Single granule pH cycling in antigen-induced mast cell secretion

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SUMMARY

The pH cycling of individual granules in secreting (serotonin-loaded) mast cells is quantitatively examined using multicolor multiphoton fluorescence microscopy. A typical exocytosis event consists of maximal calcium rise at time zero, granule alkalization a few seconds later and, finally, complete contents release at a fraction of a second after alkalization. Membrane fusion is either transient, as indicated by subsequent granule reacidification, or 'full', as indicated by a granule disappearance with a collapse of its membrane into the plasma membrane. The relative frequency of these two coexisting behaviors (the 'kiss-tocollapse' ratio) is approximately 2:1. A typical transiently fusing granule experiences multiple alkalization/

INTRODUCTION

Although the molecular players regulating secretion processes are becoming known and appear to be highly conserved in all non virus-mediated membrane fusion processes, the dynamic mechanisms of exocytosis remain elusive (Guo et al., 1998; Jahn and Sudhof, 1999). Only fixed preparations can be analyzed by electron microscopy and secretory vesicles are generally subresolution for optical microscopy. Here we the secretion behavior of serotonin describe (5hydroxytryptamine: 5-HT) loaded RBL-2H3 (RBL) cells (Maiti et al., 1997; Williams et al., 1999). This '5-HT-charged' mucosal mast cell line is an ideal model system to elucidate the basic physical and molecular mechanisms of exocytosis and recycling. It possesses optically resolvable granules, a robust and reproducible secretion physiology, wellcharacterized signaling pathways (Field et al., 1997; Kim et al., 1997; Kuchtey and Fewtrell, 1996; Oliver et al., 1988; Sheets et al., 1999) and a susceptibility to simple genetic manipulation (Miesenbock et al., 1998; Smith et al., 1997).

Within the past decade our understanding of vesicle recycling processes has expanded considerably due to experiments using FM 1-43, an amphipathic stain that labels synaptic vesicles upon cellular stimulation. These experiments have resolved characteristic internalization and priming behavior (Betz and Bewick, 1992; Klingauf et al., 1998; Ryan et al., 1993) recently down to the single vesicle level (Ryan et al., 1997). Here, with the pH indicator LysoSensor (LS), we for the first time image the single granule pH dynamics

acidification cycles after addition of exogenous antigen. Between recycling granules, coalescence events are frequent, with 80% resulting in a collapse of the formed granule complex to the plasma membrane. The full dynamics of secretion encompass a complex combination of these granule activities.

B Movies available on-line: http://www.biologists.com/JCS/movies/jcs1660.html

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accompanying antigen-stimulated RBL cell exocytosis and recycling. The LS measurements are corroborated by the use of Acridine Orange (AO), another granular pH probe, and fluorescein, a pH-sensitive tracer in the surrounding bulk solution that enters the granules during granule fusion events. We show that secreting granules exhibit both transient and 'full' fusion events (i.e. kiss and collapse, respectively). The coexistence of both behaviors reveals a dynamic physical mechanism that controls stimulation-induced endocytosis and may influence vesicle availability during repeated stimulation or the later stages of exocytosis.

The exocytotic event itself is technically more difficult to investigate due to its inherently faster time scales. Simultaneous amperometry-fluorometry (Kim et al., 1997; Smith et al., 1995; Takahashi et al., 1997) and amperometrycapacitance experiments (Albillos et al., 1997; Alvarez de Toledo et al., 1993; Chow et al., 1992) have determined, with millisecond resolution, the time of vesicle contents release with respect to other exocytotic events, such as calcium influx and fusion pore formation. Various methods for secretion imaging have recently introduced a subcellular spatial resolution to these investigations (Kaether et al., 1997; Miesenbock et al., 1998; Miesenbock and Rothman, 1997). Particularly successful has been the use of AO to mark exocytosing chromaffin cell granules (Steyer and Almers, 1999; Steyer et al., 1997). However AO accumulation into (and departure out of) the granules is extremely pH-sensitive. We show in RBL granules that AO fluorescence changes correlate specifically with granule alkalization, an event which occurs a fraction of

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a second before the actual release event. This finding is corroborated by measurements using LS. Furthermore we show that granule alkalization depends strictly on calcium and follows local maxima in oscillating cytosolic calcium after a latent period of several seconds.

MATERIALS AND METHODS

Cell preparation and dye loading

As previously described (Williams et al., 1999), RBL cells were seeded with anti-dinitrophenyl (DNP) IgE and 5-HT and allowed to adhere overnight. Whole population secretion activity was assayed by measuring supernatant concentrations of β-hexosaminidase (Williams et al., 1999). Before imaging, cells were rinsed twice and immersed in 1 ml of a Hepes-buffered saline solution (BSS): 135 mM NaCl, 1.8 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂, 20 mM Hepes, pH 7.4, 5 mM glucose, 0.1% gelatin, 1 mM ascorbic acid and 100 μ M α -tocopherol. The following fluorescent dyes were loaded in warm (37°C) BSS and rinsed twice before imaging (unless otherwise specified). (1) The acidic compartment label AO (Molecular Probes, A-1301) was loaded at a concentartion of 3 µM for 5 minutes. (2) The calcium indicator Fluo-3, AM (Molecular Probes, F-1242) was dissolved at 2 µM concentration in a loading solution of 0.012% pluronic F-127 (Molecular Probes, P-6867) and 250 µM sulfinpyrazone (Sigma, S-9509) in BSS and incubated with the cells for 20 minutes. Images were acquired in BSS with sulfinpyrazone. (3) The pH-sensitive granule probe LS (Molecular Probes, L-7545) was loaded at 3 µM concentration for at least 2 minutes. In order for dye reloading to occur during secretion, this concentration was maintained during imaging. (4) The plasma membrane label PM (Molecular Probes, H-1387) was loaded at 8 µM concentration for 5 minutes. (5) The tracer dyes Cascade Blue, carboxyfluorescein and rhodamine B 10,000 MW dextran (Molecular Probes, C-687, C-1360 and D-1824 respectively) were dissolved into BSS at a concentration of 200 µM for imaging.

Cells were stimulated at 37°C by adding 150 ng/ml antigen (DNP multiply conjugated to human serum albumin, Sigma A-6661) in BSS. BSS alone was added as a control 'blank' for the unstimulated cells.

Multiphoton microscopy

The multiphoton microscope used was similar to that previously described (Williams et al., 1999) with a solid-state-pumped Ti:Sapphire laser (Spectra Physics, Millenia-Tsunami combination) and a Bio-Rad 1024MP laser scanner and image acquisition system. A Zeiss 40×/1.3 NA F Fluar objective provided optimal transmission of the three-photon excited 320-400 nm 5-HT emission and the stage temperature was maintained at 35-37°C using a Bioptechs objective heater. The fluorescence was extracted from the excitation beam by a 670 nm long-pass dichroic filter and then further spectrally separated by one of two 3-channel (ch) multiphoton filter packs (specified at a 10⁷ rejection of stray excitation light, Chroma Technology Corp.). (1) BGRch1 (380-480 nm): 490DCXR, BGG22; BGRch2 (500-540 nm): 550DCLP, hq575/150, d525/50; BGRch3 (560-650 nm): 670DCXR, hq575/150, d605/90 or (2) UBOch1 (320-380 nm): 400DCLP, UG11+IR; UBOch2 (410-490 nm): 500DCLP, BGG22; UBOch3 (510-650 nm): 670DCXR, hq575/150. The spectrally filtered fluorescence was detected by one of three Hamamatsu photomultiplier tubes (ch1 and ch2: HC125-02 or ch3: HC125-01) and the resulting signal transmitted to the standard MRC-1024 integrators by intercepting the photomultiplier inputs on the power cable. Specific fluorophores were imaged with the following filter combinations to display pseudocolor: 5-HT (UBOch1 to grayscale), AO (BGRch3 to red, BGRch2 to green), Fluo-3 (UBOch3 line ratio to blue), LS (UBOch3 to red, UBOch2 to green or grayscale), PM with AO (BGRch1 to blue and grayscale), PM with tracers (UBOch2 to green and grayscale), Cascade Blue (BGRch1 to grayscale), fluorescein (UBOch3 to red or grayscale) and rhodamine-dextran (UBOch3 to red).

Excitation wavelengths and dosages were selected by locating regimes in which both cell spreading (characteristic of antigen stimulation in the RBL cell line) and secretion were constant and reproducible (Williams et al., 1999). The live cell image series presented were acquired with an average power at the specimen of 3-4 mW, an excitation wavelength of 750-850 nm, a beam dwell time of 0.6 microseconds and an image interval of 2-10 seconds between the 1 second image acquisitions. These excitation levels are five- to tenfold lower than those capable of exciting illumination-induced calcium oscillations in these cells (data not shown). Using 750 nm illumination and the described dye-labeling protocols, cell function was compromised at the same high excitation levels (approx. tenfold higher than those used) that caused inhibition in experiments without exogenous dyes (Williams et al., 1999). Thus at this illumination wavelength, cell viability was limited by intrinsic cellular absorbers rather than the extrinsically added fluorescent indicators. Furthermore, labeling with fluorescent indicators had no effect on secretion as assayed by β -hexosaminidase release into the supernatant (data not shown).

Image analysis

Object recognition protocol

To examine individual granule dynamics with a statistically significant sampling size, objects of interest were automatically selected and analyzed (IDL, Research Systems Inc.) by adopting an object recognition protocol (ORP) in which the image to be analyzed was thresholded by an amount given by the average background fluorescence (b) plus 3 standard deviations (σ) of this background value (b+3 σ). The resulting objects were then sequentially eroded and dilated by a 3×3 pixel kernel to remove isolated noise islands. Regions of fluorescence change were located by calculating a difference image series in which each image is subtracted by the one preceding it. The difference images were then selected and analyzed according to the ORP.

LS calibration

The fluorescence emission spectrum of the granular pH indicator LS red-shifts upon protonation. Thus the UBOch2/UBOch3 (green/red) fluorescence intensity ratio can be calibrated to the solution pH. This LS emission ratio to pH calibration is insensitive to concentrations of LS between 20 and 500 μ M, which is the range of granular staining used. The calibration, however, is sensitive to the solvent environment (LS blue-shifts with increasing hydrophobicity). We calibrated to the intragranular environment by scaling the fluorescence ratio such that granules neutralized with 15 mM NH4Cl reported an average pH of 7.4±0.9 (±s.d.; Fig. 1C, blue circles). Because the environment may vary from granule to granule, single granule LS measurements were used to indicate only relative pH changes rather than absolute pH values.

Individual granule recycling analysis

To examine the pH dynamics in single LS-labeled granules, an analysis box was chosen that contained each granule's movements throughout the experiment duration (boxes in Fig. 2A-D). Within each box the granule region was selected using the ORP applied to UBOch2 fluorescence images. At any given time the LS concentration was indicated by the sum of the average UBOch2 and UBOch3 fluorescence intensities in that region. The approximate pH was calculated as previously described, by the ratio of the two intensities.

Kiss-to-collapse ratio and determination of fractional dye changes

Decreases and increases in the UBOch2 granular fluorescence satisfying the ORP threshold were interpreted as release and recycling events, respectively. To quantify the number of release and recycling events that a given granule experiences, a difference-image series was calculated by subtracting the UBOch2 fluorescence images at 30 second intervals. (At smaller time intervals increases due to granule acidification could not be distinguished from photon noise.) Using the ORP, fluorescence changes were counted from the difference image series and normalized according to the counting redundancy (subtraction interval/frame interval). To obtain the total release or recycling rates, the fluorescence intensity changes were divided by the initial (location-specific) intensities and the subtraction interval to yield the percentage decreases and increases per second. Artifacts due to photon shot noise and granule motion were accounted for by subtracting the rates in unstimulated cells from those in stimulated cells. To determine the total amount of dye released or recycled, the rates of release and recycling were numerically integrated.

Linescan analysis

Digitized data were acquired from line scans by visually selecting and averaging across 5-20 pixel rows (2-8 μ m wide) that contained either granule or cytosol. Because the LS emission spectrum is extremely wide (400-650 nm), the fluorescein channel (Fig. 5C, grayscale) was corrected for LS bleed-through. As a result, data acquired with the LS and fluorescein were significantly noisier than those acquired with AO and Cascade Blue. In Fig. 6 the fluo-3 line ratio was calculated by averaging the first 20 pixels in each line scan to determine a basal dye level. The line scan image was then divided by this spatially specific average to indicate calcium changes (blue).

Values are given as means \pm s.d. throughout.

RESULTS

5-HT-loaded cells contain an abundance of large, secretion-competent granules, but are otherwise physiologically normal

RBL cells incorporate 5-HT from 5-HT-rich media into optically resolvable granules (Fig. 1A, grayscale), whose antigen-stimulated release can be monitored by multiphoton microscopy (Williams et al., 1999). In contrast only approx. 1% of unloaded cells contain significant levels of endogenous 5-HT (Williams et al., 1999). Because the granules in the 5-HT-loaded cells are larger than expected (Bonifacino et al., 1989), we were concerned about potential physiological differences between the two populations of cells. To compare the effect of 5-HT loading on the initial signaling events, we examined antigenstimulated tyrosine phosphorylation of whole cell lysates from loaded and unloaded cells (Sheets et al., 1999). The magnitude and time course of stimulated tyrosine phosphorylation as well as the pattern of phosphorylated proteins were similar between the two populations (R. M. Williams and E. D. Sheets, unpublished results). Furthermore, a standard assay for RBL secretion, the release of β -hexosaminidase into the supernatant, revealed that both the magnitude and kinetics of release were similar between the two populations (Fig. 1B). Although it is not known whether β -hexosaminidase and 5-HT are colocalized entirely in the same secretory granules, evidence suggests that they are to a large extent (Lloyd et al., 1998; Xu et al., 1998). The β -hexosaminidase release data are consistent with earlier work showing that 5-HT secretion, assayed by radioactive 5-HT release into the supernatant, is the same between similarly 5-HT loaded and unloaded RBL populations (Weintraub et al., 1994).

We also compared the pH of 5-HT-containing granules in both populations with LS, a dye that accumulates in the

granules and fluoresces at a wavelength that is dependent on the granule pH. Fig. 1A shows simultaneously acquired images of 5-HT (grayscale) and LS (color) in loaded and unloaded cells. The average granule pH, visually assessed by the green/red pseudocolor ratio, was calculated for 5-HTcontaining granules in both the loaded and unloaded populations (Fig. 1C). The few 5-HT-containing granules in the unloaded population (black circles, n=49) exhibit an average pH=5.1±0.5 that is somewhat acidic but similar to the pH=5.5±0.5 from granules in the loaded population (gray circles, n=1944). (Intragranular calibration of the green/red ratio to pH was accomplished by determining a scaling factor in which NH₄Cl neutralized granules (blue circles, n=726) reported an average pH of 7.4.) The slight alkalinity of the loaded cell granules is presumably due to the buffering effects (Marszalek et al., 1997) of the higher granular 5-HT concentrations in the loaded cells. Notably in the unloaded population, LS labels a variety of non-specific intracellular compartments, the majority of which do not contain 5-HT (Fig. 1A, below) and do not release their contents upon stimulation (data not shown). Taken together, these results suggest that while 5-HT loading changes the size and the fraction of acidic compartments that are secretion-competent, the physiology of the 5-HT loaded RBL cells is essentially normal as assessed by the granule pH distribution, tyrosine phosphorylation and the secretion response.

Single granule pH cycling, corresponding to release, recycling and rerelease, lasts an average of 140 seconds

Because loading with 5-HT greatly increases the fraction of acidic compartments that are secretion-competent, most of the LS, a non-selectively targeted stain, colocalizes to granules previously determined to be secretion-competent (Williams et al., 1999) in the loaded population (Fig. 1A, top panels). Thus 5-HT loading enables the use of a variety of fluorescent probes that are non-specific vesicle markers, but highly-specific chemical indicators. In particular, LS can be used to monitor secretory granule pH during exocytosis and recycling. Fig. 2A shows a representative LS-labeled granule in an unstimulated cell; during the 20 minute measurement period both the dye concentration and the granule pH remain relatively constant, varying <15% from the mean pH. However in stimulated cells (Fig. 2B-D), the granule pH instead exhibits an oscillating behavior in which rapid alkalization steps, corresponding to dye release, are superimposed upon slower acidification processes, corresponding to dye reloading. Notably, granule reacidification often results in a pH significantly lower than the initial prerelease value (Fig. 2C).

The LS fluorescence oscillations are not due to granule transport into and out of the approx. 1 μ m thick optical slice for two reasons. Firstly, identical behavior (with slower time resolution) is observed when full cell image stacks (images at 0.5 μ m axial intervals) are acquired over time (data not shown). Secondly oscillations are also observed when the granule membrane is labeled and remains stationary in the imaged field. The Fig. 2E time series (with movie f2E) shows granule dynamics in a cell labeled with PATMAN (PM), a plasma membrane stain, and stimulated within a fluorescein-containing solution. The initial release event (arrow) is indicated both by the aqueous fluorescein (red) entry into the

granule and PM (green) entry into the granule membrane. Throughout the series, the granule retains the same approximate morphology and location, but its pH-sensitive fluorescent contents (fluorescein, pK_a approx. 6.6) oscillate on a 1-2 minute time scale, indicative of successive granule fusion and reacidification cycles with kinetics similar to those shown in Fig. 2B,C.

Unlike the aqueous and membrane tracer dyes, LS cannot necessarily be used as an indicator of exocytosis because its permeability to membranes is dependent upon pH. It accumulates within granules upon protonation (pKa approx. 5.5) within their acidic environments; only neutral LS can pass through granule membranes. Thus in Fig. 2B-D, the dye loss could be attributable to stimulation-induced alkalization with no exocytosis. However, in examining cells double-labeled with PM and LS, we looked for and found no instances of a stationary granule alkalizing without plasma membrane incorporation. The 'recycling time,' therefore defined as the period from alkalization to alkalization in LS labeled granules, on average lasts 140±100 seconds and occurs 2.7±1.5 times/granule within the 20 minute experimental measurement window (n=60 granules). Fig. 2B represents a fairly typical granule behavior, showing two recycling events with an average recycling time of 250 seconds. Even though alkalization events are correlated to exocytosis events, the image series in Fig. 2D, displayed at a higher time resolution, shows that alkalization in fact slightly precedes LS release. This suggested delay between alkalization and full exocytosis is further corroborated using a variety of probes in a later section.

Granules exhibit both transient and full fusion events; the relative frequency of the two behaviors, i.e. the 'kiss-to-collapse' ratio, is approximately 2:1

Not all granules exhibit the transient fusion processes shown in Fig. 2B-E. A competing process is depicted in Fig. 2F (with movie f2F), in which the granule, after release (arrow), collapses to the plasma membrane and becomes indistinguishable from it. To determine the relative amounts of these two behaviors, we quantified release and recycling 'events' as threshold fluorescence decreases and increases in stimulated LS-stained cells. The upper panels in Fig. 3A show representative images of LS-stained cells before and after antigen stimulation. (See movies f3wAg and f3woAg for time series with and without antigen respectively.) The number of

Fig. 1. Comparison between 5-HT loaded and unloaded cells. (A) Simultaneous three-photon excited 5-HT fluorescence (grayscale) and two-photon excited LS fluorescence (red and green) of loaded (top) and unloaded (bottom) populations. The granule pH is indicated visually by the green/red ratio in the LS images. Scale bar, 15 μ m. (B) Fractional β -hexosaminidase release from loaded (gray circles) and unloaded (black circles) cells after antigen introduction. Results are means \pm s.d. from three experiments. The 0 value represents the basal release from a 40 minute incubation in buffer only. (C) Plot showing pH distribution within 5-HT containing granules in various cell populations. NH4Cl is used to neutralize the pH (blue circles, n=726 granules) for calibration of the LS curve to the granular environment (pH=7.4±0.9). The 5-HT containing granules in the loaded population (gray circles, *n*=1944 granules) report an average pH=5.5±0.5 whereas those in the unloaded population (black circles, n=49 granules) report a similar pH=5.1±0.5.

location-specific release and recycling events that occur within the interim are depicted in the lower left and right panels, respectively. For example, the granule indicated by the white arrow releases 5 times and recycles 5 times. The granule



Granular 5-HT Concentration (mM)

Fig. 2. Time series showing representative granules in (A) control unstimulated and (B-F) stimulated cells. At time 0. either antigen or a buffer 'blank' is introduced to the cells. (A-D) LS-labeled granule image series with associated pH values and LS concentrations plotted below as a function of time. (A) In unstimulated cell granules the LS fluorescence, both magnitude and color, is relatively constant. (B,C) Granules in stimulated cells exhibit a characteristic oscillating 'sawtooth' behavior in which rapid releases, indicated by alkalization and LS loss, are followed by slower recycling steps, indicated by reacidification and LS reaccumulation. At higher time resolution, the granule in (D) exhibits a granular pH jump that precedes any significant dye loss (arrows). (E with movie f2E) A PM stained (green) cell secreting with a fluorescein tracer (red) in the extracellular buffer (100-500 seconds after antigen addition). Subsequent to its release (arrow), the granule retains the same shape and location, but fluorescein fluorescence within the granule oscillates in response to changing acidity levels. (F with movie f2F) After release (arrow), a granule collapses to the plasma membrane (100-300 seconds after antigen addition). Scale bars, 5 µm.



indicated by the green arrow releases once but does not recycle, and the granule indicated by the red arrow neither releases nor recycles. Fig. 3B summarizes the granule histories for 413 stimulated cells. On average $71\pm16\%$ of the granules release, and $64\pm15\%$ of those granules that release subsequently recycle. Thus the ratio of released granules that recycle to released granules that disappear is 1.8:1. By the same analysis, less than 10% of the granules in unstimulated cells (*n*=198 cells) exhibit any events.

To determine the total rates of release and recycling, the magnitudes of these events were normalized by the location-specific initial dye concentrations. The resulting rates of change (Fig. 3C, colored) in stimulated cells minus those in control unstimulated cells (Fig. 3C, black) yield the overall release (dashed green) and recycling (dashed red) kinetics shown in Fig. 3D. The release kinetics, which are consistent with those measured by β -hexosaminidase release (Fig. 1B)

and granular 5-HT loss (Williams et al., 1999), are 4.3-fold faster than their coupled recycling kinetics. The rate of LS accumulation during recycling correlates with granule acidification processes (Fig. 2B,C) rather than simple dye transport. (LS accumulation into unstimulated cell granules is rapid, <30 seconds, data not shown). Because granule reacidification determines LS reaccumulation, it is crucial to the later stages of LS release (sustained release rate >500 seconds in Fig. 3D). The same may be true for secreted monoamines that, similar to LS, load into the secretory granule via the pH gradient established by granule membrane pumps (Albillos et al., 1997; Cao et al., 1997).

Granular alkalization jumps as measured by Acridine Orange 'flashes' correlate with exocytotic activity

For comparisons with previous secretion imaging experiments (Steyer et al., 1997), we employed AO, which accumulates into



acidic compartments because upon protonation (pK_a approx. 4.9) it becomes trapped within the granule membrane. AO also

Fig. 3. Quantification of granule recycling behavior. (A) LS-labeled cells before (upper left) and 900 seconds after antigen addition (upper right). The images at the lower left and right illustrate the total number of granule-specific release and recycling events identified. The arrows denote representative granules with diverse behavior: releasing and recycling multiple times (white), releasing only (green), or neither releasing nor recycling (red). Scale bar, 10 µm. Movie f3wAg shows the full secretion process of the same field of view. Movie f3woAg shows a representative control series of unstimulated LS-labeled cells. (B) Diagram summarizing average recycling behavior. Approx. 70% of granules release upon stimulation, of which approx. 60% reacidify immediately for eventual rerelease approx. 140 seconds later. (C) Fractional dye decreases (green lines) and increases (red lines) as calculated by normalizing the events depicted in A by the fluorescence intensities in the original dye image. In both plots the black lines represent dye changes in a control unstimulated cell population. The dotted lines show fits calculated from an average of 11 stimulated series (colored, n=413 cells) and four unstimulated series (black, n=184 cells). (D) Average release and recycling rates (dashed lines), as calculated from the average stimulated decreases and increases, minus random decreases and increases from the unstimulated populations. The total dye released or recycled at any given time (solid lines) is obtained by integrating the average release or recycling rates.

conveniently exhibits a concentration-dependent fluorescence quenching and red shift, a property that is often exploited to indicate granule pH (Barasch et al., 1988; Palmgren, 1991). AO-stained cells possess red ([AO] approx. 500 µM) granules and a slightly green ([AO] approx. 20 µM) cytoplasm (Fig. 4A). Upon stimulation with antigen, the granular AO fluorescence disappears in discrete events. Such events can result either from rapid pH jumps in the granules, in which case deprotonated (neutralized) AO rushes out of the granules and into the cytosol, or from exocytosis, in which case AO diffuses away into the surrounding bulk solution. In stimulated RBL cells the granular decreases of red fluorescence occur concurrently with cytosolic increases (i.e. 'flashes') in unquenched green fluorescence (Fig. 4A, arrow), indicative of fast granular alkalization events, the first mechanism. (See movies f4wAg and f4woAg for time series with and without antigen, respectively.)

After granule alkalization, AO often gradually reappears at the same location (arrowhead). This reappearance of AO can result either from transport of a new granule to the same 'docking site,' as interpreted by Steyer et al. (1997), or from reacidification of the original granule. We observed no evidence for any systematic antigen-induced granule transport, and our previously discussed results demonstrate a variety of evidence for single granule pH cycling.

To quantify granule alkalization (448 cells in total), we counted AO flashes and normalized by the number of cells per image. Upon addition of antigen (Fig. 4B, red lines), granules exhibit rapid alkalization steps with overall kinetics similar to those of exocytosis, as measured both by LS release (Fig. 3D, green dashed line) and the loss of granular 5-HT (Williams et al., 1999). During maximal release (the first few minutes after antigen addition), up to one third of the cells exhibit AO flashing behavior at any given time. Unstimulated cells show no significant flashing activity (Fig. 4B, black lines).

Extracellular calcium is essential to antigen-induced granule alkalization. When cells are stimulated in the absence of extracellular calcium, AO flashing is dramatically reduced



Fig. 4. Antigen-induced alkalization jumps indicated by AO fluorescence changes. (A) AO 'flashes' in an AO-labeled cell undergoing exocytosis (60-140 seconds after antigen addition). Because AO becomes permeable to the granule membrane when it is deprotonated, a rapid pH increase causes the previously quenched AO fluorescence in the red emission channel to rush out of the granule and appear as a flash (arrow) in the green channel. Refilling of the granule is evident a few frames later at the same location (arrowhead). Scale bar, 10 µm. Movies f4wAg and f4woAg show representative AO-labeled cell populations with and without introduction of antigen, respectively. (B) Granule alkalization activity under various cellular treatments (n=448 cells). The addition of antigen (Ag) at time=0 results in a tenfold enhancement in the frequency of AO flashing (red lines) as compared to unstimulated cells (black lines). Cells stimulated within a calcium-free buffer (green lines) or within a buffer in which calcium is replaced by barium (blue lines) have activities that are reduced to background levels. In both cases the restoration of calcium to the buffer 10 minutes later somewhat restores alkalization activity.

(Fig. 4B, green lines) and is restored only upon addition of calcium to the buffer. Barium, a divalent cation that can pass through calcium channels (Han et al., 1999), is not a suitable replacement for calcium; the AO flashing behavior is blocked when Ca^{2+} is replaced by Ba^{2+} in the extracellular buffer and somewhat rescued upon restoration of the Ca^{2+} (Fig. 4B, blue lines). Together these data suggest that granular alkalization is strictly dependent upon calcium influx, in a manner similar to that described previously for RBL cell exocytosis (Hide and Beaven, 1991).

Exocytosis proceeds by calcium influx, granule alkalization and finally full contents release

Although granule alkalization and exocytosis share the same overall kinetics, the previously described LS image series of Fig. 2D demonstrated that alkalization preceded LS loss, suggesting that the two processes were not necessarily coincident. Though LS concentration losses can imply exocytotic events (upon opening of the fusion pore, LS will dissolve into the surrounding bulk solution), it is not an absolute indicator because, like AO, its permeability across the granule membrane is dependent on the pH of the granule. To determine the precise temporal relationship between alkalization and the establishment of aqueous continuity, we used the pH probes AO and LS in conjunction with extracellular tracers. In the linescan images of Fig. 5A, granule



Fig. 5. Alkalization precedes full exocytosis by approx. 1 second. For the sequences depicted in (A) and (C), initial and final images are shown at the left and right sides, respectively. The middle frames are linescan 'images' in which the beam is repeatedly scanned at the indicated location (long dotted line) and the *x*dimension represents time. (A) An AO-labeled cell (red and green) is stimulated within a Cascade Blue (grayscale) containing buffer. (B) Image data, digitized and plotted as a function of time, indicate that the AO flash (green trace) occurs 1.2 seconds before Cascade Blue (black trace) enters the granule. (C) An LS-labeled cell (red and green) is stimulated within a fluorescein (grayscale) containing buffer. (D) The granular pH (red trace) jumps 1.1 seconds before extracellular fluorescein (black trace) enters the granule. Scale bars, 10 μm.

alkalization (green AO flash) occurs prior to the entrance of extracellular Cascade Blue into the granule (grayscale). Digitized data from the event (Fig. 5B) demonstrate that granule alkalization occurs 1.2 seconds before aqueous continuity. Seven such experiments reveal an average delay of 0.9 ± 0.7 seconds. This result was further tested using the combination of LS and fluorescein. In Fig. 5C the alkalization (LS red to green shift) precedes extracellular fluorescein entrance (grayscale) into the exocytosing granule by 1.1 seconds (Fig. 5D). Four such linescans report an average delay of 0.6 ± 0.4 seconds, consistent with the previous AO and Cascade Blue experiments.

Because calcium is required for the release-associated granule alkalization jumps (Fig. 4), we also examined the temporal relationship between granule pH and the antigenstimulated calcium oscillations that are well characterized in RBL cells (Kuchtey and Fewtrell, 1996). Fig. 6A shows a series of linescans through a cell stained with both LS (red, green) and the calcium indicator Fluo-3 (line ratio, blue). Although the lower granule remains inactive, the upper granule releases at 192 seconds after antigen addition (arrow), reaccumulates LS, rereleases at 313 seconds (arrowhead) and further reaccumulates LS. Throughout this process cytosolic calcium oscillations are occurring, and each release event is immediately preceded by an oscillation. Digitized data from the first release event (Fig. 6B) exhibit the temporal relationship between maximal intracellular calcium (blue), granule pH (red) and LS release (green). The latency, defined as the time from maximal calcium rise to the initiation of LS release, is 3.5 ± 0.7 seconds (n=8 linescans from six cells in which exocytotic events were captured).

Recycling granules can be structurally dynamic

While granules tend to be relatively stationary throughout the recycling process (for example Fig. 2E), they can be quite structurally dynamic, particularly during fusion/release events. In Fig. 7A, granule exocytosis, indicated by the AO flash (green) and PM entrance into the granule membrane (blue and grayscale), is followed by a collapse of the granule to half of its post-release volume. Because mast cell granule matrix swelling (and granule contents release) are dependent on pH (Curran and Brodwick, 1991), the displayed granule shrinkage might be expected to correlate to granule reacidification. However, we found no consistent correlations between granule size and pH.

Granule structure is also dynamic during intragranule fusion events. In comparing images of granules before and after stimulation (Fig. 3A, top), the average granule size clearly becomes larger as secretion progresses. Fig. 7B,C shows examples of intracellular granule-granule fusion (white arrows and arrowheads) and subsequent disappearance, presumably by collapse to the plasma membrane. In the AO- and PM-labeled cell of Fig. 7B (with movie f7B), a granule that had previously released its contents, as indicated by the stained granule membrane, fuses with an unreleased granule, causing an alkalization jump (AO flash, arrow). The resulting larger granule then fuses with another previously released granule (arrowhead) and subsequently disappears. For 19 observed granule-granule fusion events, 15 were shortly followed by a collapse of the granule complex to the plasma membrane. Fig. 7C (with movie f7C) shows a type of tubular membrane



Fig. 6. Temporal relationship between antigen-induced calcium oscillations and release events (60-480 seconds after antigen). (A) Linescan images of stimulated cells labeled with both LS (red and green) and the calcium indicator Fluo-3 (line ratio indicating calcium changes, blue). Both release events (arrow and arrowhead) are immediately preceded by calcium oscillations, which are occurring throughout the image series. After the first event (arrow), the granule experiences a partial recycling, a second release event approx. 2 minutes later (arrowhead) and a subsequent further recycling. The small images to the left show snapshots of the cell during the linescan series. Scale bar, 10 μ m. (B) LS concentration (green trace), granular pH (red trace) and the Fluo-3 ratio data (blue trace) from the first release event. In this case maximal calcium rise occurs 4.2 seconds before LS release.



Fig. 7. Granules are stationary but structurally dynamic. (A) Granule release event in an AO- (red and green) and PM- (blue and grayscale) labeled cell. (B,C with movies f7B and C) Image series of granules that coalesce (white arrows and arrowheads) and subsequently disappear, presumably by collapsing to the plasma membrane. Granule coalescence and disappearance are sometimes accompanied by dynamic tubular structures (yellow arrows). In B the cell is stained and imaged as described in A. In C the cell is stained with PM (green and grayscale) and the bulk solution contains rhodamine-dextran (red). The image series in A, B and C start at 180, 150 and 160 seconds after antigen addition, and the time interval between frames in each of these panels is 10, 10 and 20 seconds, respectively. Scale bars, 5 μm.

structure (yellow arrows) that is sometimes evident (n=7 sightings) immediately prior to granule-to-granule coalescence or granule-to-membrane collapse.

DISCUSSION

Event progression in exocytosis

In RBL mast cells, the early signaling processes resulting from crosslinking of the high affinity IgE receptor (Field et al., 1997; Oliver et al., 1988; Sheets et al., 1999) result in the production of two fundamental events, an oscillating influx of calcium into the cytosol (from extracellular and intracellular sources) and diacylglycerol production, both of which are essential for optimal secretion. The delay between antigen addition and the first few exocytotic events is typically 1-2 minutes (Kim et al., 1997). We observed that calcium mobilization was followed within a few seconds by granule alkalization, which in turn precedes granule release by a fraction of a second.

The approx. 3.5 second latency between calcium maxima and subsequent exocytosis initiation is consistent with that measured in the ATP-sensitive, slower phases of endocrine and neuroendocrine cell release (Gilon et al., 1993; Parsons et al., 1995; Smith et al., 1998; Takahashi et al., 1999), suggesting that the delay may be indicative of an ATP-dependent granule mobilization step. In contrast to our measurements, previous amperometry and microfluorimetry experiments on RBL cells have shown a correlation between calcium oscillations and release events, but no consistent delay (Kim et al., 1997).

After the rise in intracellular calcium, we observed granular alkalization, which occurred a fraction of a second before dye from the bulk solution entered the granule. Whether alkalization results from granular protons departing through the fusion pore or through granule membrane channels is still not known. The existence of a small fusion pore, the former alternative, could result in the described lag between alkalization and exocytosis. To a first approximation the molecular current of a substance through a channel whose length *l* is much greater than its radius *r* is given by $D\Delta C\pi r^2/l$ (Crank, 1975), where D is the diffusion coefficient and ΔC is the concentration difference across the channel. The half time for equilibration of a granule with its surrounds through a constricting fusion pore is thus approx. $\overline{\omega}l/2D\pi r^2$, where $\overline{\omega}$ is the volume of the granule. Because the diffusion coefficient for protons (9000 μ m²/seconds) is approx. 30 times greater than that for small dye molecules (300 μ m²/second), the granule pH is expected to equilibrate approx. 30 times faster than the dye concentration (neglecting proton buffers). If we assume a fusion pore with a radius of r=2 nm (Albillos et al., 1997) and a length l approximated by twice the membrane thickness, 8 nm, the proton and dye equilibration times for a 1 μ m³ granule would be 30 milliseconds and 1000 milliseconds, respectively. The difference between these times is consistent with our observed lag between granule alkalization and extracellular dye entry, and thus may be indicative of pseudo-stable fusion pores (Albillos et al., 1997; Chanturiya et al., 1997; Lollike et al., 1998; Spruce et al., 1990). The fact that aqueous dye diffusion has been shown to be significantly delayed by influenza hemagglutin-induced fusion pores (Zimmerberg et al., 1994) also corroborates this possible mechanism.

If the measured lag between alkalization and release is due

to a constricting fusion pore, the effect of pH buffering appears to be surprisingly insignificant. Note that in Fig. 6D the time scale of granule alkalization is fast, consistent with that calculated for a 2 μ m diameter granule. Proton buffering in the granule could be expected to slow this process by a factor of R+1, where R is the ratio of the bound to unbound protons at granule equilibrium (Crank, 1975). The proton buffering capacity of the granule is enormous due to loaded 5-HT, endogenously expressed histamine and a highly anionic proteoglycan matrix. 5-HT alone is loaded to a concentration of approx. 50 mM (Maiti et al., 1997; Williams et al., 1999) and thus its deprotonation (divalent to monovalent) with a pKa=4.9 (Marszalek et al., 1997) should by itself slow down the granule alkalization by approx. 4000 times, implying a gradual pH increase, on the minutes time scale. During a transient fusion event characteristic of a pseudo-stable fusion pore, granule alkalization could not occur until 5-HT was released from the granule. Our observed rapid alkalization preceding tracer entrance suggests either that the various proton buffers interact to inhibit 5-HT proton buffering (for example if divalent 5-HT must be dissolved from the matrix before releasing protons) or that the delay occurs by some mechanism other than simple fusion pore restriction.

Other mechanisms to explain the alkalization-exocytosis delay include the following scenarios. (1) The fusion pore may consist initially of a cationic channel, allowing protons to pass, and only become lipidic later in the fusion process, allowing other soluble species through (Spruce et al., 1990). (2) The granule pH may be elevated by granule membrane channels opened via intracellular calcium signaling (Lee et al., 1992). In support of the latter hypothesis, it has been shown (Han et al., 1999) that vesicle alkalization in PC12 cells results from intracellular calcium increases but can be pharmacologically separated from the exocytosis process.

Whatever the cause of pre-exocytosis alkalization, it may be functioning as a priming mechanism for efficient release of granular contents. The electrochemical properties of the mast cell granule matrix, a highly anionic proteoglycan polymer, have been well characterized. Increases in pH are known to induce matrix swelling and a resulting solublization of stored biogenic amines, such as 5-HT and histamine (Curran and Brodwick, 1991; Fernandez et al., 1991; Marszalek et al., 1997; Nanavati and Fernandez, 1993). We do observe granule size changes that might be attributed to matrix swelling (Fig. 7A) but have been unable to identify any consistent correlations between granule size changes and granule pH.

Single granule recycling

Although there have been exploratory experiments in RBL cells using granule-targeted pH-sensitive GFP mutants (Miesenbock et al., 1998), the majority of vesicle recycling analyses have been performed on neuronal preparations using FM 1-43. Estimates for the recycling time, ranging from 45 to 90 seconds in neurons (Betz and Wu, 1995; Ryan et al., 1993), are only twofold faster than the approx. 140 seconds reported here for mast cell recycling. This is a surprising result considering that the average volume of the granules examined here is roughly 10^4 times larger than that of synaptic vesicles. With a surface-to-volume ratio that is 100-fold smaller in the larger granules, one might expect recycling rates that are 100-fold slower. Vesicle acidification rates in neurons have recently

been shown to be 'instantaneous' with respect to endocytosis rates (Sankaranarayanan and Ryan, 2000). That is, the time for membrane retrieval is much greater than that for vesicle acidification. In large dense core granules, we have shown the opposite to be true (Fig. 2E).

Our results further show that granular acidification is intimately correlated to granule repriming before rerelease. At the single granule level, it is turned on by stimulation and interrupted only by the rapid alkalizations associated with granule release events. At the cellular level, granular acidification is known to be a requirement for the loading of biogenic amines and potentially the targeting and packing of secreted proteins (Aspinwall et al., 1997; Cao et al., 1997; Sabban et al., 1990). Granule reacidification after stimulation, possibly linked to the opening of chloride channels that relieve the membrane potential barrier to proton accumulation (Barasch et al., 1988; Barasch et al., 1991), often results in the granule pH being lower than its prerelease value. Exocytosis imaging studies interpreting AO fluorescence increases as vesicle transport into the focal plane (Steyer and Almers, 1999; Steyer et al., 1997) should be assessed with caution; exocytosis and endocytosis are inseparable processes, and vesicle AO fluorescence is dependent on acidification (Barasch et al., 1988; Palmgren, 1991). We see no systematic granule transport to the cell surface. In mast cells the plasma membrane, possibly by way of antigen-induced membrane ruffling, moves to the granule instead.

Similar to recycling in neurons (Murthy and Stevens, 1998), we find that mast cell granules in general retain their identities throughout the recycling process (Fig. 2B-E and movie f3wAg); no endosomal sorting is evident. However, unlike neurons and consistent with our earlier 5-HT imaging experiments (Williams et al., 1999), we observe some granule-granule coalescence that acts to enlarge the unit granule size during the recycling process (Fig. 7B,C and movies f7B,C).

Granule dynamics: kiss versus collapse

The physical coupling between release and recycling is an enduring controversy (Ceccarelli et al., 1973; Heuser and Reese, 1973), referred to recently as the 'kiss-and-run' versus 'collapse-to-the-membrane' debate. At issue is the fate of a postfusion granule. Does it pinch off for reacidification and recycling, or does it lose its identity by collapsing into the plasma membrane? Kiss-and-run is often used to describe an entropically economical mechanism whereby the granuleassociated molecular constituents are efficiently recycled (Fesce et al., 1994). The fact that exocytosis can be monitored by a plasma membrane stain (Fig. 7A) suggests that even in kissing granules, plasma and granule membrane lipids mix through the fusion pore. The question remains as to whether or not granule membrane proteins maintain an association with the granule after fusion; experimental results pointing to both conclusions exist (Torri-Tarelli et al., 1992; Torri-Tarelli et al., 1990; Valtorta et al., 1988).

The kiss-and-run hypothesis is consistent with experimental evidence such as reversible fusion pores (Albillos et al., 1997; Lollike et al., 1998; Spruce et al., 1990), fast vesicle recycling in neural cultures (Ryan et al., 1993) and clathrin-independent endocytosis in chromaffin cells (Artalejo et al., 1995). In contrast, the collapse-to-the-membrane hypothesis can explain

such behavior as the complete disappearance of vesicles in stimulated *shibire* (dynamin-impaired) *Drosophila* mutants (Koenig and Ikeda, 1989; Shupliakov et al., 1997) and the release of granule matrix components such as chromogranins from chromaffin cells (Eiden et al., 1987). In fact there exists substantial evidence to suggest two distinct mechanisms of membrane retrieval (Artalejo et al., 1995; Koenig and Ikeda, 1996; Smith and Neher, 1997), which may be indicative of the diversity in post-release granule behavior. Our results describe a dynamic structural balance between kiss and collapse, a direct coupling between exo- and endocytosis.

This balance may play a role in regulating the kinetics of release activity. For example cytosolic monoamines, which are loaded into granules via pH-sensitive transporters in the granule membranes (Cao et al., 1997; Sabban et al., 1990), would accumulate subsequent to release and be available for immediate rerelease. If more postfusion granules were to collapse, less would be available for monoamine reloading. We observe significant variations in the recorded release/recycling histories of individual granules. The physical mechanisms that determine the ultimate fate of a releasing granule are as yet unknown, though high extracellular calcium concentrations are reported to shift the balance toward transient fusion events in chromaffin cells (Ales et al., 1999). Because granule-toplasma-membrane collapse often follows granule-to-granule coalescence (Fig. 7B,C and movies f7B,C), the two processes may be driven by a similar mechanism. We are currently investigating the effects of extracellular ions and/or cytoskeletal modulators on the post-release behaviors of granules.

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