with the Plasma Membrane in Unstimulated Cells—In our previous experiments, we found that both PKCα and PKCδ mutants were constitutively associated with the plasma membrane (14). However, when we examined the subcellular localization of mutant constitutively active PKCδ using confocal microscopy, we found that in most unstimulated cells it displayed an apparent cytosolic localization (Fig. 3A). As described above, some cells transfected with mutant PKCδ expose LAMP on their surface in the absence of an overtly stimulated increase in intracellular Ca²⁺. When we stained intact mutant PKCδ-transfected TALL-104 cells with anti-LAMP antibody, we found PKCδ was cytosolic in most of the cells that stained, although occasional examples could be found in which it appeared to reside in the PM. There was thus no apparent correlation between localization of mutant PKCδ and exocytosis. However, when we treated transfected cells with TG, mutant PKCδ-GFP was found in the plasma membrane in most cells (Fig. 3B). This PM translocation required calcium influx, as it did not occur in the absence of extracellular Ca²⁺ (supplemental Fig. S2).

Mutant PKCδ Tethered to the Outer Mitochondrial Membrane Retains Catalytic Activity and Can Promote Exocytosis—Results presented thus far raise the possibility that PKCδ may not need to be associated with the PM to participate in exocytosis of lytic granules. To test this directly, we created a mutant construct that would be prevented from translocating to the PM. We tethered mutants to the outer mitochondrial membrane using a specific targeting sequence from BCL-x (21) inserted at the 3'-end of GFP, which was itself at the 3'-end of the mutant PKC δ-GFP construct (Fig. 4A). In this construct, were cleavage between the regulatory and catalytic domains to occur (a process reviewed in Ref. 25), the catalytic domain would remain tethered. Note that we ini-

FIGURE 2. Expressing mutant constitutively active PKCδ promotes exocytosis in TG-treated cells. A panel i, histograms of anti-LAMP fluorescence from untransfected cells that were not stimulated (UN), cells stimulated with TG alone (TG), and cells stimulated with TG + PMA. Panel ii, histograms of GFP fluorescence intensity for cells transfected with GFP or with a constitutively active mutant PKCδ-GFP. Gating regions used to analyze responses at different expression levels are shown. Panel iii, representative plots of LAMP staining intensity for GFP-transfected cells treated with TG from the different gating regions are shown. Histograms are arranged so that untransfected cells are at the back, and cells with increasing levels of expression are progressively toward the front. Panel iv, representative plots of LAMP staining intensity for mutant PKCδ-GFP-transfected cells treated with TG for the different gating regions are shown. Histograms are arranged as in panel iii. B, quantification from three such experiments. Gray bars represent GFP-transfected cells, and black bars represent PKCδ-GFP transected cells. * and # indicate that cells from gating regions 3 and 4 expressing mutant PKCδ-GFP differ significantly from non-expressers from the same sample (gating region 1) and from cells expressing comparable levels of GFP.
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FIGURE 3. Mutant PKCδ is not localized to the PM unless cells are stimulated with thapsigargin. TALL-104s were transfected with the mutant PKCδ-GFP and 6–7 h later were either left untreated or were treated with TG. The anti-LAMP antibody was present in the incubating solution, and cells were fixed after 50 min. Representative mutant PKCδ-GFP fluorescence images, anti-LAMP staining and brightfield images are shown for (A) unstimulated cells and (B) cells stimulated with TG. Scale bars are 5 μm.

We first confirmed that our targeting strategy tethered PKCδ to the outer mitochondrial membrane. We transfected TALL-104 cells with mutant PKCδ-GFP-BCL-x, and assessed subcellular localization using confocal microscopy (Fig. 4B). Representative images of cells that were not stimulated are shown in the top row, and images of cells that were treated with TG are shown in the bottom row. In these experiments, we used mitotracker orange to label mitochondria (shown in red in column 3 of Fig. 4B)). The fluorescence of mutant PKCδ-GFP-BCL-x colocalized extensively with mitochondria (a merge is shown in column 4 of Fig. 4B). Fluorescence of PKCδ-GFP-BCL-x did not overlap with anti-LAMP fluorescence (anti-LAMP fluorescence is shown in red in column 5 of Fig. 4B), and a merge between PKCδ-GFP-BCL-x and anti-LAMP is shown in column 6). Note that LAMP staining following TG treatment was not as prominent in these experiments (bottom row of Fig. 4B) as in those shown in Fig. 3B or Fig. 6B, likely due to the fact that cells were treated with mitotracker orange, which we find reduces LAMP staining intensity by ~30–40% (data not shown).

We next tested whether cleavage products of PKCδ-GFP-BCL-x were generated by proteolysis that could yield untethered catalytically active PKC fragments (Fig. 5). We used Western blotting with primary antibodies against GFP and against the catalytic domain of PKCδ to determine whether there were species that were recognized by the anti-PKCδ antibody that were not recognized by the anti-GFP antibody, or vice versa. Essentially all of the species reacted with both antibodies, consistent with the idea that all consisted of a fusion of mutant PKCδ to GFP. Thus, as the fluorescence associated with GFP was localized to mitochondria, these results suggest that all active PKCδ was likely to be properly tethered.

To confirm that the mitochondrially tethered mutant PKCδ was catalytically active, we used a commercially available antibody against the phosphorylated PKC consensus phosphorylation site to assess relative levels of PKC-dependent substrate phosphorylation in transfected cells (Fig. 4, C and D). We previously used this antibody in Western blotting experiments to test the effects of PKC inhibitors on PKC activity in TALL-104 cells (14), and evidence that the antibody works in flow cytometry is presented in supplemental Fig. S3. Cells were processed for indirect intracellular immunostaining, and the fluorescence intensity of GFP, mutant PKCδ, or mutant PKCδ-GFP-BCL-x together with anti-phospho PKC substrate antibody staining (detected with a Cy5-labeled secondary antibody) was measured with flow cytometry. For a given level of GFP fluorescence intensity, mutant PKCδ-GFP-BCL-x and untargeted mutant PKCδ-GFP produced comparable levels of anti-phospho PKC substrate antibody staining (Fig. 4, C and D), suggesting equivalent catalytic activity, while expressing GFP alone had no effect.

Next, we tested whether mutant PKCδ-GFP-BCL-x, like untethered constitutively active mutant PKCδ, can substitute for PMA in promoting lytic granule exocytosis (Fig. 4, E and F). We expressed GFP-BCL-x (which demonstrated an identical subcellular localization to PKCδ-GFP-BCL-x, data not shown) or mutant PKCδ-GFP-BCL-x, stimulated cells with TG alone, and assessed exocytosis via LAMP staining using flow cytometry. As was also the case in GFP-transfected cells (see Fig. 2), TG treatment resulted in low levels of exocytosis at all levels of expression of GFP-BCL-x (Fig. 4, E and F). In contrast, TG-stimulated cells expressing high levels of mutant PKCδ-GFP-BCL-x showed significantly increased levels of anti-LAMP staining compared with non-expressing cells or cells expressing equivalent levels of GFP-BCL-x. The enhancement of exocytosis with mutant PKCδ-GFP-BCL-x was essentially indistinguishable from that seen with the untethered PKCδ mutants.

Finally, to confirm that mutant PKCδ can function to promote lytic granule exocytosis when directed to subcellular locations other than the mitochondria, we examined the effects of tethering it to the plasma membrane via a myristoylation sequence (Fig. 6). Mutant myr-PKCδ-GFP colocalized extensively with Cy3-labeled wheat germ agglutinin (WGA) applied to cells after fixation to label the plasma membrane. However, there was some apparent overlap of myristoylated mutant PKCδ with anti-LAMP staining (Fig. 6B), and it was clear that not all of the fluorescence of either the myr-mutant-PKCδ or WGA itself was associated with the PM. Association with LAMP may reflect trafficking between the PM and intracellular organelles, including the lytic granules, and is also likely con-
founded by the fact that mutant PKCδ can promote modest levels of exocytosis without additional stimulation (supplemental Fig. S1), resulting in appearance of LAMP in the PM. As was the case for the mitochondrially tethered PKCδ mutant, cleavage fragments were detected using Western blotting, but the majority reacted with both anti-GFP and anti-PKCδ antibodies (Fig. 5). The construct was catalytically active as assessed with the anti-phospho PKC substrate antibody (Fig. 6, C and D). When we transfected cells with GFP alone or with myristoylated PH-domain mutants of PKCδ, stimulated them with TG and measured exocytosis via externalization of LAMP, there was a significant enhancement of exocytosis in cells that expressed high levels of the myristoylated mutant compared with cells that expressed comparable levels of GFP or to nonexpressing cells from the same population (Fig. 6, E and F). Similar results were obtained with a construct in which mutant PKCδ was fused at its N terminus to a truncated form of human CD4 from which most of the cytosolic sequence had been deleted (supplemental Fig. S4).
Although neither construct resulted in a pure PM localization, they would not be expected to. In the case of the myristoylated construct, the protein will likely interact with lytic granule exocytosis. This was not clear from previous work (16, 17), as in those studies granule reorientation was inhibited, and this is likely to inhibit in an obligate manner downstream steps such as exocytosis. That PKCδ can participate in granule exocytosis further underscores the fact that there is no isoform specificity to this process (14, 15). This appears not to be the case with regard to lytic granule polarization, where PKCδ plays a preferred role (16, 17). Interestingly, of the constitutively active mutant PKCs we have examined, only the PKCδ mutant was able to promote exocytosis independent of calcium influx (supplemental Fig. S1). This effect may somehow be related to the ability of PKCδ to participate in the reorientation of lytic granules (16, 17). Note that Ma et al. showed that PKCδ colocalized with lytic granules in murine CTLs (17). We did not observe this to be the case in TALL-104 cells. Instead, PKCδ exhibited a cytosolic appearance in unstimulated cells, and translocated to the membrane upon stimulation with PMA. We are not sure how to account for this discrepancy. It is apparently not due to the fact that we were examining the localization of overexpressed PKCδ in cells that endogenously express it, whereas Ma et al. were using CTLs from PKCδ knock-out animals, since they report similar localization using immunocytochemical analysis in wild-type cells (17).

Second, the results we present here, taken together with our previous work (14), demonstrate clearly that PKCs can participate in lytic granule exocytosis regardless of whether or not they are located in the plasma membrane. The result that supports this most directly is the ability of mutant PKCδ-GFP-BCL-x to support exocytosis. This is surprising, as targeting to specific membranes is thought to control the accessibility of PKCs to their substrates (26, 27). Underscoring the importance of localization, one of the key initial pieces of evidence supporting a preferential role for PKCδ in helper T cell functions was its specific localization to what is now called the immunological synapse (28). The most direct evidence to date for the importance of PKC localization in determining interactions with substrates comes from experiments that showed that PKC must be localized to the PM to phosphorylate the PM substrate MARCKs, while only a cytosolic form of PKC could phosphorylate mutant cytosolic MARCKs (29).

Having provided strong evidence that there is no preferred localization for PKCs in promoting exocytosis, it still may be informative to speculate as to how the localization of PKCs might occur. We think that wild-type PKCα, PKCδ, and PKCζ likely translocate to the PM after stimulation with PMA because this phorbol ester accumulates in the PM. Wang et al. (30) have shown that PKCδ translocates to different membranes when cells are stimulated with different phorbol ester analogs or bryostatin in CHO cells, and the effect seems to be governed by analog hydrophobicity (31). Once at the PM, PKCs might bind to phospholipids, to specific binding proteins such as RACKs (receptors for activated c kinase) and STICKs (substrates that interact with c kinase (see Ref. 26, 27), or to both lipids and protein. Membrane binding is then likely associated with activation of catalytic function.

Although we did not realize it at the time, it is not clear why constitutively active PKCα and PKCζ mutants associate with the PM, as we showed previously was the case (14). These mutants are rendered active by amino acid substitutions that displace the pseudosubstrate domain from the catalytic domain, and so do not require membrane binding for activation (22). However, it may be that they still bind lipids with high affinity. If they do, then it seems reasonable to suppose that mutant PKCα and PKCζ translocate to the membrane as a result of lipid binding. If so, then mutant PKCδ may bind to a protein that prevents membrane association, unless it alone of the isoforms does not bind lipids when rendered constitutively active. Alternatively, if mutant PKCα and PKCζ do not bind lipids, then their membrane association likely arises from binding to RACKs or STICKs. If this is the case, then we can infer that mutant PKCδ does not interact with the same protein(s). In either scenario, it seems likely that PKCα and PKCζ interact with protein(s) differently than PKCδ. Furthermore, those proteins are unlikely to be substrates for exocytosis, as in either case...
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FIGURE 6. Constitutively active myristoylated mutant PKCβ-GFP localizes to the plasma membrane, is catalytically active and can promote granule exocytosis. A, schematic representation of the myristoylation targeting construct. The amino acid sequence of the myristoylation sequence is shown in the top portion of the figure. B, myristoylation sequence targets mutant PKCβ to the plasma membrane. TALL-104s were transfected with myristoylated mutant PKCβ-GFP (shown in green in column 2). 6–7 h after transfection, the cells were fixed and stained with Cy3-labeled wheat germ agglutinin (shown in red in column 3) and Alexafluor 647-labeled anti-LAMP antibody (shown in red in column 5) then fixed. The color merge between GFP and WGA (column 4) shows more extensive colocalization than the color merge between GFP and anti-LAMP (column 6). A bright field view is shown in column 1. Images are from an unstimulated cell (top) and from a cell that was treated with TG (bottom). Scale bars are 5 μm. C, myristoylated mutant PKCβ-GFP is catalytically active. TALL-104 cells were transfected with GFP (panel i) or myristoylated mutant PKCβ-GFP (panel ii) then 6–7 h later were fixed, permeabilized, and stained with anti-phospho PKC substrate antibody. Representative dot plots of anti-phospho-PKC substrate antibody staining intensity versus GFP fluorescence intensity are shown. Numbered bars denote gating regions that were used to analyze the data subsequently. D, data from three experiments like the one in C were averaged and plotted for the different gating regions shown in C. Open circles are data obtained with myristoylated mutant PKCβ-GFP. Data from Fig. 4D are replotted, and the symbols used are the same as in that figure. E, myristoylated mutant PKCβ-GFP can substitute for PMA and support lytic granule exocytosis. Cells were either transfected with GFP (panel i) or with myristoylated mutant PKCβ-GFP (panel ii). Representative plots are shown of anti-LAMP antibody staining intensity for cells treated with TG for different gating regions as shown in (C, panels i and ii). Histograms are arranged with untransfected cells at the back, and cells with increasing levels of expression progressively toward the front. F, quantification from three experiments like the one shown in E. Gray bars represent cells transfected with GFP, and black bars represent myristoylated mutant PKCβ-GFP-transfected cells. * and # indicate that cells from gating region 3 expressing myristoylated mutant PKCβ-GFP differ significantly from non-expressers from the same sample (gating region 1) and from cells expressing high levels of GFP (cells from gating region 3).

not all of the mutants that can drive exocytosis bind to them. Thus, our results suggest that there are probably proteins to which PKCs bind in an isoform-specific manner that are not relevant substrate(s) for lytic granule exocytosis. Because mutant PKCβ, which lacks functional Ca²⁺-binding C2 domains, appears to translocate to the PM in a manner that depends on Ca²⁺, we lean toward the idea that PKCβ binds to a cytosolic protein that prevents its membrane association, but then translocates to the PM after a Ca²⁺ rise. We do not yet know whether translocation of the mutant PKCβ occurs as a result of exocytosis. However, as described above, this protein, if it exists, is not likely to be a substrate important for exocytosis.

Assuming that all of the PKC constructs we have tested have the same substrate(s) relevant for exocytosis, those substrates must be accessible to PKCs residing in the PM, tethered to the mitochondrial membrane, or, in the case of mutant untethered PKCβ, likely resident in the cytoplasm. This would seem to argue against PM-resident proteins as substrates, provided that it is correct that only PM-resident PKCs can phosphorylate PM substrates as has been reported (29). We feel that it will be important to confirm this, but so far have been unable to reliably detect a change in MARCKS localization from the PM to intracellular membranes following stimulation with PMA, and thus have been unable to investigate the effects on MARCKS localization of expressing various tethered mutant PKCs.

If the PM is not the location of the substrate(s) involved in granule exocytosis, then what is? Proteins associated with lytic granules might be accessible to cytosolic or to mitochondrially
tethered PKCs, and could become accessible to PM-resident PKCs during docking of granules with the PM. Proteins associated with the lytic granules would obviously also likely be accessible to PKCs associated with granules, as was reported to be the case for PKCθ in murine CTLs (17). Proteins resident in a recycling endosomal compartment might also be accessible to PKCs in all three locations. Ménager et al. (32) recently provided evidence that a recycling endosomal compartment had to fuse to lytic granules to form a fully functional granule capable of undergoing secretion. Alternatively, the substrate(s) could be cytosolic proteins. If so, they could bind while the PKC is in the cytoplasm. For wild-type PKCs, this might be while the mature enzyme resides in the cytosol prior to activation. For mutant PKCa and PKCθ, it could occur after synthesis but before membrane association. Generally consistent with our speculation, it has recently been proposed that in helper T cells, PKCθ binds to SPANK, a recently discovered helper T cell substrate, in the cytoplasm, and the two translocate to the PM together upon PKCθ activation (33).

We believe that the results we present here will aid in several key ways in the identification of PKC substrates important for lytic granule exocytosis. First, as the previous paragraphs indicate, our results suggest that attempts to identify substrates via identification of PKC binding partners will likely be complicated, the fact that there are almost certainly proteins to which PKCs bind that are not important substrates for exocytosis. On the other hand, our results also offer an important discriminating feature of potential substrates identified via binding: the substrates important for exocytosis must bind to wild-type and mutant PKCa, PKCθ, and PKCβ, as well as to the mitochondrially tethered and PM-tethered mutant PKCδ. Irrelevant binding partners may not bind to all of the PKC forms. Another possible strategy for identifying PKC substrates could rely on enriching phosphoproteins using tools such as immobilized metal affinity columns, followed by identification via mass spectroscopy. While the anti-phospho PKC substrate antibody we used to assess catalytic activity of mutant PKCs works in Western blotting (see e.g. Ref. 14), the antibody detects so many proteins that the result is a smear or ladder of immunoreactivity, rendering one-dimensional blotting uninformative. Two-dimensional blots might be useful. Our results indicate that PKC substrate(s) involved in lytic granule exocytosis will have to be substrates of all of the different versions of the PKC isoforms. This might serve to narrow down what otherwise could be a very long list of candidates.

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