Sequestration of phosphoinositides by mutated MARCKS effector domain inhibits stimulated Ca^{2+} mobilization and degranulation in mast cells

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ABSTRACT **Protein kinase C β (PKCβ) participates in antigen-stimulated mast cell degranulation mediated by the high-affinity receptor for immunoglobulin E, FcεRI, but the molecular basis is unclear. We investigated the hypothesis that the polybasic effector domain (ED) of the abundant intracellular substrate for protein kinase C known as myristoylated alanine-rich protein kinase C substrate (MARCKS) sequesters phosphoinositides at the inner leaflet of the plasma membrane until MARCKS dissociates after phosphorylation by activated PKC. Real-time fluorescence imaging confirms synchronization between stimulated oscillations of intracellular Ca^{2+} concentrations and oscillatory association of PKCβ–enhanced green fluorescent protein with the plasma membrane. Similarly, MARCKS-ED tagged with monomeric red fluorescent protein undergoes antigen-stimulated oscillatory dissociation and rebinding to the plasma membrane with a time course that is synchronized with reversible plasma membrane association of PKCβ. We find that MARCKS-ED dissociation is prevented by mutation of four serine residues that are potential sites of phosphorylation by PKC. Cells expressing this mutated MARCKS-ED SA4 show delayed onset of antigen-stimulated Ca^{2+} mobilization and substantial inhibition of granule exocytosis. Stimulation of degranulation by thapsigargin, which bypasses inositol 1,4,5-trisphosphate production, is also substantially reduced in the presence of MARCKS-ED SA4, but store-operated Ca^{2+} entry is not inhibited. These results show the capacity of MARCKS-ED to regulate granule exocytosis in a PKC-dependent manner, consistent with regulated sequestration of phosphoinositides that mediate granule fusion at the plasma membrane.**

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INTRODUCTION

Granule exocytosis in mast cells is stimulated by antigen-mediated cross-linking of immunoglobulin E (IgE) bound to high-affinity receptors (FcεRI), and much is known about this physiologically important process in allergic and inflammatory responses (Blank and Rivera, 2004; Gilfillan and Tkaczyk, 2006). As for many other receptor-activated exocytotic responses in other cell types, critical roles for Ca^{2+} mobilization and protein kinase C (PKC) activation are well established (Ma and Beaven, 2009; Nechushtan *et al.*, 2000). Mast cells do not exhibit voltage-gated Ca^{2+} influx, but it is known that store-operated Ca^{2+} entry participates in IgE receptor–stimulated degranulation. Previous studies revealed contributions both from Ca^{2+}-release-activated Ca^{2+} (CRAC) channels, Orai1/CRACM1 (Vig *et al.*, 2008), and from transient receptor potential canonical channels (Ma *et al.*, 2008; Suzuki *et al.*, 2010). As for exocytosis in other cell types, mast cell granules, which are secretory lysosomes (Dragonetti *et al.*, 2000; Xu *et al.*, 1998), depend on elevated intracellular [Ca^{2+}] for fusion with the plasma membrane, and this requirement has been suggested to be due to triggering of soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE)-mediated fusion by granule-associated Ca^{2+}/synaptotagmin complexes bound to polyphosphoinositides at the inner leaflet of the plasma membrane (Dai *et al.*, 2007; Paddock *et al.*, 2008).

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Abbreviations used: BSA, bovine serum albumin; BSS, buffered saline solution; CRAC, Ca^{2+}-release-activated Ca^{2+}; ED, effector domain; EGFP, enhanced green fluorescent protein; IgE, immunoglobulin E; IP_3, inositol 1,4,5-trisphosphate; MARCKS, myristoyl alanine-rich protein kinase C substrate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; SNARE, *N*-ethylmaleimide–sensitive factor attachment protein receptor.

The involvement of phosphatidylinositol 4,5-bisphosphate (PIP_2) in facilitating the membrane fusion triggered by synaptotagmin implicates this phospholipid as a key player in Ca^{2+}-dependent exocytosis (Bai *et al.*, 2004).

The mechanism of PKC participation in mast cell granule exocytosis is less clear. PKC comprises a family of at least 10 different Ser/Thr kinases that play multiple roles in cell signaling (Newton, 2010; Nishizuka, 1995). These isoforms of PKC have been classified into three categories based on structural and activation requirements: conventional and novel subfamilies are activated by diacylglycerol binding to a C1 domain, and conventional isoforms also require Ca^{2+} binding to a C2 domain. These subfamilies are activated by phorbol esters, which are structural mimics of diacylglycerol, providing early evidence for participation of PKC activity in mast cell degranulation (Sagi-Eisenberg *et al.*, 1985). Reconstitution studies with PKC isoforms in permeabilized RBL mast cells yielded the first direct evidence for positive roles for PKCβ and δ in IgE receptor–stimulated degranulation (Ozawa *et al.*, 1993a). Genetic knockout of PKCβI revealed that this isotype is important in mast cell degranulation (Nechushtan *et al.*, 2000). Despite these advances, the mechanism for PKC activity in this and other exocytotic cell processes is not well understood.

A prominent substrate for PKC isoforms is the myristoylated alanine-rich C-kinase substrate commonly called MARCKS (Aderem, 1992; Blackshear, 1993). MARCKS binds tightly to membranes containing negatively charged phospholipids via a 25–amino acid sequence that contains 13 basic amino acids and is known as its effector domain (ED; Wang *et al.*, 2001). Meyer and colleagues showed that this sequence could be displaced from the plasma membrane by depletion of polyphosphoinositides, providing evidence that these negatively charged phospholipids are the principal means by which this protein anchors to the plasma membrane (Heo *et al.*, 2006). This ED sequence contains four serine residues, three of which are phosphorylated by activated PKC to cause its dissociation from the plasma membrane (Graff *et al.*, 1989a, 1989b). MARCKS has been shown to participate in granule exocytosis in platelets and chromaffin cells (Trifaro *et al.*, 2008), but the mechanism is unknown.

Although PIP_2 constitutes only ~1% of the phospholipids in the plasma membrane, it plays a key role in regulating a large number of cellular processes, including clathrin-mediated endocytosis, activation of ion channels, actin polymerization, phagocytosis, and synaptic vesicle exocytosis (Di Paolo and De Camilli, 2006). In mast cells, antigen-stimulated activation of phospholipase Cγ (PLCγ) results in hydrolysis of PIP_2 to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol; thus, PIP_2 is an important participant in Ca^{2+} mobilization and activation of PKCs. However, the lack of specific inhibitors of PIP_2 synthesis has made it difficult to probe the mechanisms by which PIP_2 regulates multiple, diverse functions in mammalian cells. We hypothesize that one of the key roles of PIP_2—wherein it interacts with synaptotagmins to facilitate granule exocytosis— is controlled by PKC activity: PKC regulates the availability of PIP_2 because PKC phosphorylation causes MARCKS dissociation, thereby exposing PIP_2 to interact with other proteins. In the present study, we use the high avidity of the MARCKS-ED peptide, and we introduce mutations to prevent its dissociation from the plasma membrane by activated PKC. We demonstrate that expression of this mutated protein (MARCKS-ED S4A) effectively delays antigen-stimulated Ca^{2+} mobilization that is dependent on PIP_2-mediated IP_3 production. Furthermore, MARCKS-ED S4A inhibits granule exocytosis by antigen as well as by thapsigargin, which bypasses the need for IP_3 production in this process. These results provide strong evidence that this mutated MARCKS-ED construct is an effective inhibitor of PIP_2-dependent processes, and thereby it serves as a useful tool for investigating these processes.

RESULTS

To characterize the spatial and temporal changes of PKCβI in antigen-stimulated RBL-2H3 cells, we performed real-time fluorescence imaging of PKCβI–enhanced green fluorescent protein (EGFP) in transiently transfected cells. In unstimulated cells, PKCβI-EGFP is distributed homogeneously in the cytoplasm. On antigen stimulation, we observe a rapid translocation of PKCβI-EGFP to the plasma membrane in 70-80% of transfected cells with variable onset times, and this is followed by periodic dissociation and reassociation with the plasma membrane. Figure 1A and Supplemental Movie S1 show this for PKCβI-EGFP at 37°C; the accompanying plot quantifies changes in cytoplasmic fluorescence for a single representative cell. Antigen stimulation of RBL cells at 25°C also causes PKCβI-EGFP association and dissociation with the plasma membrane, as shown in Figure 1B. The periodicity of these oscillations in multiple cells is in the range of 40–60 s, similar to that at 37°C. The average lag time for the onset of this response at 37°C is 50 s (SEM = ±4.2 s; n = 28), and this lag time is 10 s longer at 25°C (60 ± 3.3 s; n = 43). Thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum ATPase, also activates PKCβI-EGFP recruitment to the plasma membrane, but this occurs more slowly and without the oscillatory association and dissociation that is seen with antigen (Figure 1C). This recruitment may be due to diacylglycerol production stimulated by thapsigargin in a pathway that uses activation of phospholipase D (Peng and Beaven, 2005).

We compared the time course of antigen-stimulated oscillations in Ca^{2+} concentrations to the periodic translocation of PKCβI-EGFP to the plasma membrane by simultaneously imaging PKCβI-EGFP and $[Ca^{2+}]$ using the Ca^{2+} indicator Fura Red, which exhibits a decrease in fluorescence when Ca^{2+} is elevated. As represented in Figure 1D, we observe a strong temporal correlation between the time course of $[Ca^{2+}]$ oscillatory increases in the cytoplasm and that for PKCβI-EGFP oscillatory translocation to the plasma membrane. Recruitment of PKCβI-EGFP to the plasma membrane follows the onset of the cytoplasmic $[Ca^{2+}]$ increase by several seconds for each of the $[Ca^{2+}]$ oscillations, and this is evident in Figure 1D as a delay in the depletion of cytoplasmic PKCβI-EGFP relative to the increase in cytoplasmic $[Ca^{2+}]$ at each oscillation.

To monitor the activity of PKC at the plasma membrane, we coexpressed MARCKS-ED labeled with monomeric red fluorescent protein (mRFP). In unstimulated RBL cells, mRFP-MARCKS-ED is concentrated at the plasma membrane as shown in Figure 2A (top). On stimulation with antigen, dissociation of mRFP–MARCKS–ED from the plasma membrane correlates with recruitment of PKCβI-EGFP (Figure 2A, bottom, and Supplemental Movies S2a and S2b). As shown in Figure 2B, there is a strong temporal correlation between the association of PKCβI-EGFP and the dissociation of mRFP-MARCKS-ED from the plasma membrane, suggesting that stimulated PKC recruitment and activation results in periodic phosphorylation and dissociation of MARCKS-ED. We observed similar temporal correlation between MARCKS-ED PKCβI oscillations in 81% of cells coexpressing these two constructs (n = 21).

To evaluate the roles of phosphoinositides in stimulated Ca^{2+} mobilization and degranulation, we developed a probe to inhibit their accessibility under conditions in which they are normally available. Heo *et al.* (2006) showed that recruitment of inositol 5′-phosphatase to the plasma membrane to hydrolyze PIP_2, together with pharmacologic inhibition of PI3 and PI4 kinases, causes MARCKS-ED to

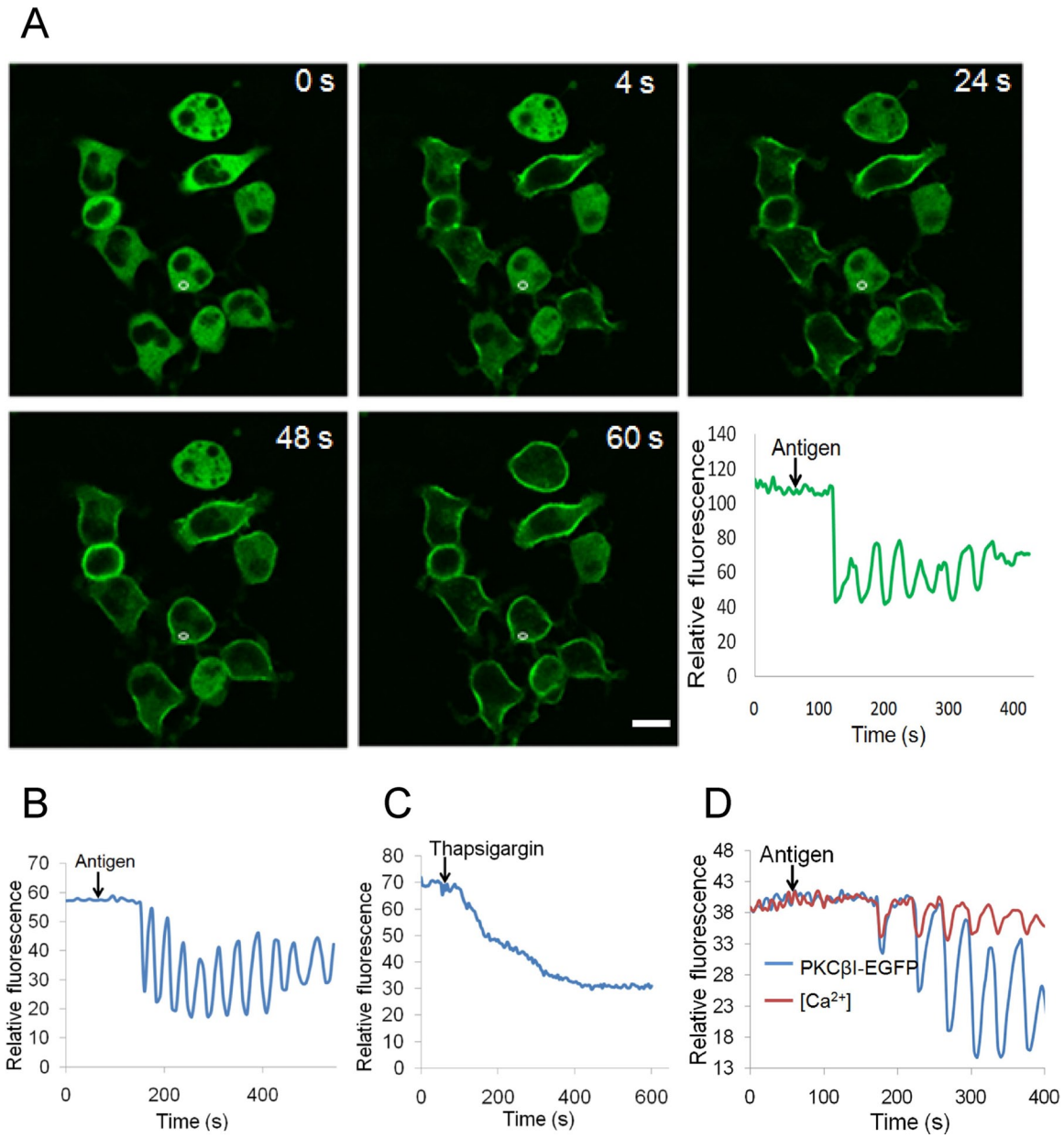

FIGURE 1: Dynamics of stimulated PKCβI-EGFP redistributions in IgE-sensitized RBL cells. (A) Time course of PKCβI-EGFP redistributions (green) in cells upon stimulation with antigen at 37°C (still frames from Supplemental Movie S1). Times in images specify intervals after antigen addition. Plot shows relative cytoplasmic fluorescence in the region of interest marked in a representative individual cell. Scale bar, 10 μm. (B) Time course of PKCβI-EGFP recruitment to the plasma membrane upon antigen stimulation at 25°C monitored as depletion of cytoplasmic fluorescence in a single representative cell. (C) Time course of PKCβI-EGFP recruitment to the plasma membrane upon stimulation with thapsigargin at 37°C monitored as depletion of cytoplasmic fluorescence for an average of six cells. (D) Time courses of PKCβI-EGFP recruitment to the plasma membrane monitored as depletion of cytoplasmic fluorescence (blue) and cytoplasmic [Ca^{2+}] changes monitored by Fura Red (red) in a single representative cell stimulated with antigen at 37°C.

dissociate from the plasma membrane. We reasoned that if we eliminated the capacity of PKC to displace MARCKS-ED from the plasma membrane under stimulating conditions, tight binding of MARCKS-ED to phosphoinositides would prevent access of proteins that use PIP_2 for their function, including phospholipase C and synaptotagmins (Chapman, 2002; Rhee and Choi, 1992). Toward this end, we mutated the four serine residues to alanine in MARCKS-ED, and we found that this prevented the periodic dissociation and rebinding of MARCKS-ED to phosphoinositides at the plasma membrane. As shown in Figure 3A, RBL cells were transfected with mRFP-MARCKS-ED SA4 and labeled with Ca^{2+} indicator Fluo-4, which increases in fluorescence with increase in Ca^{2+} concentration. Both Ca^{2+} mobilization (Figure 3A, top) and mRFP-MARCKS-ED SA4 in the cytoplasm (Figure 3A, bottom) were monitored during antigen stimulation. In contrast to results with unmutated MARCKS-ED, the SA4 mutant stays associated with the plasma membrane during stimulated Ca^{2+} responses (Figure 3, A and B). As illustrated in Figure 3C and Supplemental Movie S3, the onset of Ca^{2+} responses to antigen in cells expressing mRFP-MARCKS-ED SA4 is delayed relative to that for cells not expressing this construct. The average time to

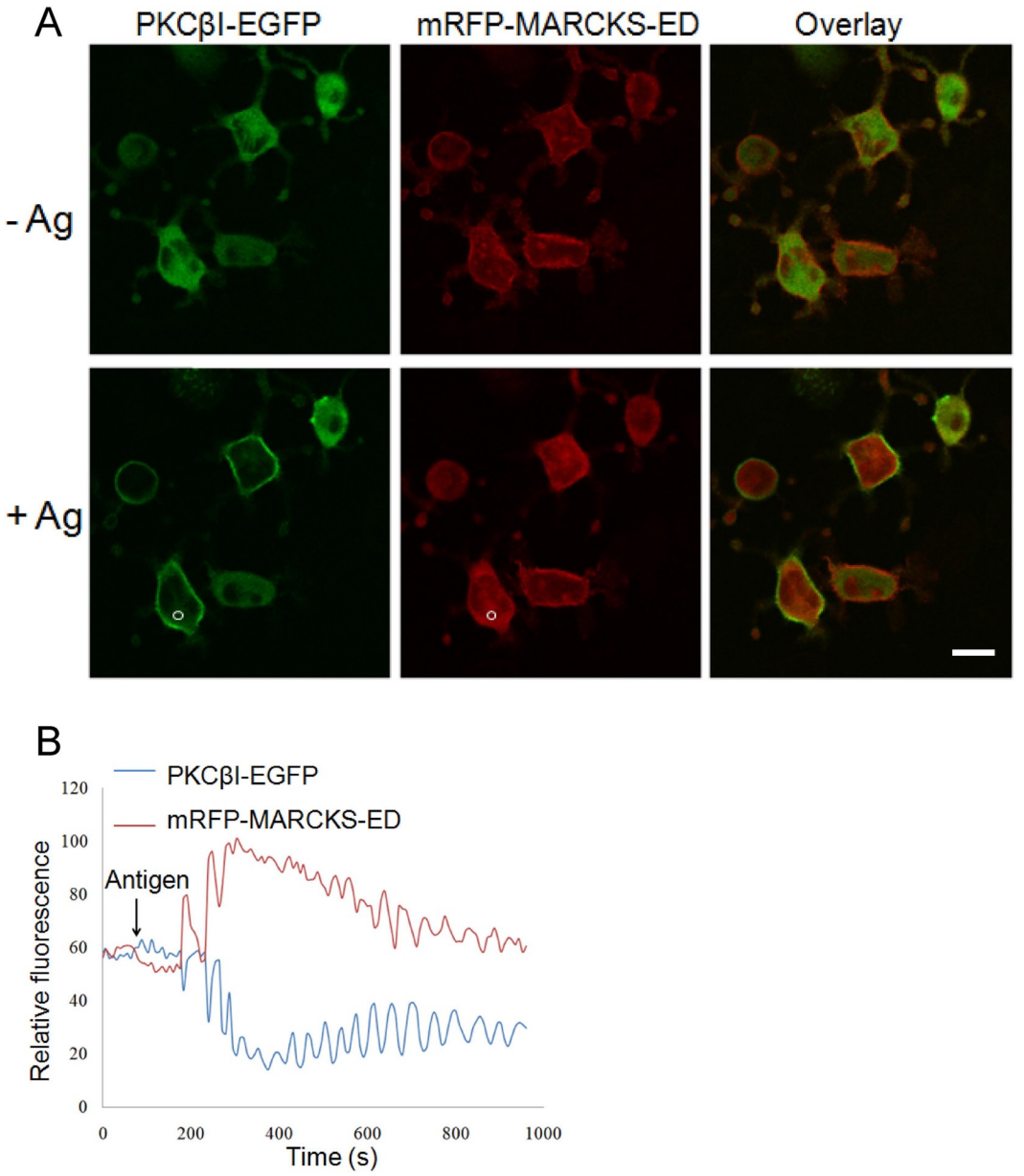

FIGURE 2: Live-cell imaging of antigen-stimulated mRFP-MARCKS-ED and PKCβI-EGFP oscillations. (A) Representative images showing the distributions of PKCβI-EGFP (green) and mRFP-MARCKS-ED (red) before (top) and 100 s after antigen addition at 25°C (bottom) (still frames from Supplemental Movies S2a and S2b). Scale bar, 10 μm. (B) Time courses of cytoplasmic changes of PKCβI-EGFP (blue) and mRFP-MARCKS-ED (red) in a region of interest indicated in representative RBL cell in A.

onset of the Ca^{2+} response is 162 s (SEM = ±20 s; n = 51) in the presence of this mutated construct, and it is 67 s (SEM = ±15 s; n = 63) for cells expressing unmutated mRFP-MARCKS-ED. To investigate further the effect of MARCKS-ED SA4 expression on Ca^{2+} mobilization, we compared the Ca^{2+} response to antigen in the absence of extracellular Ca^{2+}, conditions under which the response is restricted to IP_3-mediated Ca^{2+} release from endoplasmic reticulum (ER) stores (Lee *et al.*, 2005). As shown for a representative cell in Figure 3D, the transient Ca^{2+} response in the absence of extracellular Ca^{2+} is delayed for cells expressing mRFP-MARCKS-ED SA4 cells as compared with control cells not expressing this mutant. We observed an average delay in the onset of Ca^{2+} release from stores in MARCKS-ED SA4–expressing cells of 200 s (SEM = ±22 s; n = 18) compared with that for untransfected control cells.

To further characterize the effects of MARCKS-ED SA4 on functional responses, RBL-2H3 cells were stably transfected with mRFP-MARCKS-ED SA4, and Ca^{2+} mobilization was monitored with indo-1 in a fluorimetry-based assay of suspended cells. As shown in Figure 4A, mRFP-MARCKS-ED SA4–expressing cells exhibit a slower Ca^{2+} response to antigen when compared with the control cells, although the maximal response attained is similar for both. In the absence of extracellular Ca^{2+}, control RBL cells show transient Ca^{2+} release from stores after antigen stimulation, returning to the baseline after several hundred seconds (Figure 4B). This response is delayed for RBL cells stably expressing MARCKS-ED SA4. In both cases, addition of extracellular Ca^{2+} results in a rapid increase in intracellular Ca^{2+} to similar levels, indicating similar activation of store-operated Ca^{2+} entry. To determine the effect of expression of

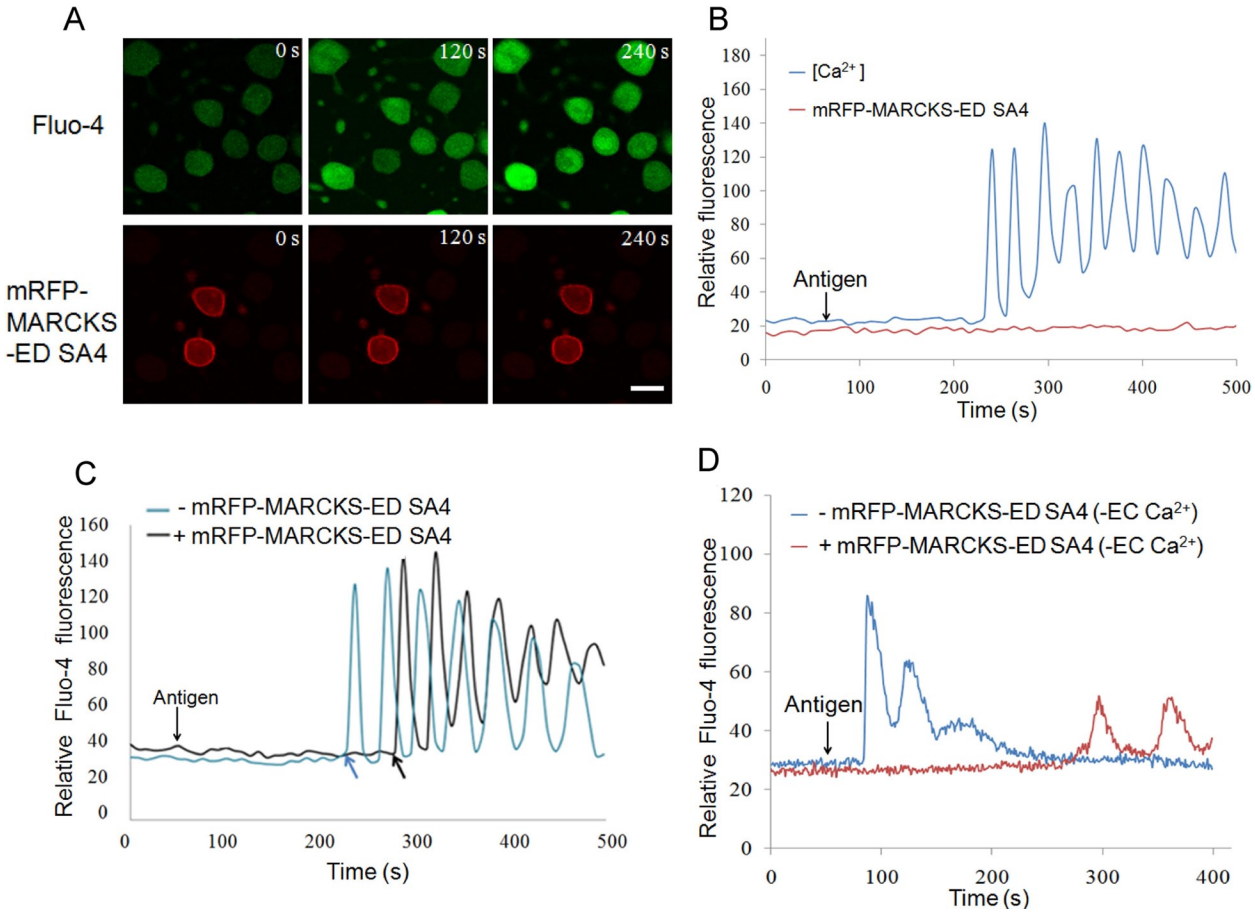

FIGURE 3: Association of mRFP-MARCKS-ED SA4 with the plasma membrane during stimulation by antigen. (A) Images of RBL cells transiently transfected with mutant mRFP-MARCKS-ED (red) and labeled with the [Ca^{2+}] indicator Fluo-4 (green) at various times of stimulation by antigen at 25°C. Images show distributions of labels at time points before and after addition of antigen. Scale bar, 10 μm. (B) Fluorescence intensities from a cytoplasmic region of interest in a representative cell show that mRFP-MARCKS-ED SA4 (red) remains concentrated at the plasma membrane while stimulated [Ca^{2+}] oscillations are observed. (C) Ca^{2+} mobilization in response to Ag at 25°C is delayed in a cell expressing mutant mRFP-MARCKS-ED (black) compared with an untransfected cell (blue); cells shown in Supplemental Movie S3. (D) Ca^{2+} response to antigen in the absence of extracellular Ca^{2+} is substantially delayed in a cell transiently expressing mutant mRFP-MARCKS-ED (red) compared with the response in an untransfected cell (blue) in the same sample.

MARCKS-ED SA4 on store-operated Ca^{2+} entry, independent of PLCγ-mediated release from stores, cells were stimulated with thapsigargin. As shown in Figure 4C, thapsigargin causes a rapid increase in cytosolic Ca^{2+} levels due to leakage of Ca^{2+} from ER, and this is followed by a more sustained phase due to store-operated Ca^{2+} entry. Cells stably expressing MARCKS-ED SA4 showed a similar rapid increase in of store-operated Ca^{2+} influx and a sustained level of store-operated Ca^{2+} entry that is somewhat larger than in control cells. These results show that the delay in the antigen-stimulated Ca^{2+} response due to MARCKS-ED SA4 expression is not observed for stimulation with thapsigargin, consistent with selective inhibition of antigen-stimulated PIP_2 hydrolysis by MARCKS-ED SA4 and consequent inhibition of IP_3-dependent Ca^{2+} release from stores.

Because of known roles for PKC as well as for Ca^{2+} mobilization in granule exocytosis, we evaluated the effect of MARCKS-ED SA4 expression on degranulation by two different methods. In one approach, we loaded granules, which are known to be secretory lysosomes in these cells (Dragonetti *et al.*, 2000; Xu *et al.*, 1998), with fluorescein isothiocyanate (FITC)–dextran by fluid-phase pinocytosis (R. Cohen, unpublished results). Live-cell imaging showed transient, single-granule fusion events as monitored by bursts of FITC fluorescence, which increases when exposed to extracellular pH upon antigen stimulation. As represented in Supplemental Movie S4, we observe that cells transfected with mRFP-MARCKS-ED have delayed onset of degranulation events and also a reduced frequency of these fusion events. As illustrated in Figure 5, still-movie frames at different time points show degranulation events starting 1.9 min (SEM = ±0.1 min; n = 22) after antigen addition to cells not expressing MARCKS-ED SA4. In contrast, we observe that the onset of granule fusion upon antigen stimulation occurs after 5.8 min (SEM = ±0.2 min; n = 22) in cells that express mRFP-MARCKS-ED SA4. Quantification of exocytosis events occurring within 10 min of antigen stimulation reveals reduced exocytosis in cells transfected with mRFP-MARCKS-ED SA4 (average number of fusion events per cell, 14.6 ± 1.4; n = 22) compared with untransfected cells in the same sample (average number of fusion events per cell, 46.1 ± 4.0; n = 22). For thapsigargin stimulation, we did not observe any significant delay in the onset of fusion events in untransfected cells (6.6 ± 0.8 min; n = 7) as compared with cells

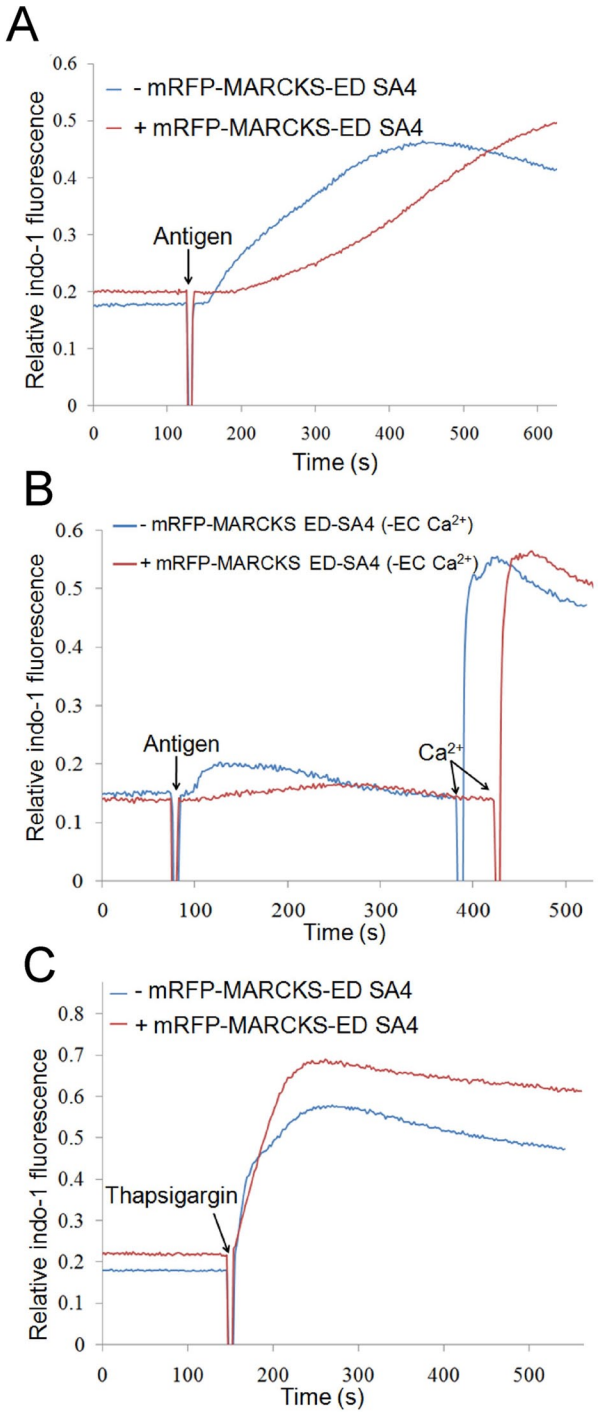

FIGURE 4: Antigen-stimulated Ca^{2+} mobilization is delayed in cells stably expressing mRFP-MARCKS-ED SA4. (A) Steady-state fluorimetry responses to antigen in control RBL cells (blue) and cells stably expressing mRFP-MARCKS-ED SA4 (red). (B) Ca^{2+} response to antigen in the absence of extracellular Ca^{2+} is substantially delayed in RBL cells stably expressing mutant mRFP-MARCKS-ED (red) compared with the response in untransfected cells (blue). Subsequent addition of 1.8 mM $[Ca^{2+}]$ results in similar Ca^{2+} entry for each. (C) Ca^{2+} mobilization in response to thapsigargin in RBL cells stably expressing mRFP-MARCKS-ED SA4 (red) is similar compared with the response in untransfected RBL cells (blue).

expressing mRFP-MARCKS-ED SA4 in the same sample (7.1 ± 0.6 min; n = 7) Under these conditions, we observed reduced exocytosis events in cells expressing mRFP-MARCKS-ED SA4 (average number of fusion events per cell, 8.4 ± 1.3; n = 7) compared with cells not expressing this mutant construct in the same sample (average number of fusion events per cell, 15.4 ± 2.4; n = 7; data not shown).

Because it is difficult to quantify differences in degranulation responses by this real-time imaging method, we also used a second method in which cells are fixed after stimulation and then labeled with anti-CD63 mAb followed by a fluorescent secondary antibody. CD63, also known as LAMP2 (Metzelaar *et al.*, 1991), is localized primarily to secretory lysosomes, but a small amount is present at the plasma membrane in unstimulated cells. In these experiments, unlabeled MARCKS-ED SA4 was cotransfected with the plasma membrane marker PM-EGFP that we used to identify transfected cells and also to define the plasma membrane for image quantification of localized Alexa 555–labeled anti-CD63. As shown in Figure 6A, labeling of CD63 increases at the plasma membrane upon antigen stimulation of RBL cells, and this is clearly stronger in nontransfected cells in the same sample. Figure 6B quantifies this analysis for multiple cells. Some CD63 is evident at the plasma membrane in unstimulated cells, and this is similar with and without expression of MARCKS-ED SA4. For antigen-stimulated cells transfected with PM-EGFP without MARCKS-ED SA4 (control cells), the plasma membrane level of CD63 increases by >2.5-fold. By comparison, antigen-stimulated cells expressing MARCKS-ED SA4 show a substantially smaller stimulated increase in CD63 at the plasma membrane: this stimulated increase was inhibited by ~60% due to expression of MARCKS-ED SA4 (n = 90–100 for each condition).

We also determined whether MARCKS-ED SA4 inhibits granule exocytosis stimulated by thapsigargin, which bypasses PIP_2 hydrolysis and IP_3-dependent Ca^{2+} mobilization. In a separate set of experiments, we again found no significant effect of MARCKS-ED SA4 on basal levels of CD63 at the plasma membrane in unstimulated cells. We observed almost a threefold increase in CD63 at the plasma membrane due to stimulation by an optimal dose of thapsigargin, and this was inhibited by 65% in cells expressing MARCKS-ED SA4 compared with cells expressing PM-EGFP alone. Together, these results provide strong evidence that MARCKS-ED SA4 exerts a direct inhibitory effect on granule exocytosis. Because MARCKS-ED SA4 has been shown to bind tightly to phosphoinositides at the plasma membrane, our results suggest that the sequestration of these lipids provides a significant barrier to granule exocytosis.

DISCUSSION

Antigen activation of IgE receptors on mast cells results in stimulated granule exocytosis, which uses Ca^{2+}-triggered membrane fusion and also depends on the activation of PKC. As shown by our results, the strong temporal synchronizations of antigen-stimulated oscillations of cytoplasmic $[Ca^{2+}]$, PKCβI, and MARCKS-ED (Figures 1 and 2) are consistent with the involvement of $[Ca^{2+}]$-controlled PKCβI activation resulting in phosphorylation of MARCKS as a pathway involved in the regulation of exocytosis. These results are reminiscent of IgE receptor–stimulated, periodic recruitment of PKCγ-EGFP to the plasma membrane previously characterized by Oancea and Meyer (1998). The temporal correlation of PKCβI-EGFP oscillations that are stimulated by antigen with $[Ca^{2+}]$ oscillations in the cytoplasm suggests that $[Ca^{2+}]$ elevation is involved in activation of this PKC isoform. Consistent with this, activation of PKCβI-EGFP recruitment by antigen is transient in the absence of extracellular Ca^{2+}, corresponding to a transient elevation in cytoplasmic $[Ca^{2+}]$ under these conditions (Supplemental Figure S1). Bypassing IP_3-dependent Ca^{2+} mobilization with thapsigargin results in slower, nonoscillatory translocation of PKCβI-EGFP to the plasma

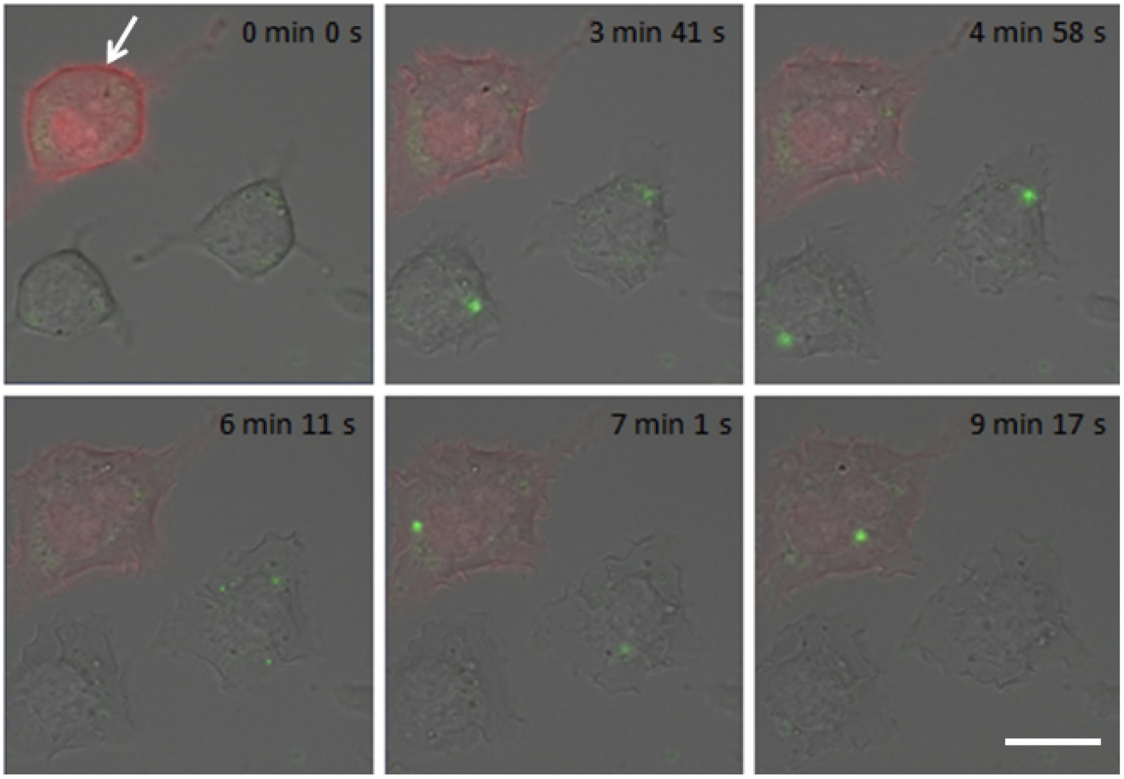

FIGURE 5: MARCKS-ED SA4 inhibits antigen-stimulated degranulation monitored by real-time imaging. Degranulation monitored as FITC-dextran bursts from antigen-stimulated RBL cells shows delayed and reduced responses in cells expressing mRFP-MARCKS-ED SA4. Times in images indicate intervals after antigen addition; white arrow identifies a cell expressing mRFP-MARCKS-ED SA4. Scale bar, 10 μm. See Supplemental Movie S4.

membrane (Figure 1C), and this is consistent with slower activation of diacylglycerol production via phospholipase D under these conditions (Peng and Beaven, 2005). Stimulated dissociation of mRFP-MARCKS-ED from and rebinding to the plasma membrane correspond to the dynamics of PKCβI-EGFP oscillatory association with the plasma membrane and are consistent with a role for PKC activation in this process.

We find that stimulated dissociation of mRFP-MARCKS-ED is prevented by our SA4 mutation of the serine residues that are phosphorylated by activated PKC (Figure 3), consistent with previous studies with MARCKS-ED (Kim *et al.*, 1994). Heo *et al.* (2006) showed that decreased phosphoinositide levels at the plasma membrane cause dissociation of MARCKS-ED, and we obtained similar results with our mutant MARCKS-ED SA4 (Smith *et al.*, 2010). Thus, the strong temporal synchronizations of oscillations of cytoplasmic [Ca^{2+}], PKCβI, and MARCKS-ED under conditions of antigen stimulation are consistent with a role for PKCβI in regulating access of PIPs by endogenous MARCKS. The positively charged, polybasic peptide sequence MARCKS-ED SA4 retains its capacity to bind tightly to highly negatively charged phosphoinositides at the plasma membrane and, of importance, does not dissociate in response to activation of PKC. As monitored with both transient and stable expression of this construct, we observed a delay in antigen-stimulated Ca^{2+} mobilization due to this expression, consistent with reduced accessibility of PIP_2 for hydrolysis by PLCγ (Figures 3 and 4). Despite this delay, the levels of sustained Ca^{2+} response to antigen are similar in the presence and absence of MARCKS-ED SA4. In contrast, stimulation by thapsigargin results in store-operated Ca^{2+} entry that is similar in magnitude and kinetics in the presence and absence of MARCKS-ED SA4.

The inhibitory effect of MARCKS-ED SA4 on the kinetics of antigen-stimulated Ca^{2+} mobilization suggests that this isolated MARCKS-ED segment functions differently in this process than when it is part of full-length, endogenous MARCKS. This inhibition is due primarily to the serine-to-alanine mutations, because unmutated MARCKS-ED does not inhibit granule exocytosis (Supplemental Figure S4). However, inhibitors of PKC activity, such as bisindolylmaleimide I, do not inhibit antigen-stimulated Ca^{2+} mobilization in RBL mast cells (data not shown; Wolfe *et al.*, 1996), implying that PKC-dependent dissociation of endogenous MARCKS is not necessary for PLCγ-mediated hydrolysis of PIP_2 in these cells. Activation of PKC by phorbol esters decreases antigen-stimulated Ca^{2+} mobilization, in part by inhibition of PLCγ (Ozawa *et al.*, 1993b), but PKC activation by antigen does not normally alter Ca^{2+} mobilization, as described earlier. These differences may reflect the degree of activation of PKC by phorbol ester versus antigen, but they also suggest some difference in the pools of PIP_2 bound by endogenous MARCKS and by MARCKS-ED SA4.

We find that MARCKS-ED SA4 effectively delays and inhibits granule exocytosis using two different approaches. Real-time monitoring of individual granule exocytosis events using FITC-dextran permits us to visualize the rate of this process (Figure 5 and Supplemental Movie S4). These results clearly reveal a delay in granule exocytosis in response to antigen that is predicted by the delay in antigen-stimulated Ca^{2+} mobilization caused by transient expression of MARCKS-ED SA4 (Figure 3C). Measurement of the stimulated increase in the granule marker CD63 at the plasma membrane permits quantification of granule exocytosis following fixation of activated cells. Inhibition of thapsigargin-stimulated degranulation to an extent similar to that for antigen demonstrates that MARCKS-ED

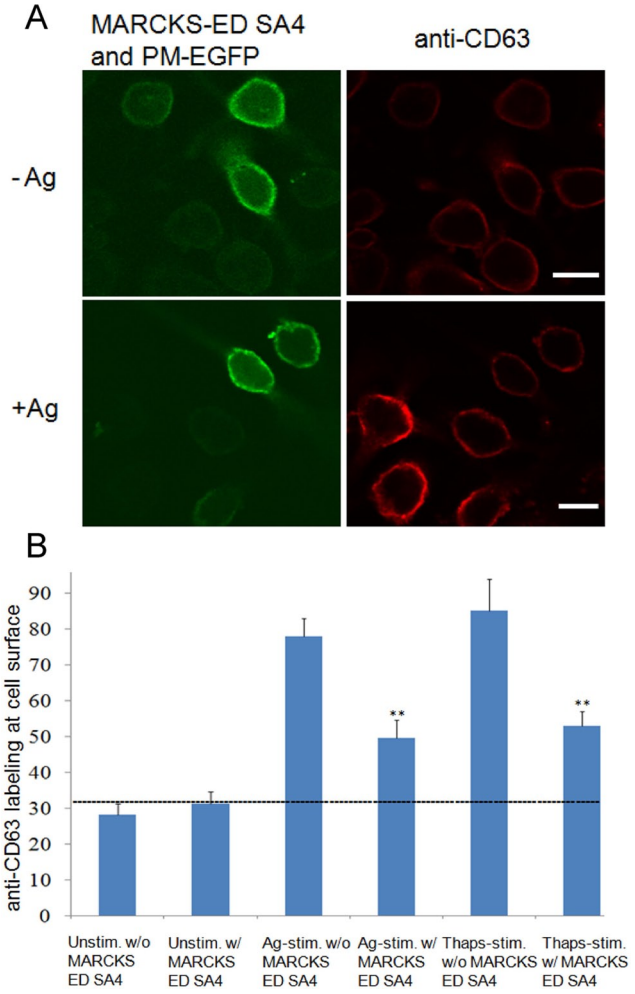

FIGURE 6: Inhibition of degranulation due to expression of MARCKS-ED SA4 as monitored by CD63 appearance at the plasma membrane. (A) Representative images showing reduced plasma membrane labeling by anti-CD63 due to antigen stimulation in cells expressing mutated MARCKS-ED expression. Scale bars, 10 μm. (B) Quantification of anti-CD63 labeling at the plasma membrane for unstimulated, antigen-stimulated, and thapsigargin-stimulated cells with and without expression of MARCKS-ED SA4. Error bars, SEM for 90–100 cells for each condition from n = 6 experiments. The p values from Student's *t*-test show **p < 0.01 for stimulated cells expressing MARCKS-ED SA4 compared with stimulated control cells.

SA4 inhibits a step in granule exocytosis that is downstream of Ca^{2+} mobilization. This step is apparently dependent on PKC activation, because expression of the nonmutated MARCKS-ED sequence does not inhibit degranulation in similar experiments (Supplemental Figure S4).

What is the mechanism of this inhibition? The finding by Heo *et al.* (2006) that MARCKS-ED binds to the plasma membrane primarily through its association with phosphoinositides, together with our previous results with MARCKS-ED SA4 (Smith *et al.*, 2010), makes it likely that the functional effects we observe in the present study are due to phosphoinositide binding by MARCKS-ED SA4. Certainly, the delay of antigen-stimulated Ca^{2+} mobilization, both in the presence and absence of extracellular Ca^{2+}, is readily accountable for by the capacity of MARCKS-ED SA4 to tie up the pool of PIP_2 that is normally hydrolyzed by activated PLCγ. Although this electrostatic binding is tight, it is not specific in the usual sense of a binding site, and it is possible that PLC or other proteins have limited access to PIP_2 under these conditions (Gambhir *et al.*, 2004).

The mechanism by which MARCKS-ED SA4 interferes with granule exocytosis is also likely to depend on its capacity to bind tightly to phosphoinositides, but the specific interactions that are prevented by this association are not yet clear. Synaptotagmins are a family of [Ca^{2+}] sensor proteins that participate in multiple exocytotic processes, including mast cell degranulation (Sagi-Eisenberg, 2007), and synaptotagmin-2 is the principal isoform that mediates granule exocytosis in mast cells (Melicoff *et al.*, 2009). Two C2 domains in synaptotagmins bind to Ca^{2+} and PIP_2 to trigger the SNARE-mediated fusion of secretory vesicles to the plasma membrane (Li *et al.*, 2006; Schwarz, 2004). A plausible mechanism for the inhibitory effects of MARCKS-ED SA4 involves its interference with this PIP_2-dependent process. A variety of SNARE proteins are expressed in RBL cells, including syntaxin-4, SNAP-23, VAMP-7 and VAMP-8 (Paumet *et al.*, 2000; Sander *et al.*, 2008), and studies in other cell types have provided evidence for a role for PIP_2 in regulating syntaxin-1 and VAMP-2–mediated membrane fusion (Daily *et al.*, 2010). Thus it is possible that MARCKS-ED SA4 affects PIP_2 binding to several different proteins in this process.

It is also possible that other PKC substrates participate in the terminal steps of mast cell degranulation. Munc18-1 is a prominent PKC substrate, and disruption of Munc18-1 association with syntaxin-1 inhibits granule fusion events in PC12 cells (Barclay, 2008). In addition to phosphorylation by PKC, binding of Ca^{2+}-activated calmodulin to MARCKS causes translocation of MARCKS from the plasma membrane and thereby releases sequestered PIP_2, as reviewed elsewhere (McLaughlin and Murray, 2005). This mechanism of MARCKS-ED displacement, which is independent of phosphorylation by PKC, may account for the incomplete inhibition of degranulation by MARCKS-ED SA4 that we observe. Overall, our results highlight a possible role for PKC in the regulation of PIP_2 accessibility, and they establish the utility of MARCKS-ED SA4 as an effective inhibitor of phosphoinositide-dependent processes. Ligand-dependent recruitment of PI 5′-phosphatases can cause acute reduction of plasma membrane PIP_2 (Stauffer *et al.*, 1998; Varnai and Balla, 1998), but this alternative approach requires coexpression of multiple constructs that can be difficult to achieve at sufficiently high levels for effective interference in some cell types.

In summary, our results provide mechanistic insights into the regulation of granule exocytosis by PKC and phosphoinositides. We showed that MARCKS-ED SA4 stably associates with the plasma membrane, delays the onset of antigen-stimulated Ca^{2+} mobilization and degranulation responses, and inhibits both antigen- and thapsigargin-stimulated granule exocytosis. These effects can be accounted for by tight association of MARCKS-ED SA4 with phosphoinositides, although contributions from interactions with other binding partners may also contribute to these functional outcomes. PIP_2 has been shown to enhance the Ca^{2+} affinity of synaptotagmins, and it may thereby facilitate triggering of secretory granule fusion with the plasma membrane. Thus, we propose that PKC is a key to regulating the access of PIP_2, such that PKC activation during stimulation results in exposure of PIP_2 necessary for granule fusion.

MATERIALS AND METHODS

cDNA plasmids

PKCβI-EGFP was from Clontech (Palo Alto, CA). mRFP-MARCKS-ED was made from yellow fluorescent protein (YFP)–MARCKS-ED by exchanging YFP for mRFP by using the *Nhe*I and *Hin*dIII restriction sites. To generate mRFP-MARCKS-ED SA4, mutation of serines 159,

163, 167, and 170 (MARCKS full-length sequence numbers; Stumpo *et al.*, 1989) to alanines was performed by using a QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) as previously described (Smith *et al.*, 2010). Mutations were confirmed by DNA sequence analysis. For some experiments, MARCKS-ED and MARCKS-ED SA4 were generated by removing mRFP cDNA from the respective fluorescent constructs. The PM-EGFP construct was prepared as previously described (Pyenta *et al.*, 2001).

Cell culture and transient/stable transfection

RBL-2H3 cells were maintained as monolayers in MEM containing 20% fetal bovine serum and 10 μg/ml gentamicin sulfate. Cells were harvested using trypsin-EDTA (Invitrogen, Carlsbad, CA) and transiently transfected using electroporation as previously described (Cohen *et al.*, 2009). Electroporated cells were plated into MatTek coverslip wells (Dover, MA) in full medium and sensitized with mouse monoclonal anti-DNP IgE (0.5 μg/ml; Posner *et al.*, 1992) during overnight culture. All experiments were done 20–24 h posttransfection. For the generation of stable RBL cell lines expressing mRFP-MARCKS-ED SA4, transfected cells were selected in MEM containing 600 μg/ml G418 (Life Technologies, Invitrogen). Cells positive for mRFP-MARCKS-ED SA4 were identified by mRFP fluorescence. Clones of cells expressing mRFP-MARCKS-ED SA4 were selected using calcium alginate fiber–tipped wood applicator swabs (Fisher HealthCare, Houston, TX). One mutant clone with a relatively low expression level and two mutant clones expressing relatively high levels of mRFP-MARCKS-ED SA4 were produced and characterized.

Live-cell imaging and analysis

Prior to imaging, cells were rinsed with buffered saline solution (BSS; 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 135 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 5.6 mM glucose, 1 mg/ml bovine serum albumin [BSA], pH 7.4) and then imaged using a Leica (Wetzlar, Germany) TCS SP2 upright confocal microscope with an Apo 63× dipping objective. Images were collected every 4 s for 1–2 min before stimulation, followed by 5–10 min of image acquisition poststimulation using DNP-BSA (0.2–1 μg/ml) or thapsigargin (0.15 μM). All live-cell imaging was carried out between 25 and 37°C as specified. For image analysis, a small cytoplasmic region of interest was chosen in each cell to monitor the fractional cytoplasmic fluorescence intensity changes with time using ImageJ (National Institutes of Health, Bethesda, MD).

Intracellular [Ca^{2+}] measurement of adherent cells

Overnight-plated and IgE-sensitized cells in MatTek dishes were loaded with Fluo-4 AM (Invitrogen) or Fura Red AM (Invitrogen) at 1 μg/ml in BSS buffer containing 0.5 mM sulfinpyrazone (Sigma-Aldrich, St. Louis, MO) for 10 min at 37°C. Cells were then washed three times in BSS/sulfinpyrazone. Fields of cells were selected to contain both transfected and untransfected cells and monitored as previously described (Calloway *et al.*, 2009). For measurement of Ca^{2+} release from endoplasmic reticulum stores alone, BSS without Ca^{2+} was used while imaging, and 2 mM $CaCl_2$ was added subsequently to observe store-operated Ca^{2+} entry.

Intracellular [Ca^{2+}] measurements of suspended cells

Cells suspended in BSS were sensitized with anti-DNP IgE and loaded with indo-1 AM (Invitrogen). Time-based acquisition of cytoplasmic [Ca^{2+}] was monitored in an SLM 8100C fluorimeter, and cells were stimulated with 0.2 μg/ml DNP-BSA or 0.15 μM thapsigargin. Stimulated [Ca^{2+}] responses were normalized using total indo-1 fluorescence following cell lysis by addition of 0.1% Triton X-100 minus total fluorescence in the presence of excess EDTA as previously described (Vasudevan *et al.*, 2009).

Granule exocytosis monitored by FITC-dextran release

Cells were transfected with mRFP-MARCKS-ED SA4 and cultured as described. They were further loaded overnight with 1 mg/ml FITC-dextran (10,000 MW from Sigma-Aldrich) and 0.2 mM 5-hydroxytryptamine (Sigma-Aldrich; R. Cohen, K. Corwith, D. Holowka, and B. Baird [unpublished data]). Cells were washed three times with BSS, and live-cell imaging was performed at 37°C on a Zeiss (Jena, Germany) 710 confocal microscope with maximum pinhole size to visualize degranulation events throughout the entire cell depth. Cells were stimulated with 0.4 μg/ml DNP-BSA, and detection of degranulation events as fluorescence bursts were recorded from movies similar to that in Supplemental Movie S4. Time of onset of degranulation was determined as the average time of first five exocytosis events.

Granule exocytosis monitored by anti-CD63 labeling

Cells were cotransfected with unlabeled MARCKS-ED SA4 (or unlabeled MARCKS-ED for Supplemental Figure S4) and palmitoylated, myristoylated EGFP (PM-EGFP) or transfected with PM-EGFP alone (control) by electroporation and then cultured in full media as described. Cells were sensitized with anti-DNP IgE (0.5 μg/ml) overnight and then stimulated with 0.4 μg/ml DNP-BSA or 1 μM thapsigargin in the presence of 1 μM cytochalasin D (Sigma-Aldrich) for 10 min at 37°C and then fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 15 min at room temperature. Fixed cells were labeled with 5 μg/ml anti-CD63 mouse antibody (AD1; BD Biosciences PharMingen, San Diego, CA) for 1 h at room temperature, followed by 10 μg/ml fluorescent secondary antibody (Alexa 555 goat anti–mouse IgGγ1; Invitrogen) for 1 h at room temperature. Imaging of fixed samples was carried out at the equatorial plane of the cells using a Leica confocal TCS SP2 microscope as described. Analysis of images was done by first generating a plasma membrane mask by thresholding the PM-EGFP image in Matlab (MathWorks, Natick, MA; Calloway *et al.*, 2009). Fluorescence of Alexa 555 goat anti–mouse IgGγ1 was determined by integrating the fluorescence intensity of all pixels in the plasma membrane mask for each cell. Control labeling was performed using nonspecific primary mouse IgG antibody under the same conditions as for AD1 labeling.

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