Temporal Separation of Vesicle Release from Vesicle Fusion during Exocytosis*

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During exocytosis, vesicles in secretory cells fuse with the cellular membrane and release their contents in a Ca2+-dependent process. Release occurs initially through a fusion pore, and its rate is limited by the dissociation of the matrix-associated contents. To determine whether this dissociation is promoted by osmotic forces, we have examined the effects of elevated osmotic pressure on release and extrusion from vesicles at mast and chromaffin cells. The identity of the molecules released and the time course of extrusion were measured with fast scan cyclic voltammetry at carbon fiber microelectrodes. In external solutions of high osmolarity, release events following entry of divalent ions (Ba2+, Ca2+) were less frequent. However, the vesicles appeared to be fused to the membrane without extruding their contents, since the maximal observed concentrations of events were less than 7% of those evoked in isotonic media. Such an isolated, intermediate fusion state, which we term “kiss-and-hold,” was confirmed by immunohistochemistry at chromaffin cells. Transient exposure of cells in the kiss and hold state to isotonic solutions evoked massive release. These results demonstrate that an osmotic gradient across the fusion pore is an important driving force for exocytotic extrusion of granule contents from secretory cells following fusion pore formation.

Vesicles in secretory cells perform two different functions: they are a storage depot for small molecules and they release these molecules during exocytosis. Storage in dense core vesicles requires stable packaging for an extended period of time. This is accomplished by association of the vesicular contents. On the other hand, release requires dissociation of this storage matrix on a relatively fast time scale so the contents can be extruded (1, 2).

In mast cells, histamine and 5-HT are associated with a negatively charged heparin matrix in the vesicles through a cation exchange type of interaction (3). These vesicles contain 150 mM histamine, which alone with its associated anion would lead to a free osmolality of ~550 mosM (4). However, the vesicles are iso-osmotic with the 300-mosM cytoplasm (5). These close associations have been documented by nuclear magnetic resonance in vesicles from both cell types (9, 10).

Upon cell stimulation, the vesicle contacts the plasma membrane and forms a fusion pore, the dynamics and control of which have been reviewed recently (11, 12). Capacitance and amperometry techniques have indicated that a portion of the vesicular contents can be released through this fusion pore (13, 14). Furthermore, at both mast and chromaffin cells, patch-amperometry measurements reveal that the fusion pore can exist for several milliseconds prior to full extrusion of vesicle contents (15, 16). Following secretory vesicle-cell membrane fusion in mast cells vesicular swelling occurs (17), which has been attributed to the displacement of associated cations in the granule with hydrated cations such as Na+ from the external solution (18). This ion exchange mechanism, with its associated hydration effects (19), has been hypothesized and experimentally supported at several cell types as reviewed by Artalejo et al. (20).

Theoretical considerations of this process have likened the extrusion and swelling to a controlled explosion that drives full release of the vesicular contents and dissociation of the vesicular matrix (18). Amperometric recordings revealed that the time course of release is longer than that expected for free diffusion (1), having a duration of several milliseconds at chromaffin cells (21, 22), and even longer at mast cells (23), consistent with a rate-limiting dissociation. The time course of extrusion can be decreased by perturbing any of the physical and chemical gradients that exist between the vesicle interior and the extracellular environment. These include osmolarity, pH, and cation concentration (24–26). For example, increasing the osmotic gradient by lowering the extracellular solution osmolarity decreases the release time course and increases the amount released, whereas high extracellular osmolarity has the opposite effect (27). In the present work, the effects of high osmolarity (using elevated NaCl) on secretory cells were evaluated in an attempt to freeze exocytotic events at the step between fusion pore formation and extrusion of vesicle contents. The results reported are consistent with such a state and reveal that extrusion of the vesicular contents is a distinct step in the sequence of events termed exocytosis.

EXPERIMENTAL PROCEDURES

Cell Culture—Bovine adrenal medullary cells, enriched in either epinephrine or norepinephrine using a Renografin gradient, were cultured as previously described and plated at a density of 3 × 10⁵ cells/plate (28). Cells were used on days 3–7 after culture. Murine peritoneal mast cells were cultured as described previously (29). Cells were plated on 25-mm glass coverslips (Carolina Biological Supply, Burlington, NC) contained in 35-mm diameter tissue culture plates (Falcon; Fisher Scientific).

Electrochemistry—Carbon fiber microelectrodes for the detection of released molecules were prepared as described (30). Electrodes were

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backfilled with a 4 mM potassium acetate, 150 mM KCl solution and calibrated using 4 mM epinephrine, norepinephrine, and histamine and 1 mM serotonin. FSCV was performed using locally written Labview software (National Instruments, Austin, TX) with an EI-400 potentiostat (Cypress Systems, Lawrence, KS) (31). Potentials were scanned from -200 mV to +1600 mV and back for catecholamines and from +100 to +1400 mV and back for histamine and serotonin (potentials versus a Ag/AgCl reference electrode, Bioanalytical Systems, Lafayette, IN). A scan rate of 2000 V/s was used with a 60-Hz repetition rate. Spike detection and analysis at the peak oxidation potentials for the molecules of interest were performed (32), and the resulting current spike amplitudes were converted to concentration based on electrode calibrations.

**Cell Experiments**—Individual cells were stimulated to release by pressure ejection (Picospritzer; General Valve Corp., Fairfield, NJ) of 5 mM BaCl2 or 60 mM KCl (chromaffin cells), 0.5 mM serotonin. FSCV was performed using locally written Labview software (National Instruments, Austin, TX) with an EI-400 potentiostat (Cypress Systems, Lawrence, KS) (31). Potentials were scanned from -200 mV to +1600 mV and back for catecholamines and from +100 to +1400 mV and back for histamine and serotonin (potentials versus a Ag/AgCl reference electrode, Bioanalytical Systems, Lafayette, IN). A scan rate of 2000 V/s was used with a 60-Hz repetition rate. Spike detection and analysis at the peak oxidation potentials for the molecules of interest were performed (32), and the resulting current spike amplitudes were converted to concentration based on electrode calibrations.

**Confocal Microscopy**—DjH labeling was used to visualize events associated with exocytosis at chromaffin cells. To measure surface-bound DjH, a measure of cell membrane-vesicle fusion, cells were exposed to 5 mM Ba2+ for 2 min in Tris-HCl buffer (no added Ca2+) in iso- or hypertonic buffer. Control cells were incubated without Ba2+. The cells were then fixed with a non-permeant protocol by quickly removing the bathing solutions and replacing them with identical buffers containing 4% formaldehyde at 4°C for 30 min (33). The cells were then rinsed twice with buffer, incubated with 0.2% bovine serum albumin for 10 min, rinsed two more times, and incubated with mouse anti-DjH monoclonal antibody (1:300; Chemicon, Temecula, CA) for 45 min at 4°C. To monitor DjH retention on the surface, an identical procedure was followed except the cells were incubated in iso- or hypertonic buffer for 20 min following Ba2+ but before fixation. To monitor vesicle recycling, the procedure was again identical except the antibody was present during the exposure to Ba2+. The cells were fixed and permeabilized with ethanol to allow visualization of endocytosed DjH.

After rinsing, DjH was visualized by incubating the cells with Alexa-Fluor 568 goat anti-mouse IgG conjugate (1:1000, Molecular Probes, Eugene, OR). The coverslips containing the cells were washed and mounted on microscope slides with Vectashield (Vector Labs, Burlingame, CA). Images were acquired using a 100× oil immersion objective on a Leica-LCS confocal microscope (Leica Instruments, Exton, PA). Analysis of images was performed using Scion Image software (Frederick, MD).

**Mast Cell Optical Imaging**—Visualization of release from mast cells was enhanced with 50 μg/ml ruthenium red (29). Individual cells in both iso-osmotic and elevated osmolarity buffers were stimulated to release and monitored using a CCD camera (Sensys, Photometrics, Tucson, AZ) through the microscope.

**Reagents and Solutions**—All chemicals were obtained from Sigma and used as received. A23187 was prepared by 1000-fold dilution of a stock solution in Me2SO. Iso-osmotic buffer contained 12.5 mM Tris-HCl, 150 mM NaCl, 4 mM KCl, 1.2 mM MgCl2, and 5 mM glucose. Hypertonic buffers were prepared by elevating the NaCl concentration to 520 mM for chromaffin cells and 350 mM for mast cells. All solutions were prepared using doubly distilled deionized water and adjusted to pH 7.4 by addition of NaOH.

### RESULTS

**Mast Cells**—Following transient exposure (5 s) to the Ca2+ ionophore A23187 in isotonic buffer (150 mM NaCl), mast cells exhibited frequent exocytotic release events that were detected by cyclic voltammetry (Table I), consistent with previous reports. Every exocytotic event resulted in the simultaneous release of 5-HT and histamine (Fig. 1) that each gave a distinct voltammetric peak (29). Each event was characterized by a simultaneous increase in the concentrations of 5-HT and histamine followed by a more gradual return to baseline. Occasionally the exocytotic spikes were preceded by a foot-like event (29) in which a transient, steady-state, low concentration of the amine was present (data not shown). Such events have been shown to be due to secretion through the fusion pore (13).

Mast cells displayed greater sensitivity to elevations in osmolality than chromaffin cells. Application of external solution with NaCl concentrations of 520 mM, as used with chromaffin cells (see below), caused either complete inhibition of Ca2+-evoked release events or rapid, spontaneous degranulation. At a lower NaCl concentration (350 mM NaCl), only a few cells degranulated spontaneously. However, for intact cells, release was quite dramatically altered. Exposure to A23187 in hypertonic medium containing Ca2+ still evoked spikes, but their maximal concentration was ~1% of that evoked in isotonic solution. Indeed, they resemble feet unaccompanied by spikes. The frequency of spikes was also reduced (Table I). Spikes with large concentrations could be obtained if the cells were subsequently exposed to an isotonic puff for a transient (5 s) period 3 min after the initial A23187 exposure (an example is shown in Fig. 1). After the exposure to isotonic buffer, the spikes continued for as long as 5 min at a frequency similar to that in isotonic buffer (Table I).

Since the average diameter of mast cell vesicles is 700 nm (29), the degranulation that occurs during exocytosis can be observed with light microscopy. In hypertonic medium, the mast cells shrank and degranulation was not apparent upon application of A23187 (Fig. 2b). This was the case despite the fact that the voltammetric results showed spikes of histamine and 5-HT (Fig. 1), albeit small ones. However, degranulation was readily observed immediately after transient application of isotonic buffer, and it continued for the time scale of the release detected with voltammetry (Fig. 2, c and d).

The lack of visual degranulation is not due to failure of Ca2+ to enter the cell. Ca2+ entry was immediately upon exposure to A23187 in hypertonic buffer as revealed by fluorescence from fura-2, the ratiometric divalent ion indicator (34). Furthermore, upon exposure to isotonic buffer, the larger spikes, with their accompanying visual degranulation, were not accompanied by a further influx of Ca2+ (Fig. 1, upper trace). Thus, in hypertonic solution, it appears that Ca2+ entry evokes vesicle-cell membrane fusion, but extrusion of vesicular contents is suppressed unless isotonic conditions are restored for a transient period.

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1. The abbreviations used are: FSCV, fast scan cyclic voltammetry; DjH, dopamine-β-hydroxylase; 5-HT, 5-hydroxytryptamine.
Chromaffin Cells—In isotonic solution without Ca\(^{2+}\), transient exposure of chromaffin cells to Ba\(^{2+}\) (5 mM, 5 s) causes exocytotic release of catecholamines (Fig. 3) (24). In isotonic solution containing Ca\(^{2+}\), transient exposure of chromaffin cells to elevated KCl (60 mM, 5 s) also causes exocytotic release of catecholamines (Fig. 4) (35). Cyclic voltammetry can be used to monitor the release and can distinguish cells that secrete norepinephrine from those that secrete epinephrine (36). Cyclic voltammetry can be used to monitor the release and can distinguish cells that secrete norepinephrine from those that secrete epinephrine (36). Cyclic voltammetry can be used to monitor the release and can distinguish cells that secrete norepinephrine from those that secrete epinephrine (36). Cyclic voltammetry can be used to monitor the release and can distinguish cells that secrete norepinephrine from those that secrete epinephrine (36).

In hyperosmotic solutions (520 mM NaCl) without Ca\(^{2+}\), transient exposure to 5 mM Ba\(^{2+}\) also results in exocytotic spikes from chromaffin cells (Fig. 3), but they are less frequent and much smaller in amplitude (Table II). Similar results are obtained for K\(^{-}\)-induced release in hypertonic solutions (Fig. 4, Table II). The maximal concentration of spikes in hypertonic medium with Ba\(^{2+}\) is only 7% and with K\(^{+}\) is only 10% of that seen in isotonic medium. In all cases the cyclic voltammograms revealed that the small, foot-like events were the same catecholamine that the cell normally secreted. Thus, the results at chromaffin cells are quite similar to those at mast cells: in hyperosmotic solutions, release events of small amplitude can be evoked, but their concentration and frequency is lower than in isotonic solution.

Transient application of an isotonic solution to chromaffin cells for 15 s at 2 min after exposure to either secretagogue in hypertonic medium induced massive release (Table II, Figs. 3 and 4). In 27% of the chromaffin cells pretreated with 60 mM KCl and 36% of the cells pretreated with Ba\(^{2+}\), this release consisted of one to two spikes with maximal concentrations greater than 40 \(\mu\)M that appeared to be compound exocytotic events. In the remainder of cells, spikes were observed shortly after exposure to isotonic buffer with a delay time of \(\sim 4\) s to the first spike. The isotonic buffer caused cell swelling on a similar time scale. The large spikes diminished immediately following cessation of transient exposure to isotonic buffer. When cells were completely restored to isotonic conditions, exposure to either secretagogue evoked spikes with a normal frequency and maximal concentrations (data not shown).

Fura-2 was used to monitor either intracellular Ba\(^{2+}\) or Ca\(^{2+}\) in these experiments. It is well established in isotonic solutions that both secretagogues cause an immediate increase in intracellular divalent ions (34), and this was replicated in this work (data not shown). In hypertonic solutions, pressure ejection of Ba\(^{2+}\) or K\(^{+}\) similarly caused an elevation in intracellular divalent ion concentrations. In the case of Ba\(^{2+}\), the signal remained elevated for greater than 3 min (Fig. 3) because intracellular Ca\(^{2+}\) stores do not effectively sequester it (38). Following K\(^{+}\) in hypertonic solution, the divalent ion signal returned to base-line levels \(-10\) s after its application. Thus, the observed, divalent ion entry accompanies the small release events obtained in hypertonic solutions.

Fura-2 measurements were also made during reintroduction of isotonic solution after exposure to secretagogues. The accom-
panying cell swelling and dye dilution complicates interpretation of the changes in divalent ions. There was no observable change in the bound to free ratio at cells that had been exposed to Ba<sup>2+</sup>; however, results in cells that had been exposed to K<sup>+</sup> were equivocal.

Confocal Microscopy—To provide evidence that vesicles fused with the plasma membrane in hypertonic solution, a fluorescently labeled antibody to DβH was employed. DβH is a vesicular enzyme that appears on the surface of chromaffin cells following exocytosis (39). Exposure to Ba<sup>2+</sup> for 2 min in

**Fig. 3.** Cyclic voltammetric monitoring of exocytosis at a chromaffin cell in isotonic (150 mM NaCl) and hypertonic solution (520 mM NaCl). Left panel, release from a cell exposed to 60 mM K<sup>+</sup> in isotonic solution. The cell in hypertonic solution was exposed to K<sup>+</sup> for 5 s during the first dashed box and then exposed to isotonic solution for 15 s during the second dashed box (right panel). Color plots: release events were monitored with FSCV. The current intensity is encoded in color as indicated by the scale bar (note change in scale bars). For epinephrine, oxidation of the catechol to its o-quinone occurs at +0.75 V with a second peak at +1.5 V from the oxidation of the secondary amine as described previously (36). Middle trace, current arising from oxidation of epinephrine at +0.75 V. Upper trace, fura-2 monitoring of intracellular divalent ion concentration at a different cell.

**Fig. 4.** Cyclic voltammetric monitoring of exocytosis at a chromaffin cell in isotonic (150 mM NaCl) and hypertonic solution (520 mM NaCl). Left panel, release from a cell exposed to Ba<sup>2+</sup> in isotonic solution upon exposure to Ba<sup>2+</sup>. The cell in hypertonic solution (middle panel) was exposed to Ba<sup>2+</sup> for 5 s during the first dashed box and then exposed to isotonic solution for 15 s during the second dashed box (right panel). Color plots: release events were monitored with FSCV. The current intensity is encoded in color as indicated by the scale bar (note change in scale bars). Middle trace, current arising from oxidation of epinephrine at +0.75 V. The experimental parameters are the same as those in Fig. 3. Upper trace, fura-2 monitoring of intracellular divalent ion concentration at a different cell.
either isotonic (Fig. 5a) or hypertonic solutions (Fig. 5b) causes DβH localization on the cell perimeter. The fluorescence at the cell surface, which is eight times more intense in cells stimulated in isotonic solution, indicates vesicle-cell membrane fusion has occurred under both conditions, exposing the vesicle interior to the external solution. In both solutions, unstimulated cells showed no fluorescence (data not shown).

A similar experiment was used to test vesicle lifetime at the cell surface. With the same treatment as in Fig. 5a, but with a 20-min incubation in buffer before the addition of DβH antibody, DβH was not found on cell surfaces when stimulated in isotonic medium (Fig. 5c). This is consistent with vesicle endocytosis that occurs on a time scale much shorter than 20 min in isotonic solutions (40). Supporting this conclusion, fluorescence was found within cells that were stimulated to release in isotonic solutions in the presence of DβH antibody (Fig. 5c). In contrast, DβH antibody was present on cell surfaces stimulated in hypertonic medium under all conditions, but was not seen to incorporate within the cell (Fig. 5d and f). The rate of endocytosis is known to be lower in hypertonic solutions, consistent with this finding (41).

**DISCUSSION**

In high osmolarity solutions, secretion from both mast and chromaffin cells is dramatically altered. The results presented here show that vesicle-cell membrane fusion can still occur under these conditions, but that full extrusion of the vesicle contents does not occur. Indeed, the fused vesicles in hypertonic solution appear to be frozen at the cell surface, and only release after swelling of the vesicle matrix is allowed to occur. Previous studies have demonstrated that both chromaffin and mast cell vesicles contract in response to hypertonic stress without disruption of their stored contents (5). Indeed, the chromaffin vesicle has been termed an ideal osmometer between 300 and 1000 mosM (6). The new finding of this work is that high osmolarity solutions prevent full extrusion of vesicle contents and allow temporal isolation of an intermediate state in the sequence of events that occur during exocytosis, a state we refer to as “kiss-and-hold.”

Hypertonic solutions clearly lower the rate of exocytosis at both mast and chromaffin cells. This could arise from an increase in actin filaments at the cell membrane as found in neutrophils in hyperosmotic solutions (42), because the rate of exocytosis in chromaffin cells depends on the degree of association of actin filaments (43). However, previous visual studies in high osmolarity solutions have shown that vesicle-cell membrane fusion is not prevented at chromaffin or mast cells (17, 44, 45), nor in sea urchin eggs (46). In this work, vesicle-cell membrane fusion is established in chromaffin cells by the presence of DβH on the cell surface in both isotonic and hypertonic medium. DβH labeling is lowered in hypertonic solutions, cor-
relating well with the decreased frequency of electrochemically detected spikes. At mast cells, visual evidence for vesicle-cell membrane fusion was not obtained in hypertonic medium, although secretion was clearly evident by the cyclic voltammetry. The secretory events that are seen are so small that they resemble the feet that precede some exocytotic events in iso-osmolar solutions at both types of cells in hypertonic media. This is the expected outcome if vesicle fusion occurred in hypertonic solutions with only minor extrusion of the contents. Despite their small size, the cyclic voltammograms establish that the substances detected are those stored in the vesicles.

All of the evidence from both cell types indicates that the small size of the spikes obtained in hypertonic medium is a consequence of inhibition of vesicle-matrix dissociation. The association of the vesicle contents in chromaffin cells has been characterized as a “dynamic viscous solution that is stabilized by ternary complex formation” (10) and is also well documented in mast cells (47). Upon exposure to the extracellular solution through the fusion pore, at the initial “kiss,” exchange between the vesicular interior and the extracellular fluid begins. Normally the matrix dissociation and extrusion of its contents relies on hydration, with its accompanying matrix swelling, that is driven by a physical or chemical gradient across the fusion pore. By removing the osmotic gradient in hypertonic solutions, one of the driving forces for dissociation and extrusion is dissipated. The small amount of secretion that does occur could arise from components originally in an unassociated state or a small amount of matrix dissociation that occurs at the fusion pore, but which is unable to propagate into the remainder of the vesicle. Because rates of endocytosis are lowered in hypertonic solutions, the fused, intact vesicle “holds” at the cell surface for an extended period of time.

The frozen, fused vesicle state established in hypertonic medium is destroyed once the osmotic gradient is re-established. This occurs with the transient isotonic exposure that evokes multiple spikes on the time scale of cellular swelling. In mast cells, the maximal concentration of these spikes is consistent with extrusion of contents from previously fused vesicles. At chromaffin cells, the maximal concentration and time course of some observed spikes are larger than under normal exocytotic conditions, consistent with multiple, simultaneous secretory events. For cells stimulated with K⁺, the original stimulus is no longer present upon isotonic solution exposure, and yet secretion is still observed. This strongly suggests that the observed spikes arise from previously fused vesicles. With Ba²⁺ at chromaffin cells and with mast cells, the isotonic-evoked spikes are accompanied by an increase in intracellular divalent ions. Thus, it is clear that the discrete processes of vesicle fusion and vesicle extrusion have been temporally separated. Concentration and pH gradients between the vesicle matrix and the external solution may exert similar effects (24, 25). Indeed, chromaffin cells previously stimulated with Ba²⁺ in hypertonic solutions did not release when exposed to isotonic buffer at pH 5.5 (data not shown). Thus, re-establishing an osmotic gradient is not sufficient to unroll the kiss and hold state; the pH gradient is necessary as well.

All of these results are consistent with recent mathematical models for the swelling of secretory granules after fusion pore formation and its role in extrusion of granule contents (18, 48). These models indicate that the hydration and swelling of the vesicle matrix cause an enlargement of the fusion pore leading to its destabilization. Thus, normally in mast and chromaffin cells the pore expansion of the vesicle occurs rapidly, driven by the irreversible swelling of the matrix, and results in full incorporation of the vesicle membrane and extrusion of vesicular contents as illustrated in the sequence of steps in Fig. 6. By removing the driving force for swelling, we were able to isolate the intermediate kiss-and-hold state. The mathematical models predict that “kiss-and-run” events, events where fusion pore formation allows escape of some transmitter followed by vesicular retrieval from the cellular membrane without fully fusing, are unlikely under normal conditions at cells with large vesicles. Consistent with these models, all fusion pore events in mast cells are followed by complete fusion of the vesicle with the cellular membrane and subsequent release (15). Patch-amperometry studies on chromaffin cells reveal only 10% of the observed fusion events are kiss-and-run (16), but these occurrences are increased with elevated external Ca²⁺ concentrations (49). In the absence of endocytosis caused by elevated osmolality, one would expect these events to be restricted to the cell membrane, as shown in this work, until acted upon by external forces. For small vesicles, the force of matrix expansion is insufficient to destabilize the fusion pore, and kiss-and-run is more likely. This has also been observed for small clear vesicles at hippocampal synapses (50).

The hypertonically constructed kiss-and-hold configuration inhibits the dissociation of matrix constituents following vesicle fusion with the cell membrane. This provides access to the vesicle interior while maintaining the viability of the cell, which may allow for a more complete understanding of the complex interaction of molecules within the vesicle matrix in their natural environment as well as a glimpse of their dissociation during exocytosis. Under normal physiological conditions, full fusion and extrusion of vesicular contents is predominant at both mast and chromaffin cells, relying on the destabilization of the granule matrix through swelling to rapidly pass through the kiss-and-hold state.

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