Spatiotemporal Resolution of Mast Cell Granule Exocytosis
Reveals Correlation with Ca$^{2+}$ Wave Initiation

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SUMMARY
Mast cell activation initiated by antigen-mediated crosslinking of IgE receptors results in stimulated exocytosis of secretory lysosomes in the process known as degranulation. Much has been learned about the molecular mechanisms important for this process, including the critical role of Ca\(^{2+}\) mobilization, but spatio-temporal relationships between stimulated Ca\(^{2+}\) mobilization and granule exocytosis are incompletely understood. Here we use a novel imaging-based method that utilizes fluorescein isothiocyanate (FITC)-dextran as a reporter for granule exocytosis in RBL mast cells and takes advantage of the pH sensitivity of FITC. We demonstrate the selectivity of FITC-dextran, accumulated by fluid phase uptake, as a marker for secretory lysosomes, and we characterize its capacity to delineate different exocytotic events, including full fusion, kiss-and-run transient fusion, and compound exocytosis. Using this method, we find strong dependence of degranulation kinetics on the duration of cell-substrate attachment. We combine imaging of degranulation and Ca\(^{2+}\) dynamics to demonstrate a spatial relationship between the sites of Ca\(^{2+}\) wave initiation in extended cell protrusions and exocytosis under conditions of limited antigen stimulation. In addition, we find that the spatially proximal Ca\(^{2+}\) signaling and secretory events correlate with participation of TRPC1 channels in Ca\(^{2+}\) mobilization.

INTRODUCTION
In response to antigen (Ag)-mediated crosslinking of cell surface IgE receptors (IgE-FceRI), mast cells undergo a series of intracellular signaling events that result in fusion of secretory granules with the plasma membrane to release a number of inflammatory mediators including histamine, proteoglycans, proteases, and lysosomal hydrolases, which trigger allergic and inflammatory reactions that can have systemic effects (Blank and Rivera, 2004), (Gilfillan and Tkaczyk, 2006). In this process, stimulated tyrosine phosphorylation of FcεRI leads to inositol 1,4,5-trisphosphate (IP\(_3\)) production by phospholipase C\(\gamma\) (PLC\(\gamma\)), which causes Ca\(^{2+}\) depletion from endoplasmic reticulum stores to induce store-operated Ca\(^{2+}\) entry (SOCE; (Di Capite and Parekh, 2009)). Activation of SOCE elicits oscillatory cytosolic Ca\(^{2+}\) elevations, that, together with activated protein kinase C, trigger granule exocytosis (Kim et al., 1997; Ma and Beaven, 2009).

Although the early biochemical events of mast cell activation have been studied extensively (Rivera and Gilfillan, 2006), the mechanisms by which degranulation occurs and is regulated in these cells are not fully understood, calling for new experimental approaches. In particular,
delineating the relationships between degranulation and Ca\textsuperscript{2+} mobilization requires high spatial and temporal resolution of both cytoplasmic Ca\textsuperscript{2+} changes and individual exocytotic events. We recently showed that Ag-mediated crosslinking of IgE-Fc\textsubscript{ε}RI initiates Ca\textsuperscript{2+} responses in mast cells in the form of a fast Ca\textsuperscript{2+} wave that usually begins in the tip of an extended protrusion, then moves down the length of the cell over a time period of several seconds (Cohen et al., 2009). At low doses of antigen, initiation of this wave depends on Ca\textsuperscript{2+} influx by the canonical transient receptor potential channel TRPC1. The initiating waves are typically followed by periodic Ca\textsuperscript{2+} oscillations that depend on Ca\textsuperscript{2+} influx via SOCE (Ma and Beaven, 2009; Vig et al., 2008). These oscillations encode temporal information relevant to degranulation events (Kim et al., 1997), but they do not manifest the spatial localization displayed by wave propagation (Cohen et al., 2009). Very little is known about the physiological function of the extended protrusions in mast cells where Ca\textsuperscript{2+} waves initiate. We hypothesize that, in vivo, mast cells protrude through epithelial cell barriers to provide distal sensors for relevant ligands. In this context, the capacity for granule exocytosis from these protrusions may be physiologically significant.

A number of methods have been developed to measure individual granule exocytotic events in mast cells (Hohman and Dreskin, 2001). Early studies utilized acridine orange, a fluorescent weak base that accumulates in acidified vesicles (Kawasaki et al., 1991; Williams and Webb, 2000). However, a recent study demonstrated that photosensitization of this dye during imaging limits its utility (Jaiswal et al., 2007). Other strategies have utilized granule-localized serotonin fluorescence imaged using multiphoton microscopy, together with complementary membrane and lysosome-labeling probes (Williams et al., 1999). Although these methods allow detection of individual stimulated granule exocytotic events in mast cells, they are technically demanding and therefore limited in their usage.

In the present study we utilize FITC-dextran, which accumulates in secretory lysosomes by fluid phase pinocytosis (Dragonetti et al., 2000), to monitor exocytosis of individual granules in RBL mast cells. The pH sensitivity of FITC (Ohkuma and Poole, 1978) reveals granule-plasma membrane fusion events that result in exposure to the extracellular medium. Such exposure relieves the low pH quenching of FITC fluorescence in the lumen of the secretory lysosomes that constitute exocytic granules in these cells (Xu et al., 1998). By this means we can simultaneously monitor granule exocytosis and cytosolic Ca\textsuperscript{2+} elevation in individual cells. We find that, at limiting concentrations of antigen, exocytosis occurs preferentially along extended cell protrusions where Ca\textsuperscript{2+} waves initiate.
RESULTS

Labeling of secretory lysosomes with fluorescent dextrans. As previously described (Dragonetti et al., 2000), fluorescent dextran conjugates accumulate in secretory lysosomes of RBL mast cells after fluid phase pinocytosis. Figure 1A shows RBL cells transfected with CD63-GFP and incubated with TxRed-dextran together with 5-hydroxytryptamine (5-HT) for 24 hr. These cells exhibit a high degree of co-localization of TxRed-dextran in the lumen of large intracellular vesicles with CD63-GFP at their periphery (see inset, Fig. 1A). CD63, also known as LAMP3, is a tetraspanin membrane protein containing a lysosome-targeting domain (Levy and Shoham, 2005), and it is commonly used as secretory granule marker in mast cells (Blott and Griffiths, 2002; Amano et al., 2001). CD63 has been used previously to monitor secretory granule exocytosis in RBL cells by total internal reflection fluorescence microscopy (Wu et al., 2007).

As evident in Figure 1A, we frequently observe a high density of granules in tips of extended protrusions. This accumulation of granules is also seen often with EGFP-VAMP7, which is a v-SNARE for secretory lysosomes (Puri et al., 2003) that is also highly co-localized with TxRed-dextran incorporated during overnight loading (Figure 1B). Cross-correlation analysis reveals a moderately high value for Pearson’s coefficient (>0.7 for n=8 cells analyzed) for this label pair. Values for Pearson’s coefficient >0.2 are usually considered to indicate significant co-localization (Pyenta et al., 2001). Co-labeling with FITC-dextran and TxRed-dextran further shows almost complete co-incidence in intracellular vesicles enlarged by 5-HT loading, (Figure S1), with a Pearson’s coefficient of 0.85 (n=12). Thus, similar localization patterns for TxRed–dextran with both CD63-GFP and EGFP-VAMP7 provide strong evidence that FITC-dextrans selectively label secretory lysosomes in these cells.

Granule fusion events. To image single exocytosis events in RBL cells that are stimulated by Ag, IgE-sensitized cells were loaded with FITC-dextran and 5-HT, and then Ag was delivered locally by micropipette as described in Materials and Methods. At a high concentration of Ag in the pipette (1.7 μg/ml), degranulation events, detected as local bursts of FITC fluorescence, are observed as soon as 20-40 sec after Ag addition, and they continue for several minutes (Supplemental Movie 1). The representative images in Figure 2 illustrate three variants of these events. Based on previous results (Williams and Webb, 2000), we interpret the rapid increase in FITC fluorescence to be pH neutralization occurring in the granule lumen as it fuses with the plasma membrane and is exposed to the extracellular medium, and the rapid decrease is due to diffusion/dilution in the extracellular
medium. No bursts of FITC-dextran release in response to Ag were observed in cells that were not sensitized with specific IgE (data not shown).

In addition to rapidly releasing individual events (Supplemental Movie 1; Figure 2A and 2B), which are the most frequent manifestation of exocytosis, a smaller percentage of the events exhibit a sharp initial burst of FITC fluorescence that more slowly returns to baseline levels. As exemplified in Figures 2C and D, a rapid rise and partial decrease in fluorescence is followed by a second increase and then a gradual decrease that lasts for >100 sec. The sustained decreases are likely due to reclosing of the partially released granule in a “kiss and run” mechanism, followed by re-acidification of the lumen (Williams and Webb, 2000). We also observe sequential or compound exocytosis (Pickett et al., 2005), resulting from granule-granule fusion that follows an initial granule-plasma membrane fusion event (Figures 2E and 2F), as previously reported for mast cells (Alvarez de Toledo and Fernandez, 1990; Guo et al., 1998).

Simultaneous detection of FITC-dextran and TxRed-dextran release from single granules. To distinguish whether a transient burst of FITC-dextran fluorescence is due to exocytotic release or transient, intracellular neutralization of acidic granules, we loaded the granules with both FITC-dextran and TxRed-dextran, then stimulated these cells as above. As shown by the images in Figure 3, the transient burst of FITC-dextran fluorescence correlates temporally and spatially with the pH-insensitive release of TxRed-dextran from the granule. Release of both fluorophores is followed by rapid diffusion and dilution in the extracellular medium. This representative example of events detected as simultaneous changes in FITC-dextran and TxRed-dextran fluorescence provide evidence that FITC-dextran transient bursts are due to exocytosis of the granule contents at the time of FITC dequenching.

Combined amperometry and FITC-dextran imaging in individual RBL cells. To evaluate further whether FITC-dextran dequenching is a reliable marker for granule exocytosis in RBL cells, we simultaneously monitored FITC-dextran fluorescence and 5-HT release that is detected amperometrically by a locally positioned carbon fiber electrode. As previously demonstrated for mast cells, amperometric detection of 5-HT release provides high temporal resolution of exocytotic events (Kim et al., 1997; Jaffe et al., 2001). As shown in Figure 4, we find a strong temporal correlation between bursts of FITC fluorescence and amperometric spikes that represent degranulation events. Multiple, spatially separated exocytic events, detected as FITC fluorescence bursts, often are seen to occur during the same amperometric spike, suggesting that the initiation of
these separate events is temporally correlated, possibly to the peak of a Ca\(^{2+}\) oscillation (Kim et al., 1997).

**Cell attachment influences RBL mast cell degranulation kinetics.** The pH sensitivity of FITC-dextran release permits measurements of exocytosis from suspended mast cells by monitoring with steady-state fluorimetry. As shown in Figure 5A, addition of Ag to IgE-sensitized RBL cells loaded with FITC-dextran causes a time-dependent increase in fluorescence that depends on extracellular Ca\(^{2+}\). Subsequent addition of the Ca\(^{2+}\) ionophore, A23187, causes a further increase in FITC fluorescence such that the combined response for antigen and ionophore approaches ~60% of the total FITC fluorescence detected following cell lysis with Triton X-100, indicating that a large percentage of FITC-dextran is released from the secretory granules under these conditions. The response to antigen is relatively slow under these conditions, with a half-time of ~10 min in multiple experiments (data not shown).

The degranulation response to Ag for cells that remain attached after overnight loading with FITC-dextran occurs somewhat more rapidly, with a half-time of ~5-6 min (data not shown). This degranulation response for attached cells is only about 2-fold slower when 1.7 ng/ml Ag is used to stimulate these adherent cells, suggesting that Ag binding and crosslinking are not rate limiting under these conditions (data not shown). These differences in Ag-stimulated degranulation kinetics for adherent compared to suspended cells prompted us to investigate their dependence on the duration of cell adhesion. As shown in Figure 5B, there is a strong dependence of both the rate and number of antigen-stimulated exocytotic events on the time that cells have been attached before stimulation, with substantial increases over 20 to 90 min of attachment. During 300 sec of stimulation, cells pre-attached for 90 min exhibit similar or even larger numbers of degranulation events than cells attached overnight, but this response was somewhat slower, with a more substantial lag time before initiation. Shorter attachment periods of 20 and 60 min resulted in substantially longer lag times. These results demonstrate that the kinetics of the degranulation response to Ag strongly depends on the duration of cell attachment.

**Simultaneous imaging of Ca\(^{2+}\) and degranulation responses.** Exocytosis and Ca\(^{2+}\) mobilization are strongly coupled, and their temporal and spatial relationships have been extensively characterized in both excitable and non-excitable cells (Kasai, 1999; Blank et al., 2001). Our previous studies demonstrated that Ca\(^{2+}\) responses to Ag in RBL mast cells usually initiate as waves that begin at the tips of extended cell protrusions (Cohen et al., 2009), and these results prompted us to investigate whether degranulation is influenced by Ca\(^{2+}\) waves. To monitor Ca\(^{2+}\) mobilization and
degranulation simultaneously in the same cells, we used Fura Red as the Ca\(^{2+}\) indicator in cells that were pre-loaded with FITC-dextran. Figure 6A shows a representative cell with an extended protrusion in which the distribution of detectable FITC-dextran is shown from a single frame taken 75 sec after the stimulation was initiated (1.7 \(\mu\)g/ml in pipette; left image). The middle image shows Fura Red from the same frame and indicates the region selected for the kymograph of Ca\(^{2+}\) changes in the cell (yellow line with arrow). The right image shows the exocytotic bursts from a series of FITC fluorescence images integrated over the stimulation time period of 75 sec. Figure 6B shows the kymograph of this cell that compares the time course of Ca\(^{2+}\) elevation (upper panel) to the time course of degranulation events corresponding to FITC-dextran bursts (middle panel). Fura Red exhibits a decrease in fluorescence when Ca\(^{2+}\) levels are elevated, such that darker intervals in this timeline correspond to increased cytoplasmic Ca\(^{2+}\), and brighter red intervals correspond to lower Ca\(^{2+}\) levels. The slanted dashed line in Figure 6B (top panel) indicates the initiation of the Ca\(^{2+}\) wave and its propagation from the protrusion towards the cell body. As seen in the lowest panel in Figure 6B, degranulation events detected with FITC-dextran initiate frequently during intervals of elevated cytoplasmic Ca\(^{2+}\) that correspond to the peaks of Ca\(^{2+}\) oscillations. To quantify this comparison, we assessed the temporal distribution of granule exocytosis events relative to the peaks of oscillatory elevations in cytoplasmic Ca\(^{2+}\) as depicted in Figure 6C. This analysis is shown in Figure 6 D and E, and it reveals that the majority of exocytotic events occur in conjunction with, and typically just following, the peaks of Ca\(^{2+}\) oscillations.

In our initial investigation of the spatial distributions of exocytotic events, we compared these distributions for cells that initiate Ca\(^{2+}\) waves from protrusions (“P-wave” cells) vs. cells initiating Ca\(^{2+}\) waves from the cell body (“B-wave” cells) when a high dose of Ag (1.7 \(\mu\)g/ml) was used for stimulation. As shown in Figures 7A, left side, only a small percentage of exocytotic events occurs along protrusions compared to the cell body at this high Ag dose, and the difference in total number of events for cells that initiate Ca\(^{2+}\) responses as P-waves vs. B-waves is small. These cells were shown to initiate Ca\(^{2+}\) waves from protrusions with a higher probability at low doses of antigen (Cohen et al., 2009), and we investigated whether a low dose of antigen (1.7 ng/ml) causes a higher probability of exocytosis along these extended protrusions than seen with a high dose of antigen. When this low dose of Ag is used to stimulate degranulation, the average number of exocytotic events per cell is less than at the high antigen dose, and this difference is particularly evident for cells with Ca\(^{2+}\) waves that initiate in the cell body (Figure 7A, right side). However, at this low dose of antigen we find that there is a substantially larger percentage of exocytotic events that occurs
along protrusions for cells that initiate Ca\textsuperscript{2+} responses at these protrusions. More than 50% of the exocytotic events occur in protrusions under these conditions, and this percentage is greater than that for cells stimulated with the high dose of antigen. When the time course for exocytosis events is compared for cells that initiate Ca\textsuperscript{2+} responses as P-waves, events in protrusions at early times (≤120 sec after stimulation) occur at similar frequencies for cells at high and low antigen doses.

Interestingly, the larger number of events along protrusions at the low dose of Ag is seen primarily at longer times after stimulation (Figure 7B). This suggests that the P-wave somehow primes that region of the cell for exocytotic events that occurs many tens of seconds after the initiating wave. In addition, we find that the increased occurrence of exocytosis events along the protrusions following P-waves correlates with appearance of one or more local Ca\textsuperscript{2+} transients, observed as an abrupt and isolated change in Fura Red fluorescence, prior to wave initiation (Figure 7C). This observation suggests that local exocytosis is facilitated by these transient and spatially restricted Ca\textsuperscript{2+} elevations.

TRPC1 knockdown inhibits Ag-stimulated granule exocytosis. We previously found that Ca\textsuperscript{2+} entry via TRPC1 is important for Ca\textsuperscript{2+}-dependent P-wave initiation, which is particularly manifest at low doses of Ag (Cohen et al., 2009). To determine whether TRPC1 contributes to Ag-stimulated degranulation in RBL cells, we compared the degranulation response of these cells in which TRPC1 is knocked down by expression of a vector containing a TRPC1-interfering small hairpin (sh) RNA sequence to that with a control shRNA sequence. As shown in Figure 8A-D, we find that TRPC1 shRNA causes a significant reduction in the degranulation response and is particularly effective in inhibiting degranulation events in protrusions. These results provide evidence that TRPC1 channels contribute to the degranulation response in RBL mast cells. Furthermore, they are consistent with results in Figure 7A showing enhanced exocytosis from protrusions at low Ag concentration, which are conditions in which TRPC1 is important for Ca\textsuperscript{2+} wave initiation from these morphological features (Cohen et al., 2009).

DISCUSSION

The present study provides several lines of evidence to establish the use of FITC-dextran as a reliable marker for live cell degranulation imaging. Our real-time imaging method permits simultaneous monitoring of Ca\textsuperscript{2+} mobilization and secretory granule exocytosis with high spatial and temporal resolution, and thus allows direct comparison of these co-stimulated processes. We find that granule exocytosis occurs more frequently along extended cell protrusions when Ca\textsuperscript{2+} waves initiate from these structures, particularly at limiting antigen concentration. Under these conditions,
Ca\(^{2+}\) influx via TRPC1 contributes significantly to granule exocytosis in these cells, consistent with our previous results indicating its participation in Ca\(^{2+}\) wave initiation from protrusions (Cohen et al., 2009) and with recent evidence for its role in stimulated degranulation in mouse bone marrow-derived mast cells (Suzuki et al., 2010).

*FITC-dextran is a reliable marker for real time detection of granule fusion in mast cells.* Consistent with previous results (Dragonetti et al., 2000), we find that pinocytosed fluorescent dextrans co-localize to a large extent with CD63 and VAMP7, two membrane markers for secretory lysosomes, and these secretory lysosomes frequently concentrate at the tips of extended protrusions (Figure 1). Stimulated exocytosis events are monitored by local increases in the pH-sensitive fluorescence of FITC-dextran, and these are manifested as either transient full fusion events (Figure 2A and B) or more sustained changes in FITC-dextran fluorescence that represents either kiss-and-run (Figure 2C and D) or compound exocytosis (Figure 2E and F) events. It is possible that these different degranulation dynamics represent distinct subsets of secretory granules that use different molecular machineries to promote the membrane fusion. However, we see little evidence for granule heterogeneity by other criteria, and the time course for stimulated FITC-dextran exocytosis events is similar to that for \(\beta\)-hexosaminidase release under optimal conditions (data not shown).

Simultaneous labeling of these granules with FITC-dextran and TxRed-dextran results in stimulated exocytosis events in which the transient increase in FITC fluorescence corresponds in time and space to the loss of TxRed fluorescence (pH insensitive) from the same granule (Figure 3), providing direct evidence for the correspondence of transient FITC fluorescence increases with the emptying of granule contents. In addition, the secretory events detected by a local burst of FITC-dextran fluorescence temporally correlate with exocytosis events detected by 5-HT amperometry (Figure 4). Together, these results provide strong evidence for the identification of stimulated FITC-dextran fluorescence bursts as granule exocytosis events.

*Degranulation is significantly influenced by the duration of cell adhesion.* The FITC-dextran loading method provides not only the means for single cell based imaging, but also provides an easy, time resolved and sensitive approach to examine population-based exocytosis studies. With that in mind, we monitored Ag-stimulated degranulation of suspended RBL cells as the net increase in FITC-dextran fluorescence detected by steady state fluorimetry (Figure 5A), which revealed a somewhat slower time course for this process relative to that observed for attached cells in imaging experiments. Therefore, we monitored the time course for Ag-stimulated exocytotic events detected as FITC-dextran fluorescence bursts as a function of RBL cell attachment time. We observed a
strong dependence of the onset and rate of granule exocytosis on the time of cell attachment in the range of 20-90 min (Figure 5B). The extent of stimulated degranulation is near-maximal after cells are adherent for 90 min, and the rate is much slower for attachment durations of 60 min or less. Because \( \text{Ca}^{2+} \) mobilization is not rate limiting for degranulation of suspended cells (Pierini et al., 1997), other aspects of signaling evidently provide this kinetic regulation that depends on cell adherence and morphology. A role for cell adherence in stimulated exocytosis was demonstrated previously for human eosinophils (Fujiu et al., 2002), as well as for RBL mast cells (Apgar, 1997), but the time dependence of this enhancement was not previously characterized. Although the molecular basis for the time needed for cell attachment to achieve optimal degranulation of RBL mast cells remains to be determined, it could be related to the time needed for development of stable cell protrusions.

Spatial localization of the initiation of \( \text{Ca}^{2+} \) mobilization influences the spatial distribution of granule exocytosis. Our simultaneous imaging approach allowed us to determine how the subcellular spatial and/or temporal properties of stimulated granule exocytosis correlate with spatially localized \( \text{Ca}^{2+} \) mobilization. Our results confirm that granule exocytosis is temporally correlated to the peaks of \( \text{Ca}^{2+} \) oscillations as previously established using \( \text{Ca}^{2+} \) indicators and amperometry (Figure 6; Kim et al., 1997). In addition, our results provide new evidence that the site of \( \text{Ca}^{2+} \) wave initiation strongly influences the region of granule exocytosis at low doses of Ag (Figure 7). Under these conditions, more than half of the exocytosis events are localized along protrusions when the initiating \( \text{Ca}^{2+} \) wave begins in this region, and this compares to less than 20% of these events along protrusions when the high dose of antigen is used. Exocytosis along protrusions is also enhanced at low doses of antigen when \( \text{Ca}^{2+} \) waves initiate from the cell body, but this effect is substantially less than that for cells with \( \text{Ca}^{2+} \) waves initiating from protrusions (Figure 7A). These results suggest that the protrusions are involved both in Ag sensing distal to the cell body and in secretion of mediators at these extended sites. It is possible, for example, that these protrusions extend through the epithelial cell layer of intestinal villi to sense luminal Ag and to deliver mediators at that location in response to IgE receptor activation.

The mechanism that correlates \( \text{Ca}^{2+} \) wave initiation in protrusions and enhanced exocytosis events in those regions is not yet clear, as these events typically occur tens of seconds after the initiating \( \text{Ca}^{2+} \) wave. It is possible that \( \text{Ca}^{2+} \) wave initiation in protrusions primes this region for enhanced exocytosis, or, alternatively, this localization of \( \text{Ca}^{2+} \) wave initiation might reflect more effective stimulus-secretion coupling in this region. The roles of TRPC1 in promoting \( \text{Ca}^{2+} \) wave
initiation along protrusions (Cohen et al., 2009), as well as in enhancing exocytosis at limiting doses of Ag (Figure 8) may underlie this correlation, and further experiments are in progress to test these possibilities.

MATERIALS AND METHODS
cDNA plasmids. The GCaMP2 construct was provided by M. Kotlikoff, Cornell University College of Veterinary Medicine. The CD63-GFP and EGFP-VAMP7 constructs were gifts from J. Bonifacino (NIH, Bethesda, MD) and P. Roche (NIH, Bethesda, MD), respectively. The shRNA constructs were from OriGene (Rockville, MD).

Chemicals and reagents. Fura Red-AM and Texas Red (TxRed)-dextran (10 kDa) were purchased from Invitrogen/Molecular Probes (Eugene, OR). FITC-dextran (150 kDa), 5-hydroxytryptamine (5-HT), thapsigargin and A23187 were purchased from Sigma-Aldrich (St. Louis, MO).

Cells. RBL-2H3 cells were maintained in monolayer culture in Minimum Essential Medium supplemented with 20% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 10 µg/ml gentamicin sulfate. All tissue culture reagents were obtained from Gibco (Grand Island, NY) unless otherwise noted.

Single cell degranulation imaging. RBL-2H3 cells were plated as previously described (Cohen et al., 2009) in 35 mm MatTek (Dover, MA) coverslip dishes in medium containing 0.5 µg/ml anti-2,4-dinitrophenyl (DNP) IgE (Posner et al., 1992) and FITC-dextran (1 mg/ml). 5-hydroxytryptamine (5-HT, 0.2 mM) was also added to the medium in some experiments to increase the diameter of secretory granules for better visualization (Williams et al., 1999); we found that this addition has no significant effects on the kinetics of granule exocytosis measured either by FITC-dextran or by β-hexosaminidase release. Transfection of RBL-2H3 cells with GFP-fusion proteins and shRNA plasmids was carried out as described previously (Cohen et al., 2009). 24 hr after transfection, cells were washed with fresh medium and incubated for 1-2 hr at 37°C. In some experiments, cells were loaded with Fura Red at 0.5 μM for 10 min at 37°C. Just prior to imaging, cells were washed into buffered salt solution (BSS: 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 20 mM Hepes, pH 7.2, 1 mg/ml BSA), and individual MatTek dishes were mounted on a Zeiss LSM 510 Meta confocal microscope with a heated 40X (NA 1.4) oil immersion objective at 37°C. The fluorophores of GFP-fusion proteins and FITC-dextran were excited using the 488 nm line of a krypton/argon laser and viewed with a 505–550 nm band-pass
filter. TxRed-dextran and Fura Red were excited using the 561 nm and 488 nm laser lines, and viewed with 575 nm long pass and 650 nm long pass filters, respectively.

Selected cells were stimulated with antigen delivered in a micropipette as previously described (Cohen et al., 2009). Briefly, cells were approached with a ~5 µm diameter pulled glass capillary, typically positioned within 100 microns from the cell and pre-filled with stimulating solution. Cells were imaged at 10-30 Hz while applying a puff of 10 seconds/5psi from the pipette. To evaluate the contribution of TRPC1 in Ag-stimulated degranulation, RBL cells were transfected with either TRPC1 shRNA or a control shRNA vector as previously described (Cohen et al., 2009), together with a monomeric red fluorescent protein (mRFP)-containing vector at a 2.5:1 DNA ratio. Transfected cells were sensitized with IgE, loaded with FITC-dextran and 5-HT, and mRFP-positive cells were evaluated for degranulation responses stimulated with 7 ng/ml Ag in the micropipette.

**Fluorimetry-based degranulation assay.** 10^6 cells in 2 ml of full medium were cultured in the presence of 1-2 mg/ml FITC-dextran and IgE (0.5 µg/ml) for 24 h at 37°C, then harvested, washed, and resuspended in 2 ml of BSS. 0.5 ml of these cells was diluted to 2 ml in a stirred acrylic cuvette, and FITC fluorescence (ex 490 nm, em 520 nm) was monitored at 37°C using an SLM 8100C steady-state fluorimeter (SLM Instruments, Urbana, IL) in a time-based acquisition mode. Lysis of cells by addition of 0.1% Triton X-100 at the end of each experiment provided unquenched FITC fluorescence that was used to normalize the other fluorescence measurements in the same sample.

**Combined single cell amperometry and imaging.** Cells were sensitized with IgE and loaded with FITC-dextran and 5-HT in overnight culture as described above. Amperometry was performed using custom-made carbon fiber electrodes and a patch-clamp amplifier (EPC-8, HEKA Elektronik). Electrodes were positioned in contact with the cell surface, and voltage was maintained at +700 mV using a reference Ag/AgCl electrode. The measured current was low pass-filtered at 500 Hz using the built-in analog filter of the EPC-8 amplifier. Cells were stimulated using a glass pipette containing 1.7 µg/ml Ag positioned ~40 µm away from the cell in conjunction with a pressure application system (PicoSpritzer II, Parker-Hannifin/General Valve Corporation). Amperometric recordings were collected for up to 10 min after stimulation, and the data were digitized at a rate of 2 kHz by a 16-bit resolution NIDAQ board (BNC-2090, National Instruments). Recordings were analyzed as previously described (Mosharov and Sulzer, 2005). Spikes with amplitude <10 pA, with half-width >300 ms, or with overlapping areas were excluded from the analysis.

**Image analysis.** Fluorescence images of individual cells used in the analyses are representative from multiple experiments. Off-line image analysis was carried out using Zeiss ZEN
image analysis software (Carl Zeiss, Inc) and ImageJ (NIH, Bethesda, MD). Image processing of FITC-dextran degranulation for noise and background reduction was achieved in two steps as previously described (Demuro and Parker, 2005). Briefly, ratio images were formed by dividing each “raw image” frame in the time series stack by an average of the initial 10-20 frames acquired before applying the stimulus. The fluorescence signal at any pixel in the resulting ratio image thus represents the change in fluorescence as a fraction of the resting fluorescence (ΔF/F₀). To further minimize background noise, a highly smoothed copy of each frame was formed by applying a Gaussian blur with a width of 10 pixels, and this was subtracted from its original ratio image to form the final, corrected image in the time sequence.

Degranulation kinetics were analyzed either by manual counting of FITC fluorescence bursts, or by using the SparkFinder plug-in for ImageJ (Babraham Bioinformatics). Data were processed and plotted using Origin 8 (OriginLab) and Excel (Microsoft Corp.). Statistical comparisons between experiments were performed using Student’s t-test.

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FIGURE LEGENDS

Figure 1. TxRed-dextran accumulates in secretory granules that are labeled with CD63-GFP (A) and EGFP-VAMP7 (B) in RBL-2H3 cells. Representative confocal images show each component separately, as well as merged image with brightfield illumination. Insets show high magnification of regions marked by yellow boxes. Cells in (A) were labeled with TxRed-dextran in the presence of 5-HT; cells in (B) were labeled without 5-HT and were fixed prior to imaging. Analysis of multiple cells demonstrates high co-localization of EGFP-VAMP7 and TxRed-dextran (Pearson's coefficient > 0.7; n = 8).

Figure 2. Individual antigen-stimulated granule exocytosis events are imaged using FITC-dextran to label secretory granules. The top panel for each case (A, C and E) shows the progression in time-resolved frames. Bottom panels (B, D and F) show the time trace of fluorescence for depicted events. A kymograph of granule secretion (right D and F), where x-axis represents time and y-axis represents distance along the arrow indicated by d in upper panel. A and B) “Full Fusion” exocytosis event occurring proximal to the cell body (indicated by yellow box in right panel of B). Note release of FITC-dextran that diffuses away in the external medium. C and D) “Kiss and Run” exocytosis, where an initial transient granule fusion is reversed then followed by a prolonged secretion of FITC-dextran. E and F) “Compound Exocytosis”, where three adjacent granules fuse with the plasma membrane in a continuous sequential manner.

Figure 3. Stimulation by Ag causes simultaneous release of FITC-dextran and TxRed-dextran from individual granules in co-loaded RBL cells. Ag (1.7 µg/ml in the pipette) was puffed at t=0, and fluorescence changes in the red and green channels were monitored. A) Individual red and green frames showing the time course of granule fusion at the specified ROI (yellow circle) on the same cell. B) Time traces of integrated fluorescence for ROI highlighted in (A) demonstrate concurrent FITC-dextran dequenching and TxRed-dextran release, followed by diffusion/dilution of both probes in the external medium.

Figure 4. Simultaneous amperometry and FITC-dextran imaging of an individual cell after Ag stimulation. RBL cells, which were incubated overnight with IgE, FITC-dextran, and 5-HT, were approached individually with an amperometric carbon fiber and imaged after puffing Ag (1.7 µg/ml in micropipette) at t=0. Amperometric signal (black trace) is due to 5-HT oxidation on the carbon
fiber, and FITC fluorescence increase (color traces) is due to dequenching; both of these events occur upon granule release. Fluorescence can be visualized from individual granules and are represented by different colors (see inset; the imaging frame rate was 4 Hz.). The amperometric signal does not distinguish among granules. Inset: Micrograph showing cell outlined in yellow and carbon fiber outlined in black; location of individual granule exocytosis events monitored by fluorescence increases are indicated by corresponding colors in the time trace.

Figure 5. A) Degranulation of FITC-dextran in suspended RBL cells, monitored by fluorimetry, depends on extracellular Ca\(^{2+}\). Cells (~10^6/ml) in a cuvette were stimulated by addition of Ag (1.7 µg/ml) in the presence (black trace) or absence (grey trace) of extracellular Ca\(^{2+}\) as indicated. Subsequently, A23187 (1 µM) was added to the sample with extracellular Ca\(^{2+}\) as indicated. Fluorescence for both samples is normalized to total FITC-dextran fluorescence detected following addition of 0.1% Triton X-100 at the end of each experiment (not shown). B) Initiation of degranulation responses of attached cells depends on time of adherence. Cells were allowed to adhere for indicated times, then stimulated with delivery of Ag (1.7 µg/ml) from a micro-pipette, and exocytotic events were monitored in individual cells by enumerating bursts of FITC-dextran fluorescence.

Figure 6. Cellular Ca\(^{2+}\) and degranulation responses, monitored by independent fluorescent probes, can be compared in time and space. A) Representative images of an RBL cell loaded with FITC-dextran and Fura Red. A-left panel: Bright field and FITC-dextran images 75 seconds after initiation of stimulation (1.7 µg/ml in pipette). A-middle panel: Fura Red image at same time point and ROI (yellow line) evaluated in B. A-right panel: overlay of Fura Red image and Z projection of FITC-dextran images indicates degranulation events (green spots) over time. B) Kymograph of Ca\(^{2+}\) dynamics and degranulation events for the cell in A. The x axis represents the time dimension (time bar indicates the first 30 seconds of image acquisition); the y-axis corresponds to the distance along the yellow line specified in A-middle panel (wave direction indicated by arrowhead). B-top panel: Ca\(^{2+}\) changes along the cell as imaged with Fura Red; dashed yellow line indicates Ca\(^{2+}\) wave direction from protrusion toward cell body. B-middle panel: FITC-dextran channel, showing individual degranulation events as detected in time and along cell length. B-bottom panel: overlay of Fura Red and FITC channels, showing the spatial and temporal relationship between Ca\(^{2+}\) dynamics and degranulation events. C) Timing of degranulation compared to Ca\(^{2+}\) oscillations in an individual
cell. Degranulation events (green, blue, red), measured as bursts of dequenched FITC-dextran, are superimposed on traces of Ca\(^{2+}\) oscillations (black) that were imaged in the same cell using Fura Red and plotted as the inverse of the fluorescence intensity (b marks Ca\(^{2+}\) peak). D) Histogram for multiple cells of timing for peaks in Ca\(^{2+}\) oscillations: The width of individual Ca\(^{2+}\) peaks, normalized by the distance between two troughs (e.g., a=0, c=1 in C), was used to quantify the relative time of the peak (e.g., b corresponds to ~0.5 in C). Relative times for peak Ca\(^{2+}\) were calculated and plotted as a histogram in binned intervals for multiple events. E) Histogram for multiple cells of timing for degranulation events: Using the same normalized time scale based on Ca\(^{2+}\) peaks as for D, the relative times of exocytosis bursts in respective cells were calculated, binned, and plotted as a histogram. The histograms in D and E were derived from 20 stimulated cells showing oscillatory Ca\(^{2+}\) with a total of 51 exocytosis events. The smooth curves are normal distribution fits. The analysis of D and E confirms visual impression for the representative cell in C that degranulation events usually follow just after the peak of the Ca\(^{2+}\) oscillations.

Figure 7. Spatial distributions of exocytotic events stimulated by high (1.7 µg/ml) and low (1.7 ng/ml) doses of Ag. Cells loaded with FITC-dextran and Fura Red were stimulated by puffs of designated doses of Ag in the micropipette, and images were collected as in Figure 6. A) Summary of the numbers and relative distributions of exocytosis events in protrusions (PRTS) compared to the cell body as functions of Ag dose and initiation site for Ca\(^{2+}\) waves (B-waves originate in cell body; P-waves originate in extended cell protrusions; ** p=0.008 refers to the total number of events). For 1.7 µg/ml DNP-BSA, n=30 cells: 19 cells had P-waves (80 P-events and 350 B-events), and 11 cells had B-waves (29 P-events and 208 B-events). For 1.7 ng/ml DNP-BSA, n=25 cells: 19 cells had P-waves (169 P-events and 143 B-events), and 6 cells had B-waves (13 P-events and 22 B-events). B) Time courses for distributions of degranulation events in protrusions, following P-waves, for high and low Ag doses. C) Number of degranulation events per cell occurring in protrusions as a function of the number of preceding Ca\(^{2+}\) transients in that location for stimulation with 1.7 ng/ml. Error bars show SEM.

Figure 8. TRPC1 knockdown reduces the frequency and delays the onset of exocytotic events. A-C) Cells were transfected with shTRPC1 or control vector, loaded with FITC-dextran, and stimulated with 7 ng/ml Ag in the micropipette. Time courses show average numbers of exocytotic events per cell: total events (A), events in cell body (B), and events along protrusions (C). D) Cumulative
exocytotic events over 300 seconds from (A-C). Error bars show SEM (n=33 for shTRPC1; n=26 for shRNA control). Percentage values above shTRPC1 columns represent the decrease of exocytosis due to TRPC1 knock-down. Differences between shTRPC1 and control: total events, \( p=0.0099 \); cell body events, \( p=0.051 \); protrusion events, \( p=0.050 \).

Supplemental Figure S1. Confocal microscopy images compare the distributions of FITC-dextran (A), TxRed-dextran (B) and merged images with brightfield (C) for RBL mast cells labeled overnight in the presence of 5-HT to enhance granule size. D) 2-D representation of TxRed-dextran and FITC-dextran spatial overlap as calculated from pixel intensity correlations. Axis values indicate relative fluorescence intensities.

Supplemental Movie. Exocytotic events imaged in RBL-2H3 cells loaded with FITC-dextran and stimulated with \( 1.7 \mu g/ml \) Ag. Movie is x20 real time, and the Ag puff starts at \( t=0 \).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

![Graph showing fluorescence and stimulation time]
Figure 6
Figure 7

Figure 8
Figure S1