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Observations of Calcium Dynamics in Cortical Secretory Vesicles

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SUMMARY

Calcium (Ca^{2+}) dynamics were evaluated in fluorescently labeled sea urchin secretory vesicles using confocal microscopy. 71% of the vesicles examined exhibited one or more transient increases in the fluorescence signal that was damped in time. The detection of transient increases in signal was dependent upon the affinity of the fluorescence indicator; the free Ca^{2+} concentration in the secretory vesicles was estimated to be in the range of $\sim 10 - 100 \mu\text{M}$. Non-linear stochastic analysis revealed the presence of extra variance in the Ca^{2+} dependent fluorescence signal. This noise process increased linearly with the amplitude of the Ca^{2+} signal. Both the magnitude and spatial properties of this noise process were dependent upon the activity of vesicle p-type ($\text{Ca}_v2.1$) Ca^{2+} channels. Blocking the p-type Ca^{2+} channels with ω -agatoxin decreased signal variance, and altered the spatial noise pattern within the vesicle. These fluorescence signal properties are consistent with vesicle Ca^{2+} dynamics and not simply due to obvious physical properties such as gross movement artifacts or pH driven changes in Ca^{2+} indicator fluorescence. The results suggest that the free Ca^{2+} content of cortical secretory vesicles is dynamic; this property may modulate the exocytotic fusion process.

Keywords

Secretory Vesicles; Calcium Dynamics; Confocal Microscopy; Fluctuation Analysis; Super-Poisson Statistics

INTRODUCTION

Many cell functions are regulated by the intracellular Ca^{2+} concentration, among them secretion/exocytosis/endocytosis (i.e. neurotransmitter release), fertilization, programmed

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*This work is dedicated to our mentor, colleague, and friend, the late Professor Rami Rahamimoff whose absence is felt throughout and beyond the scientific community.

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cell death, and gene expression [1–5]. The intracellular Ca^{2+} concentration, in turn, depends upon the amount of Ca^{2+} transported through the plasma membrane, the Ca^{2+} released from intracellular organelles, and the endogenous buffering mechanisms available. Among the intracellular organelles that can store and release Ca^{2+} , the roles of the endoplasmic reticulum and the mitochondria have been established [2, 6, 7]; the involvement of the secretory vesicle in the regulation of intracellular Ca^{2+} has received increasing attention [8–16], in part, because the Ca^{2+} content of vesicles is high. For example, the total Ca^{2+} content of the cholinergic synaptic vesicles of the electric ray is ~ 120 mM [17]. Of interest is whether this high Ca^{2+} content affects the local intracellular environment surrounding the vesicle. Intra-vesicular Ca^{2+} could affect the Ca^{2+} concentration adjacent to vesicles because vesicles contain Ca^{2+} channels [15, 18, 19] and other Ca^{2+} transport mechanisms [20–22]. These transport mechanisms may contribute to the function of the vesicle by supplying Ca^{2+} to critical sites close to the release machinery; even small changes of the local Ca^{2+} concentration can have a profound effect on the release of transmitter [23]. It has been demonstrated that changes in the vesicular Ca^{2+} concentration can have effects on exocytotic release [9, 11, 12, 14, 24–27]. Intra-vesicular Ca^{2+} dynamics may be an inherent vesicle property that is important to secretion and signaling.

Some vesicle types have the necessary machinery to support dynamic Ca^{2+} behavior including Ca^{2+} oscillations [8, 10, 14, 28]. The dynamic behavior can be stimulated by inositol 1,4,5-trisphosphate (InsP_3), and is pH and potassium dependent, showing many similarities to other internal storage compartments involved in Ca^{2+} release and uptake [29]. At the cellular level, Ca^{2+} oscillations are also observed in both space and time, and the relationship between these dynamic properties, signaling and coupled biochemical pathways, is the subject of both experimental and theoretical study [2, 30–34]. At the cellular level, Ca^{2+} dynamics have been evaluated using deterministic, stochastic, and chaotic models [30].

Large docked (i.e. ‘stationary’), fusion-ready secretory vesicles, amenable to confocal microscopy, are found in the sea urchin egg; there is also a striking similarity in the Ca^{2+} dependence of their release and the release of synaptic and other secretory vesicles [35]. Here we demonstrate that Ca^{2+} oscillations occur in sea urchin secretory vesicles and these oscillations have super-Poisson noise properties. The super-Poisson component is dependent upon the magnitude of the Ca^{2+} signal and p-type ($\text{Ca}_v2.1$) Ca^{2+} channel activity. These Ca^{2+} properties may have a role in the regulation of the secretory/exocytotic pathway.

Materials and Methods

Solutions and Calibration Beads

All reagents (unless noted otherwise) were purchased from Sigma (Sigma Chemicals Co., MO, USA). The osmotic strength of all solutions (~ 1000 mOsm) were verified using an osmometer and in agreement with solution composition. All buffer concentrations were calculated using WINMAX (Stanford University, CA, USA) and verified using Ca^{2+} electrode measurements [36]. Artificial seawater contained, in mM, 338.35 NaCl, 30.73 MgSO_4 , 40 MgCl_2 , 11 CaCl_2 , 10.2 KCl, 9.98 HEPES, 1 EDTA, pH 8.0 titrated with NaOH. Baseline Intracellular Medium (BIM), used for shearing eggs, contains, in mM, 210 Glutamate (free acid or K-salt), 500 Glycine, 10 NaCl, 1 EGTA, 10 PIPES, 0.05 CaCl_2 , 1 MgCl_2 , pH 6.7 titrated with KOH; the free Ca^{2+} concentration is ~ 100 nM, comparable to the basal intracellular concentration. Exocytotic release, when required, was triggered by perfusion with a high Ca^{2+} solution (estimated $[\text{Ca}^{2+}]_{\text{free}} > 1$ mM), containing, in mM, 210 Glutamate (free acid or K-salt), 500 Glycine, 10 NaCl, 10 EGTA, 5 HEDTA, 10 PIPES, 15–23.05 CaCl_2 , 2 MgCl_2 , pH 6.7 titrated with KOH.

Calibration beads (InSpeck Green) were purchased from Molecular Probes (Invitrogen); the bead specifications are 6 μm diameter with excitation/emission maxima at 505/515 nm.

Animal Handling, Collection of Eggs, and Cortex Preparation

Strongylocentrotus purpuratus and *Lytechinus pictus* sea urchins were purchased from Marinus, Inc. (Long beach, CA, USA) and maintained in seawater aquaria cooled to 12°C and 15°C, respectively. The urchins were fed with *Ulva lactula* seaweed (generously supplied by SeaOr Marine, Michmoret, Israel) grown in a separate aquarium. Eggs were obtained by intra-coelomic injection of ~ 3 ml of 0.5 M KCl, and collected in artificial seawater. Eggs were dejellied mechanically by passage through a 90 (*S. purpuratus*), or 110 (*L. pictus*) μm pore size nylon mesh, washed in a large volume of artificial sea water, and allowed to settle under gravity, with minimum packing, in a large beaker. After concentrating the eggs at the bottom of 50 ml tubes, a few aliquots of the egg suspension were deposited onto cover-slips (Marienfeld, Germany, No. 0) coated with poly-L-lysine hydrobromide (MW >300,000, 250 $\mu\text{g ml}^{-1}$) that were attached to the bottom of Nunc culture dishes (Nalge Nunc, NY, USA) using Sylgard silicone elastomer (Dow Corning, MI, USA). In some experiments, poly-L-lysine coated 35 mm glass bottom culture dishes (Mattek, MA, USA) were used. The eggs were allowed to settle and attach to the coverslips over a 2–5 min period. The cover slips were rinsed once with BIM. The attached eggs were sheared using a stream of 2–5 ml of BIM solution. The stream was produced using a hand-held syringe with a 19-gauge needle. This procedure creates an attached layer of plasma membrane with bound cortical vesicles [37], known as the planar isolated cortex. The cover slips were rinsed again with BIM.

Indicator loading

All indicators were purchased from Molecular Probes Inc. (OR, USA). Fluo-4, Fluo-4FF, and Fluo-5N (pentapotassium salts) non-ratiometric indicators were used for the detection of free Ca^{2+} ; FM 4-64 (a styryl indicator), was used for membrane labeling, and cSNAFL-2 was used as a non-ratiometric indicator for the evaluation of pH dynamics. Cortices were labeled with Fluo-4, Fluo-4FF, Fluo-5N and cSNAFL-2 indicators (prepared as 50 μM stocks in BIM), by incubation for 45–60 min at room temperature, covered and in the dark. Labeling vesicles with non-permeant fluorophores requires Mg-ATP (2.5 mM) in the indicator solution. Cortices were labeled with 4 μM solutions of FM 4-64, prepared in BIM, for 15 minutes at room temperature. Labeled cortices were washed with BIM solution. For double staining with FM 4-64 and Fluo-4, cortices were first labeled with FM 4-64 for 15 min. Cortices were then washed with BIM and followed by labeling with Fluo-4 for ~1 h. Double labeled cortices were washed with BIM solution.

Microscopy

Confocal microscopy was performed using a Molecular Dynamics Phoibos 2001 Confocal microscope, built on a Nikon Diaphot-TMD inverted microscope with a 100 $\times$, 1.25 NA, Plan oil-immersion (index of refraction, $n = 1.515$) objective (Zeiss, Oberkochen, Germany). All filters were obtained from Omega Optical Inc. (VT, USA). The 488 nm line of a 25 mW argon-ion laser (Melles Griot, CA, USA) was used as the excitation light source. The selected line (in the line scan mode) was repetitively scanned at a rate of 100 Hz. Excitation light was first reflected by a primary 510 nm dichroic beam-splitter (510 DRLP), and used to excite the fluorescence indicator in the specimen. The emitted fluorescence light passed through the primary beam-splitter, and through a 50 μm diameter pinhole. For all Ca^{2+} indicators a 510 nm long-pass emission filter (510 ALP) was used. For pH imaging using cSNAFL-2 as a non-ratiometric indicator (emission isosbestic wavelength ~ 630 nm) a 545AF75 band-pass emission filter (510 nm–590 nm) and a 100 μm diameter pinhole were used. This emission range yielded a pH-dependent fluorescence signal. For FM 4-64 and

Fluo-4 double staining a 595 nm secondary dichroic beam-splitter (595 DRLB B/S) was used. Then, a 610 nm long-pass emission filter and a 545AF75 band-pass emission filter were used for the FM 4-64 and Fluo-4 signals, respectively.

Various methods were used to distinguish the vesicles from other cellular structures. First, image sections were collected from above and below the focal plane of the investigated plane of interest. Knowing the calculated axial resolution of the confocal optics (0.945 μm), the step size between two adjacent series, and the vesicle diameter ($\sim 1 \mu\text{m}$) data collection from structures that differed in the expected size (vesicle diameter), geometry (circular), and position (membrane plane) for vesicles, was avoided. The Fluo-4 indicator (the penta-potassium form) was shown to label secretory vesicles without labeling other membranous compartments that might survive the shearing, such as residual ER. To further demonstrate that secretory vesicles were labeled, Ca^{2+} activation solutions were used to induce a massive release/fusion of all cortical secretory vesicles; the observed loss of fluorescence signal is consistent with labeling of fully docked, fusion-ready vesicles.

Microscope Calibration

Fluorescence photon statistics, in the absence of other processes and expressed in photo-electron events, is described by a Poisson process where the ratio of intensity variance to intensity mean is one. The presence of other noise processes, both instrumental and biological can alter this ratio. However, confocal microscopes are typically not single photon counting instruments and rely on a number of signal processing steps (photomultiplier output integration, amplification, and digitization) to produce instrument dependent, analog digital units (ADU). The ratio of the intensity variance over mean intensity, in ADUs, may differ significantly from one because the output is the sum of offsets (O) and Gain (G) according to the relationship $I(\text{ADU}) = O + G \cdot n_e$ where n_e represents the photo-electron events in photon counts. A model, tested below, for the output of the microscope electronics, under the simplifying assumption that the offset and gain are constant, is that the ratio of variance/mean equals G and not one [38]. The gain of the system was evaluated using two procedures. Fluo-4 solutions (4 μM) containing saturating and minimal free Ca^{2+} were imaged, using the same laser power, in line scan mode and the resulting time series analyzed. Alternatively, a single Fluo-4 solution (saturating free Ca^{2+}) was imaged, in line scan mode, using a series of acquisitions made with decreasing laser power such that the 8 bit dynamic range of the instrument was sampled. Neutral density filters were used to change the laser power; this ensures that the properties of the laser light were the same for all acquisitions. Both procedures gave the same result for the instrumental gain, $G = 0.65 \pm 0.17$ (mean $\pm$ std. dev.); readout noise was less than 1 ADU.

Data analysis and statistics

Data were analyzed using MATLAB (The MathWorks Inc., MA, USA). Row-vector data was collected using a Silicon Graphics Iris Indigo computer controlling the confocal microscope and transferred to a second computer. Row-vector data was then transformed into matrices representing the physical image according to its scanning parameters. Vesicles were identified using the section series of the cortex. A line-scan, passing through one or several vesicles, was performed. From this line scan, regions of interest were extracted. After verifying the vesicle morphology and characteristic size, line-scan data of the vesicle were collected into a matrix that contained data from all the vesicles present in the line-scan. All vesicles showed significant loss of signal upon repetitive scanning. Frequently, the loss of signal resulted in the disappearance of almost all the detectable indicator fluorescence within a vesicle.

Non-Stationary Signal Processing

A simple procedure for estimating the noise in a stationary signal is to calculate the variance. However, the time varying fluorescence signals acquired from vesicles and beads (Figure 1) required calculating the variance around a non-stationary signal. Group regression analysis was used to calculate the variance. Typically, 4992 scans (~ 50 sec) were divided into 156 segments consisting of 32 scans each (~ 0.3 sec). Linear regression was applied to each group of 32 scans; the variance was calculated around each segment regression line and not the mean. To evaluate the effect of segmentation on signal processing, different segmentation values were used. Segmenting the data using 25, 32, 50, or 64 scans resulted in the same qualitative dependencies reported; outside of small differences, the results were invariant with these levels of segmentation. The noise properties of each data segment were characterized using the Fano factor which is the ratio of the variance to the mean. The Fano factor is a useful descriptor of stochastic processes because deviations from Poissonian behavior are revealed by Fano factor values that differ from one; a Fano factor of one is consistent with a Poisson process [39]. Fano factors greater than one represent super-Poissonian stochastic processes [39–41].

Vesicle Spatial Profile

Vesicle diameter were calculated by first identifying the plane that maximized the vesicle cross-section and then determining the number of pixels two standard deviations above the mean background or, for the treatments with Ca^{2+} channel blockers, by subtracting the background so that the edges of the initial scan were clear for subsequent alignment. The Matlab Image Analyzer zoom was increased and the number of pixels for each vesicle was determined. 120 vesicles from 5 different preparations were used to calculate vesicle size using the theoretical value for the pixel size ($0.25 \mu\text{m}$); the average vesicle diameter was $1.3 \pm 0.3 \mu\text{m}$ (mean $\pm$ std. dev.) or 5 ± 1 pixel in agreement with previous work [42]. The same approach was used to find the spatial center of each vesicle. Vesicles spanning the same number of pixels were aligned, using the center pixel (maximum intensity), and subsequent statistics calculated for the aligned population of vesicles. For these calculations, the data was segmented using 50 scans or 0.5 sec.

Results

Figure 1A and 1B illustrates the time dependent fluorescence intensity measured from sea urchin egg secretory vesicles labeled with the Ca^{2+} indicator Fluo-4 during repetitive line scan confocal microscopy. Two types of behaviors are observed. In 29% of 189 cortical vesicles studied from 13 preparations, the fluorescence signal decayed monotonically (Figure 1A) while the remaining 71% of vesicles exhibited a more complex temporal process with one or more transient increases in fluorescence in addition to a decaying fluorescence signal (Figure 1B). The transient increases in fluorescence either occurred at the start of scanning or appeared later during the scanning process. A monotonic decrease in signal would be expected if some combination of laser induced, irreversible, photo-degradation/bleaching of fluorescent molecules, fluorophore extrusion, and Ca^{2+} efflux were the only active processes occurring. The first type of behavior is designated 'simple' while the wave-like behavior is designated 'complex'. The observed fluorescence behavior is not due to temporal properties of the scanning laser because both simple and complex fluorescence behavior can be observed when two or more vesicles within the same line scan are analyzed. To assess the possibility that complex behavior is the result of other, non-biological factors, fluorescent beads were imaged using the same experimental procedures and analyses used to characterize Fluo-4 fluorescence. Figure 1C illustrates the time dependent signal measured using a fluorescent bead (representative of 50 such analyses of separate beads). Only monotonic decreases in bead fluorescence were ever observed. This

simple bead fluorescence behavior also supports the hypothesis that complex signal behavior is not due to scanning laser intensity variations and/or gross movement. If the complex Fluo-4 fluorescence behavior is induced by the laser illumination, then the effect should be larger at increased laser power. There was no significant correlation between the transient increases in Fluo-4 intensity as a function of laser power over the range $\sim 46 - 55 \mu\text{W}$ at the sample (correlation coefficient 0.07); these power levels are representative of normal instrument operation. The detection of complex vesicle fluorescence behavior is consistent with the existence of a temporally active, intra-vesicular process capable of altering the fluorescence of the Ca^{2+} indicator, Fluo-4.

If each vesicle is an independent, asynchronous originator of complex fluorescence behavior that originates from a common process or pathway, then each repetitive line scan series begins at a different temporal start or phase of the process and ends with the loss of signal due to indicator photo-degradation/bleaching as a consequence of continuous observation. Line scan series were segmented and the time to peak signal for the segmented means determined. The time to peak signal frequency distribution is exponential with a decay time of $\tau \sim 4.40 \pm 0.35 \text{ sec}$ (value $\pm$ std. err.). Exponential behavior is consistent with complex Fluo-4 fluorescence behavior occurring independently with the appearance of waves occurring at a constant rate, $1/\tau \sim 0.23 \text{ sec}^{-1}$. However, the probability of observing complex events decreases from the start of observation due to indicator photo-bleaching.

The observed complex Fluo-4 fluorescence behavior is consistent with a temporally active, Ca^{2+} -dependent, intra-vesicular process. Non-stationary statistical analysis was used to evaluate the temporal properties of the Fluo-4 fluorescence signal. Figure 2A and 2B illustrates the time dependent behavior of the Fano factor, $F_{\text{ADU}} = \text{Variance}(\text{Intensity}_{\text{ADU}}) / \langle \text{Intensity}_{\text{ADU}} \rangle$, using the Fluo-4 fluorescence intensities, in analog digital units (ADU), for the simple and complex fluorescence behavior shown in Figures 1A and 1B, respectively. For a purely Poisson random process, F_{ADU} is expected to equal the gain of the microscope system, 0.65 ± 0.17 (ADU mean $\pm$ std. dev). However, for both simple and complex vesicle fluorescence, F_{ADU} , averaged over time, is significantly greater than the gain of the system ($F_{\text{ADU}}^{\text{simple}} = 1.05 \pm 0.08$ and $F_{\text{ADU}}^{\text{complex}} = 1.62 \pm 0.08$; mean $\pm$ 99% confidence); both types of intra-vesicular fluorescence signals exhibit greater than expected variance. Figure 2C illustrates the time dependent behavior of F_{ADU} for a bead using the fluorescence intensity shown in Figure 1C. For this fluorescent bead, $F_{\text{ADU}} = 0.97 \pm 0.04$ (mean $\pm$ 99% confidence). The bead also exhibits greater than expected variance in the fluorescence signal consistent with the existence of additional, non-biological noise factors — but these factors clearly cannot explain all of the observed variance in intra-vesicular Fluo-4 fluorescence signals. The statistical characteristic of the simple and complex Fluo-4 fluorescence signals is consistent with the existence of additional noise processes beyond those observed with beads. Since F_{ADU} is greater than what is expected for a Poisson process these signals can be described as originating from super-Poisson distributions having a greater than expected variance; F_{ADU} is significantly greater than one.

Figures 1 and 2 illustrate the fluorescence behaviors of single vesicles and fluorescent beads as a function of time. Mean F_{ADU} values were determined for each vesicular Fluo-4 and fluorescent bead scan segment (Figure 3A). Mean F_{ADU} values calculated from both solution Fluo-4 fluorescence signals, used to calculate system gain and bead fluorescence signals were invariant with time; however, the vesicular mean F_{ADU} values calculated from Fluo-4 fluorescence signals was not, and asymptotically approached the value for beads with an exponential decay of $22.1 \pm 0.8 \text{ sec}$. The asymptotic behavior is consistent with the observed loss of Fluo-4 signal with continuous observation. To evaluate the relationship between excess variance and signal intensity, parametric plots of the mean segment intensity and mean F_{ADU} for intra-vesicular Fluo-4 signal and that of beads were compared using

scan time as the parametric variable (Figure 3B). The signal statistics of fluorescent beads are consistent with an additional, non-biological noise process that adds to the expected Poisson statistics. This noise process is both time invariant (Figure 3A) and intensity (signal amplitude) invariant (Figure 3B); this is a constant, additive, stationary noise process. However, the noise process associated with vesicular Fluo-4 fluorescence, above a lower intensity level, is linearly dependent upon the fluorescence intensity ($r^2 = 0.96$). At the lowest levels of intensity, the noise characteristics are consistent with those observed in beads. The dashed line in Figure 3B represents the most extreme 99% confidence value for all segmented bead scans and it is below this range that the excess noise process associated with vesicular Fluo-4 fluorescence is no longer dominant and the vesicle fluorescence begins to behave, statistically, as that of fluorescent beads.

Increased variance can originate from Fluo-4 fluorescence behavior inside secretory vesicles and it can originate from other, non-biological factors. For example, excitation light variation, temperature fluctuations, motion, and signal electronics, are additional processes that can contribute to creating a super-Poisson intensity distribution [43]. To further evaluate biologically correlated and non-biological noise processes as the origin of the complex behavior, isolated planar cortices were labeled with either the pH indicator cSNAFL-2 or the membrane labeling lipophilic indicator, FM 4-64; cSNAFL-2 labels the interior of the vesicle, analogous to Fluo-4, while FM 4-64 is localized in the external vesicle membrane and the adherent plasma membrane. If the complex behavior observed with Fluo-4 is due to complex, dynamic changes in the internal pH of the vesicle, then cSNAFL-2 should also show complex fluorescence signal behavior. Only monotonic decreases in cSNAFL-2 fluorescence were observed in 93 vesicles from 13 different preparations. Thus, cSNAFL-2 fluorescence is not consistent with complex Fluo-4 fluorescence behavior arising from pH dependent changes in the fluorescence properties of the Ca^{2+} indicator because complex cSNAFL-2 vesicle behavior was never observed. Vesicles were impermeable to the lipophilic indicator FM 4-64 and appeared as ring-like structures. Monotonic decreases in fluorescence were observed in 98% of FM 4-64 labeled pixels (323 edge pixels representing 109 vesicles from 2 preparations); complex FM 4-64 behavior was not observed. The observed complex vesicular Fluo-4 fluorescence behavior is therefore inconsistent with either complex pH correlated changes in Fluo-4 fluorescence or preparation movement (as both cSNAFL-2 and FM 4-64 labeled vesicles exhibit simple fluorescence behavior when imaged identically as Fluo-4 labeled vesicles).

Mean F_{ADU} values were determined for each scan segment for cSNAFL-2 and FM 4-64 labeled vesicles (Figure 4A). Two classes of FM 4-64 fluorescence signals were identified; both classes are invariant with time but have different mean F_{ADU} values (0.57 ± 0.17 and 1.11 ± 0.45 mean $\pm$ std. dev.). FM 4-64 labels both vesicles and cortex (i.e. plasma) membrane; it was difficult to ensure that the pixels imaged represented only vesicles and not contributions from other membranes. The lower mean F_{ADU} value is comparable to the Fluo-4 solution value while the higher mean F_{ADU} value is close to that observed in fluorescence beads. These data are consistent with the collection of both vesicle and cortex membrane derived FM 4-64 signals and supports the hypothesis that a non-biological, additive noise process exists when imaging either ~ 1 or $6 \mu\text{m}$ sized objects in this confocal microscope system. However, the vesicular cSNAFL-2 signal is not invariant with time and monotonically decreased with an exponential decay of 32.9 ± 0.6 sec. To evaluate the relationship between excess variance and signal intensity, parametric plots of the mean segment intensity and mean F_{ADU} for cSNAFL-2 labeled vesicles and the solution-like FM 4-64 class were compared using scan time as the parametric variable (Figure 4B). The signal statistics of these FM 4-64 preparations are consistent with Poisson statistics. This noise process is both time invariant (Figure 4A) and intensity (signal amplitude) invariant (Figure 4B) with a value not significantly different from the instrument gain. However, the noise

process associated with vesicular cSNAFL-2 fluorescence, above a lower intensity level (7 ADU), is linearly dependent upon the fluorescence intensity ($r^2 = 0.73$). Below 7ADU of intensity, the noise characteristics are consistent with those observed in beads; in this range the excess noise process associated with vesicular cSNAFL-2 fluorescence is no longer dominant and the vesicles behave, statistically, as fluorescent beads.

The F_{ADU} values for fluorescent beads and, cSNAFL-2, and Fluo-4 labeled vesicles were 0.98 ± 0.01 , 1.00 ± 0.00 , and 1.33 ± 0.01 (mean $\pm$ 99% confidence). If the threshold for super-Poisson behavior is defined as the $\langle \text{Gain} \rangle + 3\sigma$ ($F_{ADU} = 1.13$) then the fraction of super-Poisson distributed time segments for beads and, cSNAFL-2, and Fluo-4 labeled vesicles, is 9%, 33%, and 55%. The intensity fluctuations measured with beads do contain a small fraction of super-Poisson data segments and the cumulative distribution function is shifted to higher F_{ADU} values relative to Ca^{2+} solutions and FM 4-64. Both Fluo-4 and cSNAFL-2 labeled vesicles exhibit super-Poisson behavior; however the fraction of cSNAFL-2 data is lower (33% vs. 55%).

If Fluo-4 Ca^{2+} binding reactions, driven by complex Ca^{2+} fluctuations, are the origin of super-Poisson fluorescence intensity statistics and complex fluorescence behavior then lower affinity indicators may be less sensitive to intra-vesicular fluctuations in the Ca^{2+} bound fluorophore concentration because the bound fraction is dependent upon affinity and the Ca^{2+} bound fluorophore is the dominant contributor to the observed fluorescence. Both simple and complex fluorescence behaviors are observed in the time dependent fluorescence intensity measured from sea urchin egg secretory vesicles labeled with the Ca^{2+} indicator Fluo-4 FF during repetitive line scan confocal microscopy. However, only 15% (6 of 39 vesicles from 3 preparations) exhibited complex behavior while the remaining 85% decayed monotonically. Three preparations were labeled with Fluo-5N but only 6 vesicles provided sufficient fluorescence signal for evaluation; all exhibited simple behavior. The K_d , in solution, for Fluo-4, Fluo-4 FF, and Fluo-5N are, 0.54, 9.7, and 90 μM respectively. The fraction of complex fluorescence behavior observed depends upon the K_d of the Ca^{2+} indicator used to label vesicles; complex fluorescence behavior is estimated to occur in 50% of the vesicle population for $K_d \sim 5 \mu\text{M}$. The acidic environment of a vesicle (pH ~ 5.5 [44]) is expected to shift the intra-vesicular K_d of BAPTA-derived Ca^{2+} indicators to ~ 10 fold lower affinity values [45–50]; this is consistent with the detection of μM Ca^{2+} dynamics using Fluo-4 and Fluo-4 FF and maximum dynamics of $\sim 100 \mu\text{M}$ Ca^{2+} (a 10-fold lower limit for the pH adjusted K_d of Fluo-5N). Furthermore, the fraction of super-Poisson distributed Fluo-4 FF time segments is 37% compared to 55% for Fluo-4. Mean F_{ADU} values were determined for each scan segment for Fluo-4 FF labeled vesicles (Figure 4A). The vesicular Fluo-4 FF signal is not invariant with time and monotonically decreased with an exponential decay of 27.6 ± 2.5 sec. The noise process associated with vesicular Fluo-4 FF fluorescence, above a lower intensity level (7 ADU), is linearly dependent upon the fluorescence intensity ($r^2 = 0.79$). Below 7ADU of intensity, the noise characteristics are consistent with those observed in beads; in this range the excess noise process associated with vesicular Fluo-4 FF fluorescence is no longer dominant and the vesicles behave, statistically, as fluorescent beads (Figure 4B). That complex fluorescence behavior and the fraction of super-Poisson distributed time segments are dependent upon the K_d of the Ca^{2+} indicator supports the hypothesis that fluctuation in the intra-vesicular free Ca^{2+} concentration represents an intrinsic biological process that increases the variance of the measured fluorescence signal.

If Ca^{2+} fluctuations are the origin of super-Poisson fluorescence intensity statistics then vesicle Ca^{2+} channels may be involved; this was tested using vesicles treated with ω -agatoxin ($0.2 \mu\text{M}$), a blocker of p-type Ca^{2+} channels, known to be present [19]. Line scan fluorescence intensity measured in untreated and toxin treated vesicles ($N = 96$ and 34) were

pixel aligned and averaged using the maximum intensity as the origin. The first 1000 scans were used to evaluate the signal statistics in order to minimize the contribution of low intensity Poisson behaviour. Average fluorescence intensity scans were comparable for both preparations; the profile for untreated vesicles is shown in Figure 5A. However, the average noise characteristics for these two preparations are different (Figure 5B). Untreated vesicles have larger F_{ADU} values with greater individual pixel variance; the F_{ADU} values are lowest at the center of the vesicle and increase towards the edge. In contrast, ω -agatoxin treated vesicles have lower F_{ADU} values, lower individual pixel variance, and have maximal values in the interior of the vesicle. Blocking the p-type Ca^{2+} channel alters the noise characteristics of vesicular Fluo-4 fluorescence. This result further supports the hypothesis that a biological process involving Ca^{2+} channel dependent fluctuations in intra-vesicular free Ca^{2+} concentration is responsible for both complex Fluo-4 fluorescence behavior and super-Poisson fluorescence intensity statistics.

Discussion

Here we identify damped, oscillatory behavior of intra-vesicular free Ca^{2+} concentrations, evaluated using fluorescence indicators, and show that the noise characteristic of this signal was super-Poisson (i.e. inconsistent with an average, equilibrium, background signal). Furthermore, the level of super-Poisson behavior was dependent upon both the magnitude of the fluorescence signal and the activity of p-type Ca^{2+} channels. The demonstration that changes in the vesicular Ca^{2+} concentration can have significant effects on secretion [9, 11, 12, 14, 24–27] and that vesicles contain the necessary machinery to alter intra-vesicular Ca^{2+} [15, 18–22] is consistent with the hypothesis that free intra-vesicular Ca^{2+} can regulate or modify vesicle secretion. The goal of this study was to determine if sea urchin egg secretory vesicles possess dynamic Ca^{2+} behavior, evaluate the stochastic properties associated with this dynamic behavior, and begin to assess their role as a potential intracellular Ca^{2+} modulatory site. The results of these studies suggest an evolutionarily conserved vesicular Ca^{2+} handling mechanism that, along with those of the endoplasmic reticulum and mitochondria, has a role in Ca^{2+} homeostasis and signaling.

Controls for Contributing Noise Sources

An increase in variance in excess of that predicted by a Poisson process can originate from both biochemical and instrumental/non-biological processes. Care must be taken in evaluating the origin of contributing noise sources; NADH fluorescence in muscle cells, although having super-Poisson properties was shown to be no different from the contributing instrumental noise [43]. However, the noise properties of Ca^{2+} signals derived from nerve terminals and mitochondria have an excess noise component attributed to biological processes analogous to those present in vesicles [51, 52]. One statistical tool that is used to evaluate the noise properties of stochastic signals is the Fano factor (variance/mean). In Fluo-4 labeled vesicles, both the magnitude and intensity dependence of the Fano factor are consistent with a super-Poisson process that increases linearly with the Ca^{2+} concentration. This contrasts with the behavior of both fluorescent beads and Fluo-4 in solution. While solution Fluo-4 is consistent with the steady-state Poisson statistics of chemical binding reactions, fluorescent beads and low signal vesicles (Fluo-4 and cSNAFL-2) had a greater than expected Fano value compared to the instrumental properties of the microscope system. We hypothesize that this is a time and amplitude independent additive noise process, perhaps due to vesicle jitter. Vesicle jitter has been shown to be a feature of Ca^{2+} -triggered exocytosis [53, 54]. However, if vesicle motion were the origin of all complex signal behavior, then all fluorescence indicators should behave similarly; this was not observed. At higher Fluo-4 and cSNAFL-2 signals, the noise is dominated by Ca^{2+} and pH dynamics.

Laser induced perturbations in the concentration of reactive oxygen species (ROS) within intact cells have been implicated in the induction of intracellular cytosolic Ca^{2+} transients [55]. However, the planar cortex is an isolated membrane preparation lacking the cytosolic components implicated in ROS intracellular signaling pathways. Furthermore, the local fluctuations occur on a time scale 1 – 2 orders of magnitude shorter than the oscillatory Ca^{2+} transients observed in vivo [55]. Another possibility is that the ROS signaling pathway is present within the vesicle and drives the local fluctuations. In such a case, it would be expected that it will increase with the laser power, which was not observed. That the fluorescence signal within the intra-vesicular space decays with continuous scanning may be consistent with free radical formation; a pathway for photo-degradation of Ca^{2+} indicators has been proposed [56]. However, the presence of ROS is associated with both a decrease in the dynamic range and affinity of Ca^{2+} indicators [56, 57]. We observed increases in the fluorescence signal in the presence of a decaying process suggesting that our measurements may be underestimating the actual magnitude of the intra-vesicular Ca^{2+} dynamics. Hence, we conclude that the fluorescence signal observed from secretory vesicles is coupled to changes in the free Ca^{2+} concentrations.

Vesicular Ca^{2+} Dynamics

The characteristic time of the damped, oscillatory Ca^{2+} signal was $\sim 4.40 \pm 0.35$ sec. This value is the same order of magnitude as Ca^{2+} oscillations observed in inositol 1,4,5-triphosphate (InsP_3) triggered mast cell granules (8 – 10 sec; [8]) and airway goblet cells (10 – 15 sec; [10]). Vesicular Ca^{2+} oscillations are hypothesized to be the result of both a $\text{Ca}^{2+}/\text{K}^+$ exchange process in which matrix bound Ca^{2+} is displaced by potassium, and an InsP_3 -receptor- Ca^{2+} channel dependent release process [8, 10]. The coupling of vesicular Ca^{2+} dependent biochemical processes to the Ca^{2+} readout (i.e. fluorescence, via indicator- Ca^{2+} binding reactions), occur under conditions in which the noise properties of the Ca^{2+} signal may be greater than expected compared to steady-state or equilibrium concentration fluctuations.

Coupled or cascading biochemical processes can support unique properties including threshold or switch-like activity [58, 59]. Depending upon the coupling, the output of these processes could be sensitive to biochemical noise arising from the finite numbers of reactants participating as discrete entities [60]. The role of stochastic fluctuations in regulated biochemical networks is an active area of research [61].

Although we do not know the absolute concentrations of free Ca^{2+} within the vesicles, we estimate that the free Ca^{2+} concentration transients are in the range of 1 – 10 μM judging from the behavior of the Ca^{2+} indicators with different K_d values. While total Ca^{2+} content of the secretory vesicle has been estimated to be ~ 100 mM [20] the free Ca^{2+} concentration from our estimation is definitely lower than 100 μM and taking into account the pH dependence of the Ca^{2+} indicators [45–50] and the ability of Fluo-4 to follow the oscillatory behavior, suggests values typically less than 10 μM . Hence, the ratio between the total and the dynamic Ca^{2+} in the secretory vesicles is at least 3 orders of magnitude. This estimation is consistent with the results of Mitchell et al.(2001) from dense core secretory vesicles of pancreatic islet β -cells, that appear to have the highest Ca^{2+} -buffering capacity (0.1% free/bound) of all sub-cellular organelles so far examined [15].

In order for the fluorescence signal, a “reporter” of the free Ca^{2+} concentration, to have non-Poissonian behavior, the following conditions must exist: 1) the local, free Ca^{2+} concentration can not be at a steady-state with respect to indicator binding and 2) the availability of free Ca^{2+} to bind to the indicator must be coupled to additional biochemical processes such as the conversion of matrix-bound Ca^{2+} to free Ca^{2+} in bursts [58, 62]. When p-type Ca^{2+} channels are blocked with ω -agatoxin the vesicle noise properties change; this

is consistent with a coupled or cascading series of biochemical steps that includes Ca^{2+} release from the vesicle matrix, indicator binding, and Ca^{2+} loss through vesicle membrane channel activity. Super-Poisson Ca^{2+} noise may be a characteristic feature driving oscillatory behavior. In these experiments the Ca^{2+} concentration surrounding the vesicle was kept low to avoid triggering exocytosis; sustained oscillatory behavior would not be expected if the vesicle refilling or triggering pathways were not maintained. The presence of super-Poisson statistics in the decaying signal suggests that photo-bleaching is not the only process that leads to signal loss. Labeling vesicles with the anionic form of the indicators was dependent on the presence of Mg-ATP in the bathing medium, consistent with 1) indicator entry facilitated by an ATP dependent transporter or 2) anionic indicators are negatively charged molecule whose transport into the vesicle requires the presence of an ATP dependent electrical potential. For such movement to occur, it is necessary that the lumen of the vesicle be positively charged as shown for a number of secretory vesicles [63, 64], and that there is an anion permeating channel in the vesicle membrane. Such channels have been described for a number of secretory vesicles [65–67]. The presence of pathways capable of altering the Ca^{2+} -matrix and Ca^{2+} transport properties supports coupled biochemistry as a factor in the observed super-Poisson behavior.

The changes in intra-vesicular Ca^{2+} concentration may be due to efflux of Ca^{2+} ions or to transients in the buffering capacity, following changes in the concentration of other intra-vesicular components. The dynamics of the Ca^{2+} in the secretory vesicles could affect Ca^{2+} dynamics in the cytosol through the activity of known ion channels in secretory vesicles [18, 19, 68–71]. Factors that open Ca^{2+} permeable channels in secretory vesicles may release Ca^{2+} from the secretory vesicles into the cytosol, affect the Ca^{2+} concentration immediately around the secretory vesicles, and modulate the fusion process and vesicle content release. If this mechanism were expressed in synaptic vesicles, then transmitter release would be affected, particularly because the synaptic vesicle is the predominant membrane in the active zone. Studies have revealed that a major intracellular system for handling calcium, the endoplasmic reticulum, possesses proteins whose mutation causes disease, presumably due to defective calcium regulation and/or signaling [72]. Since secretory vesicles are derived from the endoplasmic reticulum and Golgi, it remains to be seen whether the activities demonstrated in this paper emerged as a result of optimizing the regulation of secretion or are vestigial elements of the secretory pathway that developed in these organelles.

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References

1. Allbritton NL, Meyer T. Localized calcium spikes and propagating calcium waves. *Cell Calcium*. 1993; 14:691–697. [PubMed: 8131187]
2. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol*. 2000; 1:11–21. [PubMed: 11413485]
3. Bootman MD, Collins TJ, Peppiatt CM, Prothero LS, MacKenzie L, De Smet P, Travers M, Tovey SC, Seo JT, Berridge MJ, Ciccolini F, Lipp P. Calcium signalling--an overview. *Semin Cell Dev Biol*. 2001; 12:3–10. [PubMed: 11162741]
4. Petersen OH, Michalak M, Verkhratsky A. Calcium signalling: Past, present and future. *Cell Calcium*. 2005; 38:161–169. [PubMed: 16076488]

5. Petersen OH, Tepikin AV. Polarized calcium signaling in exocrine gland cells. *Annu Rev Physiol.* 2008; 70:273–299. [PubMed: 17850212]
6. Parekh AB, Penner R. Store depletion and calcium influx. *Physiol Rev.* 1997; 77:901–930. [PubMed: 9354808]
7. Budd SL, Nicholls DG. A reevaluation of the role of mitochondria in neuronal Ca^{2+} homeostasis. *J Neurochem.* 1996; 66:403–411. [PubMed: 8522981]
8. Quesada I, Chin WC, Steed J, Campos-Bedolla P, Verdugo P. Mouse mast cell secretory granules can function as intracellular ionic oscillators. *Biophys J.* 2001; 80:2133–2139. [PubMed: 11325716]
9. Quesada I, Chin WC, Verdugo P. ATP-independent luminal oscillations and release of Ca^{2+} and H^{+} from mast cell secretory granules: Implications for signal transduction. *Biophys J.* 2003; 85:963–970. [PubMed: 12885643]
10. Nguyen T, Chin WC, Verdugo P. Role of $\text{Ca}^{2+}/\text{K}^{+}$ ion exchange in intracellular storage and release of Ca^{2+} Nature. 1998; 395:908–912. [PubMed: 9804425]
11. Moreno A, Lobaton CD, SantoDomingo J, Vay L, Hernandez-SanMiguel E, Rizzuto R, Montero M, Alvarez J. Calcium dynamics in catecholamine-containing secretory vesicles. *Cell Calcium.* 2005; 37:555–564. [PubMed: 15862346]
12. Mahapatra NR, Mahata M, Hazra PP, McDonough PM, O'Connor DT, Mahata SK. A dynamic pool of calcium in catecholamine storage vesicles - Exploration in living cells by a novel vesicle-targeted chromogranin A-aequorin chimeric photoprotein. *Journal of Biological Chemistry.* 2004; 279:51107–51121. [PubMed: 15358782]
13. Davis LC, Morgan AJ, Ruas M, Wong JL, Graeff RM, Poustka AJ, Lee HC, Wessel GM, Parrington J, Galione A. Ca^{2+} Signaling Occurs via Second Messenger Release from Intraorganelle Synthesis Sites. *Current Biology.* 2008; 18:1612–1618. [PubMed: 18951023]
14. SantoDomingo J, Vay L, Camacho M, Hernandez-SanMiguel E, Fonteriz RI, Lobaton CD, Montero M, Moreno A, Alvarez J. Calcium dynamics in bovine adrenal medulla chromaffin cell secretory granules. *European Journal of Neuroscience.* 2008; 28:1265–1274. [PubMed: 18973554]
15. Mitchell KJ, Pinton P, Varadi A, Tacchetti C, Ainscow EK, Pozzan T, Rizzuto R, Rutter GA. Dense core secretory vesicles revealed as a dynamic Ca^{2+} store in neuroendocrine cells with a vesicle-associated membrane protein aequorin chimera. *Journal of Cell Biology.* 2001; 155:41–51. [PubMed: 11571310]
16. Mitchell KJ, Lai FA, Rutter GA. Ryanodine receptor type I and nicotinic acid adenine dinucleotide phosphate receptors mediate Ca^{2+} release from insulin-containing vesicles in living pancreatic beta-cells (MIN6). *J Biol Chem.* 2003; 278:11057–11064. [PubMed: 12538591]
17. Schmidt R, Zimmermann H, Whittaker VP. Metal ion content of cholinergic synaptic vesicles isolated from the electric organ of *Torpedo*: effect of stimulation-induced transmitter release. *Neuroscience.* 1980; 5:625–638. [PubMed: 7374962]
18. Vogel SS, Smith RM, Baibakov B, Ikebuchi Y, Lambert NA. Calcium influx is required for endocytotic membrane retrieval. *Proc Natl Acad Sci U S A.* 1999; 96:5019–5024. [PubMed: 10220411]
19. Smith RM, Baibakov B, Ikebuchi Y, White BH, Lambert NA, Kaczmarek LK, Vogel SS. Exocytotic insertion of calcium channels constrains compensatory endocytosis to sites of exocytosis. *J Cell Biol.* 2000; 148:755–767. [PubMed: 10684256]
20. Gillot I, Ciapa B, Payan P, Sardet C. The calcium content of cortical granules and the loss of calcium from sea urchin eggs at fertilization. *Dev Biol.* 1991; 146:396–405. [PubMed: 1864463]
21. Oberdorf JA, Head JF, Kaminer B. Calcium uptake and release by isolated cortices and microsomes from the unfertilized egg of the sea urchin *Strongylocentrotus droebachiensis*. *J Cell Biol.* 1986; 102:2205–2210. [PubMed: 2423535]
22. Payan P, Girard JP, Sardet C, Whitaker M, Zimmerberg J. Uptake and release of calcium by isolated egg cortices of the sea urchin *Paracentrotus lividus*. *Biol Cell.* 1986; 58:87–90. [PubMed: 2952202]
23. Schneggenburger R, Neher E. Presynaptic calcium and control of vesicle fusion. *Curr Opin Neurobiol.* 2005; 15:266–274. [PubMed: 15919191]

24. Fossier P, Diebler MF, Mothet JP, Israel M, Tauc L, Baux G. Control of the calcium concentration involved in acetylcholine release and its facilitation: An additional role for synaptic vesicles? *Neuroscience*. 1998; 85:85–91. [PubMed: 9607705]
25. Haynes CL, Buhler LA, Wightman RM. Vesicular Ca²⁺-induced secretion promoted by intracellular pH-gradient disruption. *Biophysical Chemistry*. 2006; 123:20–24. [PubMed: 16678962]
26. Mundorf ML, Troyer KP, Hochstetler SE, Near JA, Wightman RM. Vesicular Ca²⁺ participates in the catalysis of exocytosis. *Journal of Biological Chemistry*. 2000; 275:9136–9142. [PubMed: 10734047]
27. Scheenen WJ, Wollheim CB, Pozzan T, Fasolato C. Ca²⁺ depletion from granules inhibits exocytosis. A study with insulin-secreting cells. *J Biol Chem*. 1998; 273:19002–19008. [PubMed: 9668080]
28. Malgaroli A, Fesce R, Meldolesi J. Spontaneous [Ca-2+]I Fluctuations in Rat Chromaffin Cells Do Not Require Inositol 1,4,5-Trisphosphate Elevations but Are Generated by a Caffeine-Sensitive and Ryanodine-Sensitive Intracellular Ca-2+ Store. *Journal of Biological Chemistry*. 1990; 265:3005–3008. [PubMed: 2105938]
29. Quesada I, Verdugo P. InsP3 signaling induces pulse-modulated Ca²⁺ signals in the nucleus of airway epithelial ciliated cells. *Biophys J*. 2005; 88:3946–3953. [PubMed: 15792976]
30. Falcke M. Introduction to Focus Issue: Intracellular Ca²⁺ Dynamics- A Change of Modeling Paradigm? *Chaos*. 2009; 19
31. Skupin A, Falcke M. Statistical properties and information content of calcium oscillations. *Genome Informatics*. 2007; 18:44–53. [PubMed: 18546473]
32. Skupin A, Falcke M. From puffs to global Ca²⁺ signals: How molecular properties shape global signals. *Chaos*. 2009; 19:17.
33. Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: Dynamics, homeostasis and remodelling. *Nature Reviews Molecular Cell Biology*. 2003; 4:517–529.
34. Bootman MD, Lipp P, Berridge MJ. The organisation and functions of local Ca²⁺ signals. *J Cell Sci*. 2001; 114:2213–2222. [PubMed: 11493661]
35. Zimmerberg J, Coorssen JR, Vogel SS, Blank PS. Sea urchin egg preparations as systems for the study of calcium-triggered exocytosis. *J Physiol*. 1999; 520(Pt 1):15–21. [PubMed: 10517796]
36. Blank PS, Cho MS, Vogel SS, Kaplan D, Kang A, Malley J, Zimmerberg J. Submaximal responses in calcium-triggered exocytosis are explained by differences in the calcium sensitivity of individual secretory vesicles. *J Gen Physiol*. 1998; 112:559–567. [PubMed: 9806965]
37. Vacquier VD. The isolation of intact cortical granules from sea urchin eggs: calcium ions trigger granule discharge. *Dev Biol*. 1975; 43:62–74. [PubMed: 1171035]
38. Lidke KA, Rieger B, Lidke DS, Jovin TM. The role of photon statistics in fluorescence anisotropy imaging. *IEEE Trans Image Process*. 2005; 14:1237–1245. [PubMed: 16190460]
39. Kobayashi M, Devaraj B, Inaba H. Observation of super-Poisson statistics of bacterial (Photobacterium phosphoreum) bioluminescence during the early stage of cell proliferation. *Physical Review E*. 1998; 57:2129–2133.
40. Kobayashi M, Inaba H. Photon statistics and correlation analysis of ultraweak light originating from living organisms for extraction of biological information. *Appl Opt*. 2000; 39:183–192. [PubMed: 18337887]
41. Thattai M, van Oudenaarden A. Intrinsic noise in gene regulatory networks. *Proc Natl Acad Sci U S A*. 2001; 98:8614–8619. [PubMed: 11438714]
42. Sardet C. The Ultrastructure of the Sea-Urchin Egg Cortex Isolated before and after Fertilization. *Developmental Biology*. 1984; 105:196–210. [PubMed: 6468759]
43. Blinova K, Combs C, Kellman P, Balaban RS. Fluctuation analysis of mitochondrial NADH fluorescence signals in confocal and two-photon microscopy images of living cardiac myocytes. *Journal of Microscopy-Oxford*. 2004; 213:70–75.
44. Demaurex N. pH Homeostasis of cellular organelles. *News Physiol Sci*. 2002; 17:1–5. [PubMed: 11821527]
45. Christensen KA, Myers JT, Swanson JA. pH-dependent regulation of lysosomal calcium in macrophages. *J Cell Sci*. 2002; 115:599–607. [PubMed: 11861766]

46. Eberhard M, Erne P. Kinetics of calcium binding to fluo-3 determined by stopped-flow fluorescence. *Biochem Biophys Res Commun.* 1989; 163:309–314. [PubMed: 2775268]
47. Lattanzio FA Jr. The effects of pH and temperature on fluorescent calcium indicators as determined with Chelex-100 and EDTA buffer systems. *Biochem Biophys Res Commun.* 1990; 171:102–108. [PubMed: 2118341]
48. Lattanzio FA Jr, Bartschat DK. The effect of pH on rate constants, ion selectivity and thermodynamic properties of fluorescent calcium and magnesium indicators. *Biochem Biophys Res Commun.* 1991; 177:184–191. [PubMed: 2043105]
49. Lanzer M, Rohrbach P. Subcellular pH and Ca²⁺ in *Plasmodium falciparum*: Implications for understanding drug resistance mechanisms. *Current Science.* 2007; 92:1561–1570.
50. Minta A, Kao JP, Tsien RY. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J Biol Chem.* 1989; 264:8171–8178. [PubMed: 2498308]
51. Melamed-Book N, Kachalsky SG, Kaiserman I, Rahamimoff R. Neuronal calcium sparks and intracellular calcium “noise”. *Proc Natl Acad Sci U S A.* 1999; 96:15217–15221. [PubMed: 10611365]
52. Melamed N, Helm PJ, Rahamimoff R. Confocal microscopy reveals coordinated calcium fluctuations and oscillations in synaptic boutons. *J Neurosci.* 1993; 13:632–649. [PubMed: 8426230]
53. Machado JD, Camacho M, Alvarez J, Borges R. On the role of intravesicular calcium in the motion and exocytosis of secretory organelles. *Commun Integr Biol.* 2009; 2:71–73. [PubMed: 19704891]
54. Degtyar VE, Allersma MW, Axelrod D, Holz RW. Increased motion and travel, rather than stable docking, characterize the last moments before secretory granule fusion. *Proc Natl Acad Sci U S A.* 2007; 104:15929–15934. [PubMed: 17893335]
55. Knight MM, Roberts SR, Lee DA, Bader DL. Live cell imaging using confocal microscopy induces intracellular calcium transients and cell death. *Am J Physiol-Cell Physiol.* 2003; 284:C1083–C1089. [PubMed: 12661552]
56. Scheenen W, Makings LR, Gross LR, Pozzan T, Tsien RY. Photodegradation of indo-1 and its effect on apparent Ca²⁺ concentrations. *Chem Biol.* 1996; 3:765–774. [PubMed: 8939693]
57. Sarvazyan N, Swift L, Martinez-Zaguilan R. Effects of oxidants on properties of fluorescent calcium indicators. *Arch Biochem Biophys.* 1998; 350:132–136. [PubMed: 9466830]
58. Thattai M, van Oudenaarden A. Attenuation of noise in ultrasensitive signaling cascades. *Biophys J.* 2002; 82:2943–2950. [PubMed: 12023217]
59. Ferrell JE Jr. Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. *Trends Biochem Sci.* 1996; 21:460–466. [PubMed: 9009826]
60. Ozbudak EM, Thattai M, Kurtser I, Grossman AD, van Oudenaarden A. Regulation of noise in the expression of a single gene. *Nature Genet.* 2002; 31:69–73. [PubMed: 11967532]
61. Ziv E, Nemenman I, Wiggins CH. Optimal Signal Processing in Small Stochastic Biochemical Networks. *PLoS One.* 2007; 2:16.
62. Thattai M, van Oudenaarden A. Intrinsic noise in gene regulatory networks. *Proc Natl Acad Sci U S A.* 2001; 98:8614–8619. [PubMed: 11438714]
63. Kelly RB. Storage and release of neurotransmitters. *Cell.* 1993; 72(Suppl):43–53. [PubMed: 8094036]
64. Tabb JS, Kish PE, Van Dyke R, Ueda T. Glutamate transport into synaptic vesicles. Roles of membrane potential, pH gradient, and intravesicular pH. *J Biol Chem.* 1992; 267:15412–15418. [PubMed: 1353494]
65. Meir A, Ginsburg S, Butkevich A, Kachalsky SG, Kaiserman I, Ahdut R, Demigoren S, Rahamimoff R. Ion channels in presynaptic nerve terminals and control of transmitter release. *Physiol Rev.* 1999; 79:1019–1088. [PubMed: 10390521]
66. Stanley EF, Ehrenstein G, Russell JT. Evidence for anion channels in secretory vesicles. *Neuroscience.* 1988; 25:1035–1039. [PubMed: 2457183]
67. Yakir N, Rahamimoff R. The non-specific ion channel in *Torpedo ocellata* fused synaptic vesicles. *J Physiol.* 1995; 485(Pt 3):683–697. [PubMed: 7562610]

68. Lii T, Lee HC, Gleason FK, Levitt DG. Characterization of a large conductance, cation-selective channel from sea urchin eggs that is sensitive to sulfhydryl reducing agents. *J Membr Biol.* 1996; 150:27–35. [PubMed: 8699477]
69. Yakir N, Rahamimoff R. The non-specific ion channel in Torpedo ocellata fused synaptic vesicles. *J Physiol.* 1995; 485:683–697. [PubMed: 7562610]
70. Meir A, Butkevich A, Demigoren S, Yakir N, Rahamimoff R. Ion channels in transmitter release. *Cell Biology.* 1997; 100:11–37.
71. Rahamimoff R, DeRiemer SA, Sakmann B, Stadler H, Yakir N. Ion channels in synaptic vesicles from Torpedo electric organ. *Proc Natl Acad Sci U S A.* 1988; 85:5310–5314. [PubMed: 2455900]
72. Sammels E, Parys JB, Missiaen L, De Smedt H, Bultynck G. Intracellular Ca^{2+} storage in health and disease: A dynamic equilibrium. *Cell Calcium.* 2010; 47:297–314. [PubMed: 20189643]

Figure 1.

Fluo-4 labeled vesicles exhibit varied time dependent fluorescence intensity profiles. Fluo-4 labeled cortex preparation (A). Fluo-4 fluorescence line scan signals collected from secretory vesicles showing either simple (B) or complex (C) behavior; representative of a total of 147 and 476 scans, of 189 vesicles, respectively. D is a fluorescence line scan signal collected from a fluorescent bead; representative of 50 beads analyzed. Line scans were acquired at 100 Hz; the total observation time was ~ 50 sec.

Figure 2.

The noise behavior of Fluo-4 labeled vesicles is super-Poissonian. Time dependent behavior of the Fano factor, $F_{ADU} = \text{Variance}/\text{Mean}$ for the typical time dependent fluorescence intensity profiles shown in Figure 1; vesicular Fluo-4 signal corresponding to simple (A) and complex (B) fluorescence behavior, while (C) corresponds to a fluorescent bead. The solid red line represents the microscope system gain and corresponds to the F_{ADU} value expected for a purely Poisson process.

Figure 3.

The average noise behavior of Fluo-4 labeled vesicles is super-Poissonian. Mean F_{ADU} values were calculated for every vesicular Fluo-4 and fluorescent bead scan segment and plotted as either a function of time (A) or mean intensity (B) using time as the parametric variable. F_{ADU} of intra-vesicular Fluo-4 exponentially decays with time, ($F_{ADU} = A \cdot \exp(-t/\tau) + B$; $A = 1.02 \pm 0.01$, $B = 0.76 \pm 0.01$, $\tau = 22.1 \pm 0.8$ sec, mean $\pm$ std. err.) to the values observed in beads, which were invariant with time ($F_{ADU} = A + B \cdot t$; $A = 0.98 \pm 0.01$, $B = -2.7 \pm 1.9 \times 10^{-6}$ (not significantly different from zero slope, $p = 0.16$). F_{ADU} of intra-vesicular Fluo-4 increased linearly with mean intensity ($F_{ADU} = A + B \cdot \text{Intensity}_{\text{Mean}}$; $A = 1.22 \pm 0.01$, $B = 8.6 \pm 0.2 \times 10^{-3}$, mean $\pm$ std. err.) while that of beads was invariant with mean intensity ($F_{ADU} = A + B \cdot \text{Intensity}_{\text{Mean}}$; $A = 0.95 \pm 0.02$, $B = 2.9 \pm 2.2 \times 10^{-4}$, mean $\pm$ std. err. (not significantly different from zero slope, $p =$

0.19). The slopes describing the F_{ADU} dependence on mean intensity for beads and intravesicular Fluo-4 was significantly different ($p < 0.001$). The solid red line represents the microscope gain while the dashed red line represents the 99% confidence value for the largest bead F_{ADU} value.

Figure 4.

The noise characteristics of Fluo-4FF, cSNAFL-2, and FM4-64 labeled vesicles differ from Fluo-4 labeled vesicles. Both Fluo-4 FF and cSNAFL-2 vesicles F_{ADU} exponentially decayed with time ($F_{ADU} = A \cdot \exp(-t/\tau) + B$; $A = 0.59 \pm 0.02$ and 0.68 ± 0.01 , $B = 0.78 \pm 0.02$ and 0.65 , $\tau = 27.5 \pm 2.5$ and 32.9 ± 0.6 mean $\pm$ std. err. respectively (A) and, above ~ 10 ADU intensity level, were linearly correlated with mean segment intensity ($F_{ADU} = A + B \cdot \text{Intensity}_{\text{Mean}}$; $A = 1.15 \pm 0.01$ and 1.17 ± 0.01 , $B = 3.8 \pm 0.4 \times 10^{-3}$ and $2.8 \pm 0.4 \times 10^{-3}$ mean $\pm$ std. err., respectively). Note, when fitting the cSNAFL-2 data, the parameter B was set equal to the instrument gain, 0.65, in order to achieve convergence. One class of FM4-64, attributed to cortex membrane, behaved similarly to Fluo-4 in solution with noise properties comparable to those assigned to the Poisson behavior of the instrument ($F_{ADU} = A + B \cdot t$; $A = 0.59 \pm 0.00$ and $B = -1.1 \pm 0.1 \times 10^{-5}$ and $F_{ADU} = A + B \cdot \text{Intensity}_{\text{Mean}}$; $A = 0.52 \pm 0.00$ and $B = 1.1 \pm 0.1 \times 10^{-3}$ mean $\pm$ std. err.). The fitted behavior of Fluo-4, Figure 4, is included for comparison.

Figure 5. ω -agatoxin, a p-type Ca^{2+} channel blocker, alters the noise characteristics of Fluo-4 labeled vesicles. Typical average intensity profiles using the maximum intensity to pixel align data (A). The spatial dependence of mean F_{ADU} was dependent upon treatment with the p-type Ca^{2+} channel blocker, ω -agatoxin (B). ω -agatoxin treated vesicles have lower F_{ADU} values, lower individual pixel variance, and have maximal values in the interior of the vesicle. Data are plotted as mean $\pm$ 99% confidence.