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Detection of Secretion from Single Pancreatic β-Cells Using Extracellular Fluorogenic Reactions and Confocal Fluorescence Microscopy

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Confocal microscopy with Zinquin, a fluorogenic Zn^{2+}-specific indicator, was used for spatially and temporally resolved measurement of Zn^{2+} efflux from single pancreatic β-cells. When cells were incubated in buffer containing Zinquin, application of insulin secretagogues evoked an increase in fluorescence around the surface of the cell, indicative of detection of Zn^{2+} efflux from the cell. The fluorescence increases corresponded spatially and temporally with measurements of exocytosis obtained simultaneously by amperometry. When images were taken at 266-ms intervals, the detection limit for Zn^{2+} was ∼0.5 μM. With this image frequency, it was possible to observe bursts of fluorescence which were interpreted as fluctuations of Zn^{2+} level due to exocytosis. The average intensity of these fluorescence bursts corresponded to a Zn^{2+} concentration of ∼7 μM. Since insulin is co-stored with Zn^{2+} in secretory vesicles, it was concluded that the Zn^{2+} efflux corresponded to exocytosis of insulin/Zn^{2+}-containing granules from the β-cell. Exocytosis sites identified by this technique were frequently localized to one portion of the cell, indicative of active areas of release.

Insulin produced and stored in pancreatic β-cells is released by exocytosis in response to glucose and other stimuli. A greater understanding of stimulus-secretion coupling and exocytosis at β-cells is of intense interest due to mounting evidence for the importance of defective insulin secretion in type II diabetes.[1,2] Studies of stimulus-secretion coupling and exocytosis in these cells are greatly aided by techniques that allow high-resolution monitoring of secretion. Amperometry has proven useful in monitoring secretion from single cells, even at the level of single exocytotic events, because it allows detection of attomole quantities with millisecond temporal resolution.[3,4] Amperometry has been applied to β-cells either by using a ruthenium oxide/cyanoruthenate-coated microelectrode to directly detect insulin[5,6] or by using a carbon fiber microelectrode to detect 5-hydroxytryptamine (5-HT) that had been allowed to accumulate in the secretory vesicles.[7,8] A limitation of amperometry is that a microelectrode is inherently a single-point sensor, meaning that measurements are made at one location on a cell with a detection area determined by the size of the electrode. In principle, this limitation of amperometry may be overcome by using microelectrode arrays; however, present microelectrode technology does not allow the combination of high spatial and temporal resolution possible with microscopy. Spatially resolved measurements of release, such as those possible with imaging techniques, may be a useful complement to single-point measurements to facilitate probing spatial organization of secretion.

Recent advances in fluorescence imaging have resulted in several new approaches to monitoring exocytosis that may be applicable to β-cells. Styryl dyes, such as FM 1-43, which display enhanced fluorescence when partitioned into cellular membranes, allow detection of membrane-area changes associated with exocytosis and endocytosis.[9] In addition, individual vesicles can be detected and their intracellular movements tracked by labeling secretory vesicles either with dyes that accumulate into acidic vesicles[10] or with fusions of green-fluorescent proteins with naturally occurring vesicular proteins.[11−13] Native fluorescence imaging by UV or multiphoton excitation has also been used to detect intracellular levels of certain hormones such as serotonin or catecholamines.[14−18] Changes in intracellular fluorescence corresponding to secretion of the compounds have been detected.[15−18]

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As a complement to these other fluorescent imaging techniques, we have explored imaging secretion at β-cells by direct detection of endogenous Zn^{2+} efflux from single pancreatic β-cells. Insulin is stored in secretory vesicles as a solid hexamer bound with two Zn^{2+} per hexamer[19−22] so that during exocytosis the zinc−insulin complex dissolves and dissociates, co-releasing free Zn^{2+} and insulin.[6,23,24] Detection of Zn^{2+} efflux, therefore, could allow secretion in the β-cell to be monitored. Zn^{2+} is an attractive target for fluorescent measurement because several Zn^{2+}-specific chelating fluorogenic dyes have already been developed. In this investigation, Zinquin [2-methyl-8-*p*-toluenesulfonamido-6-quinolyloxy acetic acid], a dye that chelates Zn^{2+} with a K_d of 370 nM (1:1 complex) and enhances its fluorescence by 20-fold upon binding to Zn^{2+},[25,26] was used with laser-scanning confocal fluorescence microscopy to image Zn^{2+} released by exocytosis. This is the first example of using fluorescence microscopy to detect extracellular concentration changes associated with secretion. Imaging of Zn^{2+} efflux reveals that β-cell secretion is frequently constrained to only a portion of the cell suggesting spatial organization of the secretory machinery within the cell.

EXPERIMENTAL SECTION

Chemicals. Zinquin and its ester form (Zinquin ester) were synthesized and characterized according to an established protocol.[27] Unless otherwise stated, all chemicals for islet and cell culture were obtained from Life Technologies. All other chemicals for buffers and stimulants were from Sigma.

Cell Culture. Mouse islets were isolated from CD-1 mice following ductal injection with collagenase and were dispersed into single cells by shaking in dilute trypsin for 8 min at 37 °C.[28] Cells were plated onto 25-mm-diameter coverslips in tissue culture dishes (Corning) and incubated in RPMI 1640 containing 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C, 5% CO_2, pH 7.4. Cells were used for experiments 2−3 days after isolation.

Confocal Imaging and Analysis. All imaging experiments were performed on a Nikon RCM 8000 laser scanning confocal microscope with a Coherent argon-ion laser (model 622) for fluorescence excitation.[29] All confocal images were obtained as a 1-μm-thick slice through the cell. A Nikon 40X, 1.15 numerical aperture, UV-corrected water-immersion objective was used for all the experiments. The imaging field of view with a 40X objective is 150 μm, corresponding to ~0.3 μm/pixel in both x and y directions with a 512 × 480 pixel image. A full $x-y$ laser scan requires 33.3 ms ($x-y$ scans completed at 30 Hz), which yields a dwell time for an individual pixel of ~135 ns. A full $x-y$ laser scan is defined as one frame, so the frame acquistion rate of this system is 30 Hz. Typically, a number of frames will be averaged to reduce noise in the images. Thus, an image acquisition time of 266 ms corresponds to averaging 8 frames collected at 30 Hz.

Zinc images were taken with 351-nm excitation and 480 ± 10 nm band-pass emission filters. FM 1-43 images were acquired using 488-nm excitation and 595-nm long-pass emission filters. All images were stored on an optical disk cartridge (TQ-FH332, Panasonic) by an optical disk recorder (TQ-3038F, Panasonic). For analysis, images were played back from the optical disk cartridge and captured and analyzed using Simca image analysis software (C-IMAGING Systems, Cranberry Township, PA). For plots of fluorescence as a function of time, a region of interest around a cell was highlighted and the average intensity within this region was calculated for a series of images.

Cells to be imaged were bathed in Krebs-Ringer buffer (KRB), pH 7.4, containing (in mM) 118 NaCl, 5.4 KCl, 2.4 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 20 HEPES, 3.0 D-glucose and maintained at 37 °C on the stage of the microscope by a microincubator (Medical Systems, Inc.). For intracellular Zn^{2+} measurements, cells were loaded with 15 μM Zinquin ester in RPMI media for 30 min at 37 °C and washed with dye-free KRB twice. For extracellular Zn^{2+} measurements, cells were bathed in KRB containing 10 μM Zinquin (acid form). For FM 1-43 experiments, the bathing buffer contained 1 μM FM 1-43.[9] For Ca^{2+}-dependency experiments, $CaCl_2$ in the KRB was replaced by the same concentration of $MgCl_2$. Secretagogues and control KRB were applied to individual cells by pressure ejection at 3−6 psi from micropipets positioned ~60 μm from the cells.[6] Stimulants (30 mM K^+, 20 mM glucose, 200 μM carbachol or 20 μM digitonin) were dissolved in solutions identical to that that the buffer cells were bathed in. For the K^+ stimulating solution, the NaCl was decreased by 30 mM to maintain ionic strength. For digitonin stimulation, extracellular Ca^{2+} was reduced to 0.2 mM. For controls, the identical buffer minus the stimulant was applied to the cell from a different pipet.

Simultaneous Fluorescence and Amperometric Measurement. Cells used for simultaneous measurement were loaded with 5-HT by incubating them in RPMI media containing 0.5 mM 5-hydroxytryptophan (5-HTP) for 16 h at 37 °C in 5% CO_2, pH 7.40.[6] The 5-HTP is enzymatically coverted to 5-HT once it enters the cell. After loading, cells were treated as for fluorescence measurements. Amperometric measurements of secretion were performed using either small (total tip diameter of 1−3 μm) or larger (total tip diameter of 30 μm) electrodes that were constructed as previously described.[30,31] The larger electrodes consisted of 9-μm carbon fiber (P-55S, Amoco Performance Products) sealed with epoxy (Miller Stephenson) in the tip of a glass pipet. The smaller electrodes consisted of the same type of C fiber, flame-etched to a point and coated with an insulating layer of polymer.[31] Both types of electrodes were polished before use at a 45° angle using a pipet beveler (BV-10 with 104D polishing wheel, Sutter Instruments). The larger electrodes were positioned over the top of the cell ~1 μm away to measure secretion from the top face of

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the cell while the smaller electrodes directly touched the cell to measure secretion from a small area of cell membrane.

Amperometry was performed using a two-electrode system and a battery to apply −0.65 V to the sodium-saturated calomel reference electrode. Current was monitored at the C-fiber working electrode using an AI-403 current amplifier and CyberAmp 320 programmable signal conditioner (Axon Instruments, Foster City, CA). Data were low pass filtered at 100 Hz and collected at 500 Hz using a personal computer (Gateway 2000 P6-266) via a data acquisition board (Digidata 1200B, Axon Instruments). During simultaneous measurements, the confocal scanning caused fluctuations in the amperometric trace with a frequency that matched the laser scanning (30 Hz). These fluctuations were large enough to obscure all amperometric signals; therefore, the amperometric data were notch-filtered at 30 Hz by Fourier filtering software, written in-house, to remove this noise.

RESULTS

Intracellular Zn^{2+} Measurements. Zinquin has previously been used to measure intracellular and intravesicular labile Zn^{2+} by incubating cells with the ester form which crosses the plasma membrane and is hydrolyzed by intracellular esterases to the carboxylate anion which is relatively impermeable to the membrane causing intracellular accumulation of the dye.[26] Used in this way at β-cells, we would expect that stimulation of exocytosis would cause a decrease of fluorescence as Zn^{2+} is released similar to what has been observed in Zn^{2+}-secreting hippocampal neurons.[32] In experiments done with this approach, we found that intracellular fluorescence signals did decrease during stimulation as shown in Figure 1. As shown in the figure, even though intracellular fluorescence decreases also occurred during application of control buffers to the cell, the decrease, measured as the slope of the time-dependent fluorescence signal, was significantly greater during stimulation than during control stimulations or basal conditions. The decreases that occurred during basal or control conditions could be caused by photobleaching of the dye or "leaking" of the dye out of the cell. While the intracellular fluorescence decreases during stimulation were significant, it was apparent that such decreases could be caused by other events such as changes in native fluorescence of the cell during stimulation and other decreases in intracellular Zn^{2+} level besides those associated with secretion. These confounding factors prevented sensitive and selective measurements of secretion in these cells.

Extracellular Zn^{2+} Measurements with Zinquin Acid. To avoid problems associated with intracellular Zn^{2+} measurements, we explored measurements of extracellular Zn^{2+} concentration changes. For these measurements, the cell was bathed in a solution containing Zinquin, which is relatively impermeable to the membrane, so that Zn^{2+} released from the cell is expected to react with dye, resulting in increased fluorescence outside the cell as illustrated in Figure 2. This approach is expected to avoid the difficulties of intracellular measurements because intracellular fluorescence does not interfere and photobleached dye can be quickly replaced by dye in the bath.

Figure 3 illustrates images of a single cell stimulated with 20 mM glucose in the presence of Zinquin. The data show that

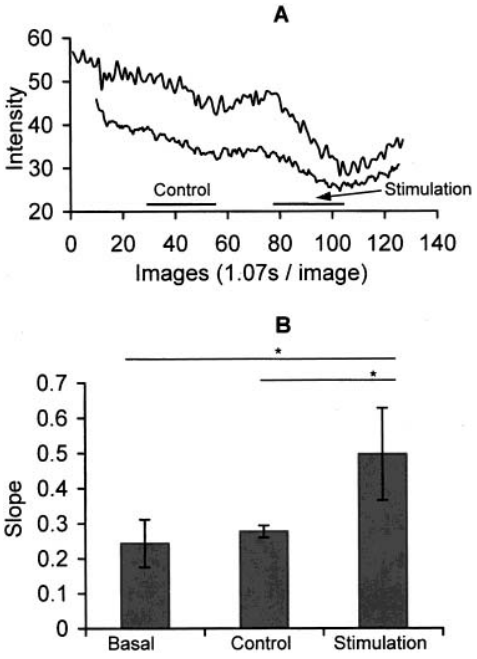

Figure 1. (A) Intracellular Zn^{2+} fluorescence changes of two different cells. Cells were preloaded with 15 μM Zinquin ester. Images were taken continuously at 1.07s intervals (32 frames averaged per image). A control buffer and carbachol stimulating solution were applied for 30 s as indicated by the bars underneath the curves. (B) Slopes of fluorescence decrease during three periods, (1) basal (before the application of buffer); (2) control (during the application of buffer); (3) stimulation (during the application of carbachol). Slopes were estimated by linear regression and shown as mean ± S. D.(n = 5). Statistical significance of * is $p < 0.05$, which is evaluated by Student's t test.

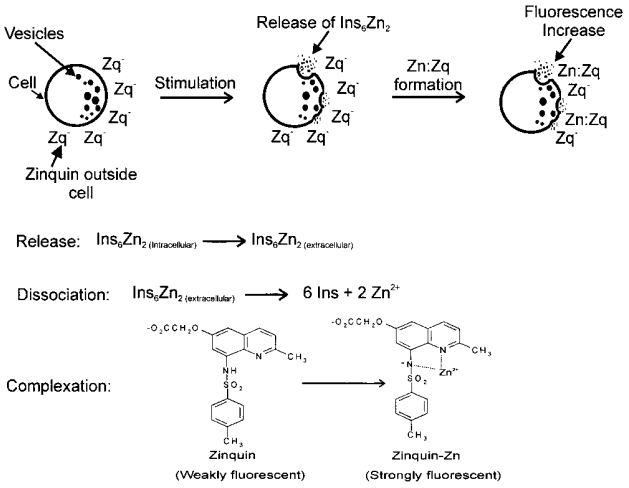

Figure 2. Scheme of extracellular Zn^{2+} efflux measurement. Zn^{2+} co-stored in vesicles with insulin is released by exocytosis. Once exposed to the extracellular milieu, the insulin−Zn^{2+} granule (Ins_6-Zn_2) dissolves and dissociates, allowing the Zn^{2+} to react with Zinquin acid (Zq^-) to form a fluorescent complex. The detailed sequence of reactions is shown above.

fluorescence outside the cell dramatically increased at the surface of the cell, primarily in a semicircular region covering about 50% of the circumference, during the stimulation. In cells that exhibited fluorescence increases (n = 22), 80% showed heterogeneous increases with fluorescence enhancements occurring around <60%

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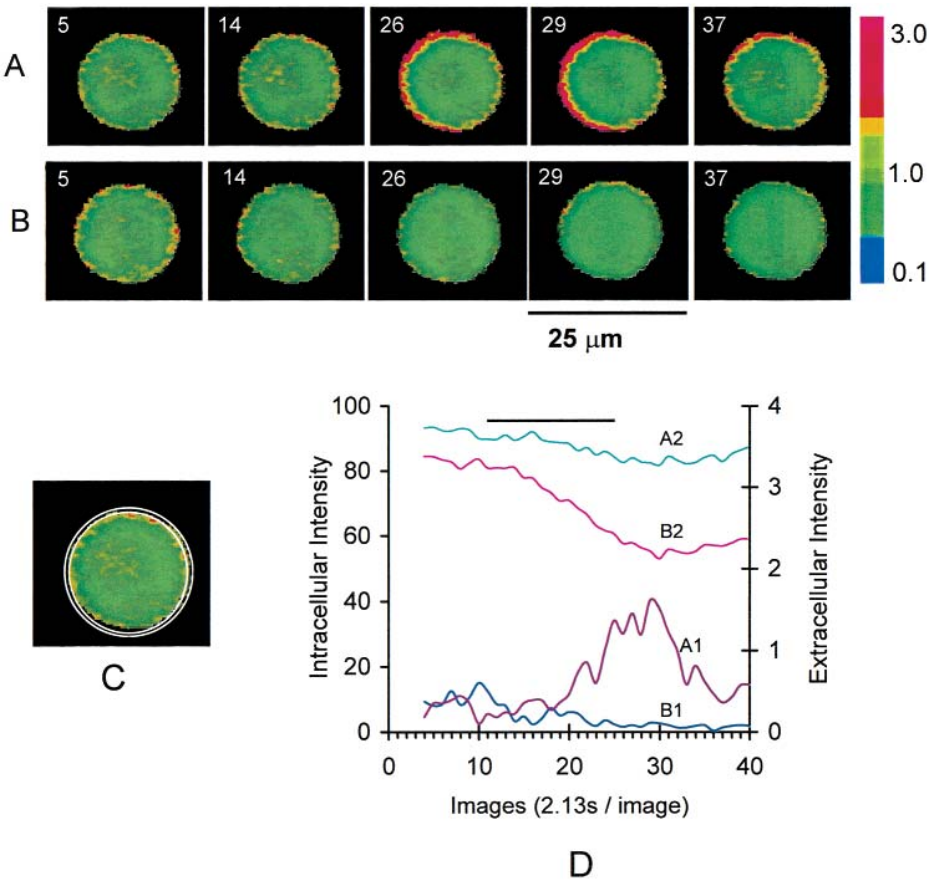

Figure 3. Extracellular measurements of Zn^{2+} release. Images are fluorescence intensity ratioed against the first image collected in the series. Cells were bathed in KRB containing 10 μM Zinquin acid. Scans were taken continuously, and 64 frames were averaged per image (2.13 s per image). The image number is given for each picture. (A) Glucose stimulation where 20 mM glucose was applied to the cell by a micropipet (stimulus applied from images 15 to 29). (B) Control experiment where control solution was applied to the cell by a micropipet (stimulus applied for the same images as (A)). (C) Regions of interest (ROI). The ROI were defined by drawing two circles around the cell. The inner circle defines the region inside the cell. The region between the two circles defines the region outside the cell. (D) Average fluorescence intensities within the ROI as a function of image. A1 and B1 are extracellular fluorescence for glucose and the control stimulation, respectively. A2 and B2 are intracellular fluorescence for glucose and the control stimulation, respectively. Stimulation periods are indicated by the bar over the curves. Results are typical of experiments at 22 separate cells.

of the cell circumference. The extracellular fluorescence enhancement was not observed in control experiments where the cell was stimulated with KRB (Figure 3B). With both control and glucose application, fluorescence inside the cell decreased which may be due to a decrease of native cellular fluorescence or Zinquin fluorescence from a small amount of Zinquin that permeated the cell.

To test the hypothesis that increases in fluorescence outside the cell were due to detection of Zn^{2+} efflux, control experiments were performed in which a cell was stimulated in the presence of tetrakis-(2−pyridylmethyl)ethylenediamine (TPEN), a nonfluorescent Zn^{2+} chelator with higher affinity for Zn^{2+} than Zinquin.[33] In these experiments, no fluorescent changes occurred outside the cells even though the same cells exhibited strong signals in the absence of TPEN as illustrated in Figure 4 ($n = 4$). Thus, the signals observed were dependent upon the presence of free Zn^{2+} outside the cell. In addition, no extracellular fluorescence changes were observed in the absence of Zinquin. Finally, the fluorescence changes were not due to the cells moving or membrane swelling, as indicated by visual inspection and imaging of the membrane

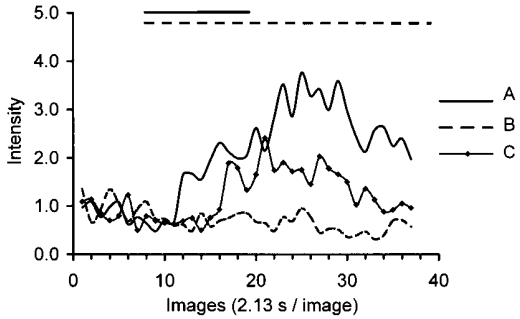

Figure 4. Effects of TPEN on detection of Zn^{2+} efflux. Images were taken and analyzed as in Figure 3 and intensity is normalized against the first image in each serial. Traces were recorded from the same cell which was bathed in KRB with 10 μM Zinquin acid. (A) Stimulated with 20 mM glucose (solid bar). (B) Stimulated with solution containing 20 mM glucose and 50 μM TPEN after a 5-min interval (dashed bar). (C) Stimulated with 20 mM glucose again after 5 min. A longer stimulus was used with (B) to maintain TPEN around the cell during the course of secretion.

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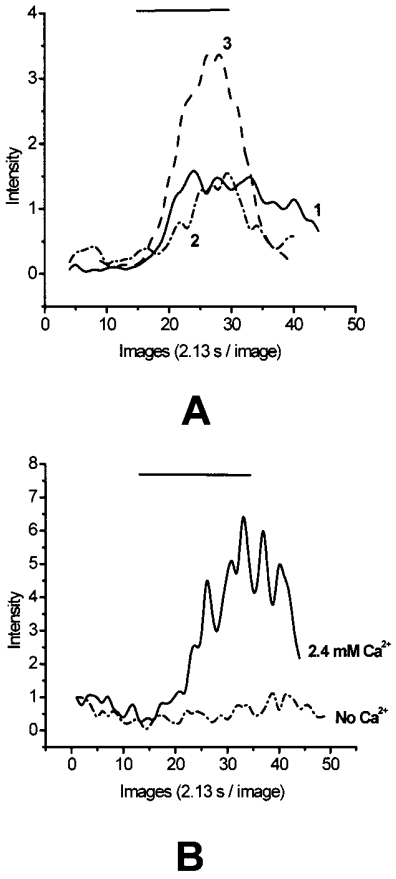

Figure 5. (A) Extracellular changes in fluorescence in the presence of 10 μM Zinquin evoked by application of different insulin secretogogues. Images were acquired and analyzed as in Figure 3. Stimulants used were (1) 30 mM K^+, (2) 20 mM glucose, and (3) 200 μM carbachol. The bar above traces indicates the stimulation period. Traces for different stimulants were not from the same cell. Results are representative for at least 10 separate experiments for each stimulant. (B) Ca^{2+}-dependency of Zn^{2+} efflux. Traces were recorded from the same cell which was bathed in Ca^{2+}-free KRB containing 10 μM Zinquin acid. The cell was first stimulated by Ca^{2+}-free glucose and then glucose containing 2.4 mM Ca^{2+} 5 min after the first stimulation. The bar on the top indicates the stimulation period.

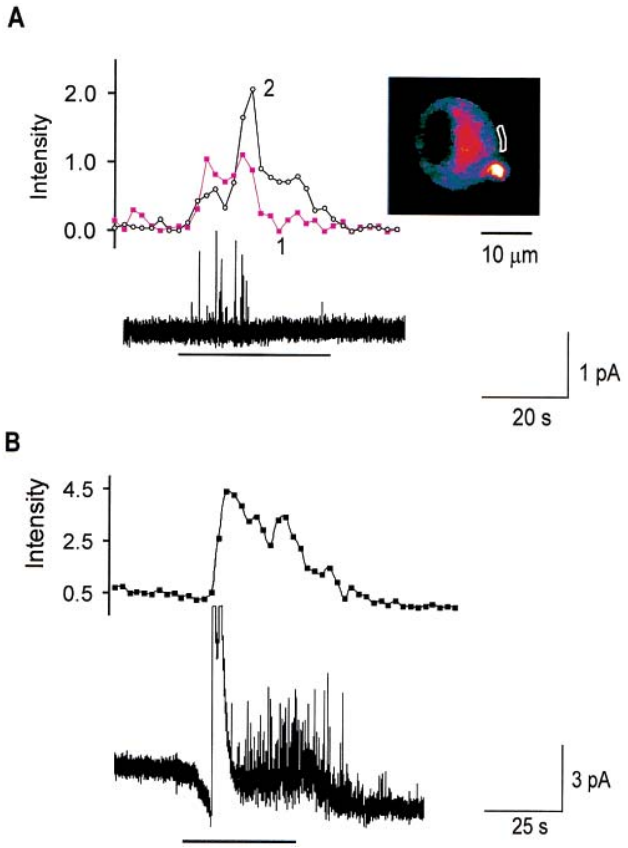

Figure 6. Simultaneous fluorescence and amperometric measurements. Images were acquired as in Figure 3. Fluorescence and amperometric traces are aligned on the same time axis for a given experiment, and bars underneath traces indicate the periods of stimulation. (A) K^+ stimulation during which 5-HT secretion was monitored using a C-fiber electrode with an ~3-μm tip diameter. The inset image is a FM 1-43 labeled cell image. The relatively bright area in the lower right corner indicates the position of the electrode. Fluorescence trace 1 (pink) is the fluorescence intensity measured in a region close to the electrode and indicated by the white outline on the image. Fluorescence trace 2 (black) is obtained from the extracellular region around the entire cell, as in Figure 3 (excluding the electrode). (B) Digitonin stimulation during which a carbon fiber electrode with total tip diameter of 30 μm was positioned over the top of the cell. Extracellular fluorescence was measured in an optical section below the electrode. Fluorescence data was from the entire circumference of the cell and presented as in Figure 3.

with FM1−43 during stimulation. Taken together, these results support the hypothesis that the fluorescent signals observed were due to Zn^{2+} efflux from the cell.

Correlation between Zn^{2+} Efflux and Exocytosis. The initial observations of Zn^{2+} efflux from single cells evoked by glucose led us to develop the hypothesis that detected Zn^{2+} corresponded to Zn^{2+} co-released with insulin by exocytosis. This hypothesis was tested by using other insulin secretogogues, Ca^{2+}-free experiments, simultaneous amperometric and fluorescence measurements of secretion, and higher temporal resolution measurements.

Other Insulin Secretagogues and Ca^{2+}-Dependency. Treatment of cells with 30 mM K^+, a depolarizing agent, and 200 μM carbachol, a secretogogue that releases intracellular Ca^{2+} stores to induce exocytosis, both caused increases in extracellular Zn^{2+} as illustrated in Figure 5A. The increases in Zn^{2+} fluorescence induced by glucose and K^+ were dependent upon having Ca^{2+} in the extracellular media as expected for exocytosis evoked by these secretogogues (Figure 5B, $n = 8$).

Simultaneous Amperometry and Fluorescence. To further explore the apparent correlation between Zn^{2+} secretion and exocytosis, we attempted simultaneous fluorescence measurements of Zn^{2+} and amperometric measurements of 5-HT secretion at the same cell. (Amperometric detection of 5-HT has been shown to correspond to exocytosis of insulin granules at these cells[8].) These measurements were technically difficult because the electrode blocked observation of Zinquin fluorescence at the cell membrane in the immediate vicinity of the electrode because of the high fluorescence background caused by the electrode (see Figure 6A), preventing Zn^{2+} efflux detection at exactly the same location as 5-HT secretion. In addition, the laser scanning induced significant noise at the electrode which could only be partially removed by filtering; therefore, the noise level in the amperometric measurements was higher than what is typically achieved. Despite these

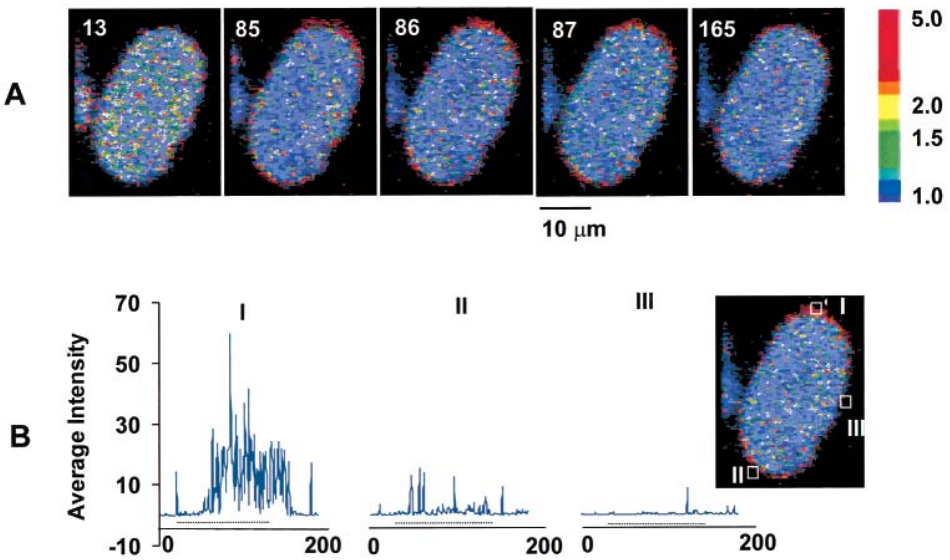

Figure 7. Measurements of zinc release with images acquired continuously at 266 ms/image (8 frames averaged per image). (A) Montage of 30 × 40 μm ratio images. Images are ratioed against the first image in the entire series and image number is indicated in each image. Images actually show two cells that are touching each other resulting in the oblong shape. Cells were stimulated with 200 μM carbachol from images 30 to 143. Image 13 is a typical image before stimulation. Images 85−87 are a typical series during stimulation, and image 165 is a typical image after stimulation. The color bar is indicative of the fluorescence ratio against the first image so that the red color at the edge of the cells are indicative of Zn^{2+} release. (B) Zn^{2+} release signal measured in three small extracellular regions (4.3 μm^2) as shown in the inset image. Bars underneath each trace indicate application of 200 μM carbachol.

limitations, it was possible to simultaneously observe both Zn^{2+} efflux and 5-HT secretion from the same cell using either small electrodes that only covered a portion of the cell (Figure 6A) or larger electrodes that covered the top face of the cell (Figure 6B).

If fluorescence was measured in the immediate vicinity of the electrode, the fluorescence and amperometric signals (amperometric signals are current spikes corresponding to detection of bursts of 5-HT released by exocytosis) began and ended in tandem as shown by the pink trace in Figure 6A ($n = 4$). Interestingly, if fluorescence around the whole cell was measured, we observed a poorer correlation with the fluorescence signal lasting 2−10 s longer than the amperometric trace (see black trace in Figure 6A). The requirement of detecting fluorescence near the electrode to obtain a good correlation between the Zn^{2+} and 5-HT signals is attributed to the spatially heterogeneous nature of the Zn^{2+} signal (Figure 3). As a complement to these positive correlations, we also observed that if the electrode was positioned around a zone of a cell that did not exhibit Zn^{2+} efflux, no serotonin secretion was observed ($n = 5$).

Further correlations were attempted using a 9-μm-diameter electrode which could be positioned over the top of the cell and measure release from the entire top face of the cell. When using electrodes of this dimension, good correlations between onset of 5-HT detection and Zn^{2+} detection were also achieved as illustrated in Figure 6B for a digitonin treatment (digitonin induces secretion by permeabilizing the membrane, allowing Ca^{2+} entry and stimulation of exocytosis). For simultaneous measurements, secretion was observed at 21 different cells using K^+ ($n = 3$), glucose ($n = 8$), carbachol ($n = 6$), or digitonin ($n = 4$) as the stimulant. In all cases, fluorescence and amperometric signals began and ended at the same time within the temporal resolution of the fluorescence measurements.

High-Temporal Resolution Measurements of Zn^{2+} Efflux. If Zn^{2+} release is primarily due to exocytosis, then we would expect to detect sharp fluctuations in Zn^{2+} concentration near the membrane if the temporal resolution of the measurement was high enough. To attempt detection of such fluctuations, Zn^{2+} secretion from cells was measured with image acquistion rates of 4 Hz as shown in Figure 7. With this rate of image collection, the detection limit was estimated to be 0.5 μM (S/N of 3). The montage of images in Figure 7A show that, during the course of stimulation, strong increases in fluorescence are observed immediately outside the cell and these signals end soon after the stimulation. As with images obtained at lower temporal resolution, Zn^{2+} release is heterogeneous across the surface of the cell with some portions of the cell not generating any signal and others nearly continuous signals.

Another feature of the data is that fluorescence measured in small locations on the cell surface occurs in bursts which is expected if Zn^{2+} is released by exocytosis (Figure 7B). These temporal plots from different parts of the cell further illustrate the highly localized nature of the release. Individual fluorescence bursts appear to have the time course expected for detection of exocytotic events. In amperometric measurements of insulin release by exocytosis, we have previously observed that individual current spikes had a full-width at half-height of 37 ± 27 ms and total duration of 270 ± 165 ms.[34] On the basis of these observations, we would expect Zn^{2+} bursts, if due to single exocytosis events, to occur in one or two images at 266 ms/image. Indeed, 50% of the bursts that were detected in these small windows lasted only one image and 34% lasted two images. These burst widths should not be taken to represent the time of exocytosis events,

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as the temporal resolution using this approach is at present too poor to adequately sample the spikes or to resolve multiple bursts occurring within a single image. Higher temporal resolution will be be required to adequately characterize the spike widths and correlate them to isolated exocytosis events. These data simply show that the spikes are narrow enough, within the temporal resolution of the measurement, to be due to exocytosis events. The signals observed in Figure 7 are typical of experiments at 7 different cells.

In addition to time course, the amplitude of the spikes is approximately what would be expected for exocytotic events. The average signal intensity for spikes collected at 4 Hz corresponds to a Zn^{2+} concentration of $\sim$7 μM. (This concentration is nearly as large as the indicator concentration (10 μM Zinquin); therefore, the released Zn^{2+} may consume most of the Zinquin near the release site, resulting in an underestimation of the actual Zn^{2+} concentration. This situation may be exacerbated by the fact that this concentration represents the average within the measuring window so some regions may have an even higher concentration of Zn^{2+}. Therefore, the estimate of 7 μM is considered a lower boundary to the concentration generated at the cell surface.) Each vesicle is estimated to contain 1 amol Zn^{2+} on the basis of previous measurements that determined the Zn^{2+}/insulin ratio in vesicles to be 1:1.5[22] and measurements of insulin release that demonstrated 1.6 amol insulin/vesicle.[5] If a single vesicle is released at the cell surface and the released Zn^{2+} has 125 ms to diffuse (assuming that the vesicle exocytosed during the middle of the image collection), then it could diffuse an average of 5 μm (diffusion coefficient of the complex assumed to be 10^{-6} cm^2/s) resulting in an average concentration of $\sim$4 μM around the release site. This calculation is approximate because it neglects delays associated with dissolution of the Zn^{2+}/insulin granule, the uneven concentration generated by diffusing from a point source, and the possibility of multiple events occurring in time and space of the measurement; however, it does illustrate that the size of fluorescence bursts observed are reasonable for exocytotic events given the amount of Zn^{2+} expected to be released. This calculation also illustrates a limitation of the technique, which is that the convolution of diffusion and reaction of the analyte prevent identifying the exact location of exocytosis sites.

DISCUSSION

Zn^{2+} Efflux As a Measure of Insulin Secretion. The results support the conclusion that Zn^{2+} co-released with insulin by exocytosis from single pancreatic β-cells can be monitored by reaction of Zn^{2+} with Zinquin at the cell surface and subsequent detection of the fluorescent product by confocal fluorescence microscopy. This conclusion is based on the use of insulin secretogogues to elicit Zn^{2+} signals, the Ca^{2+}-dependence of such signals, good temporal correlation of such signals with amperometric measurements of 5-HT secretion, and detection of fluctuations of Zn^{2+} level on the time scale and magnitude expected for exocytotic events.

This technique should be valuable in physiological studies of insulin secretion. For example, these initial observations of imaging Zn^{2+} release reveal that β-cells are spatially heterogeneous with respect to secretion around single cells. In 80% of the cells that were studied, secretion occurred over 60% or less of the circumference of the cell being observed (Figures 3 and 7). Several previous observations have suggested that β-cells may be polarized with respect to release. For example, vesicles have been found to preferentially accumulate at one portion of the cell,[35] and intracellular Ca^{2+} changes, necessary for secretion, have been found to be localized in the cells.[36] The physiological significance of spatially heterogeneous release is not clear; however, such an organization may be beneficial in directing secretion directly into blood vessels for rapid and large responses to glucose challenges. Supporting this idea, it has previously been observed that blood vessels within an islet are arranged so that most β-cells have a single face against a capillary.[35]

Other Applications. In addition to studies of pancreatic β-cells, this method may be of value in the study of Zn^{2+} secretion in other cells such as hippocampal mossy fiber neurons. In these cells, Zn^{2+} appears to act as a neurotransmitter or neuromodulator and its release to play a role in development of Alzheimer's disease as well as excito-toxicity.[37,38] Therefore, monitoring the dynamics of Zn^{2+} secretion would be important in the study of these cells.

While these experiments have been directed at Zn^{2+} measurements, these results demonstrate a novel approach to detection of secretion based on rapid, fluorogenic reactions at the cell surface. Development of other reaction schemes may allow this approach to be a general route to detection of secretion from cells. Besides physiological studies, the speed and simple setup of such confocal methods may also have application in drug screening. For example, the technique applied to β-cells may prove to be a valuable cell-based assay for rapidly screening compounds as potential diabetes drugs that elicit insulin secretion.

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