Secretory vesicles in chromaffin cells of the adrenal medulla sequester a remarkably high concentration of molecules. The major component, catecholamines, is present at a concentration of 550 mM, but the other vesicular constituents such as ATP (122 mM), Ca\(^{2+}\) (17–30 mM), Mg\(^{2+}\) (5 mM), ascorbate (22 mM), and the acidic protein chromogranin A are also present at high concentrations (1). Because the total soluble concentration of intravesicular components is more than 750 mM, it has long been of interest how these vesicles are able to form stable entities that are isotonic with 300 mosM solutions. It seems from nuclear magnetic resonance studies of intact vesicles that the contents form a dynamic viscous solution that is stabilized by ternary complex formation (2). Solution studies have revealed that catecholamines and ATP associate, resulting in a remarkable opportunity to temporally separate exocytotic secretion from entry of divalent ions.

Whereas intravesicular association stabilizes vesicular storage, upon exocytosis the vesicular contents must dissociate to be extruded from the cell in their soluble form. The osmotic gradient that exists immediately upon cell vesicle fusion between the isotonic extracellular milieu and the vesicle contents certainly plays a role in the overall exocytotic process. Indeed, Knight and Baker (9) showed that sucrose-based hypertonic solutions inhibit the amount of Ca\(^{2+}\)-dependent catecholamine secretion, whereas incubation with hypertonic media increases it. Similar results were obtained in other laboratories with cultured cells (10–13) and in perfused adrenal glands (14) as reviewed by Holz (1). The inhibition of release in hypertonic media seems to be due to a direct effect on the exocytotic process because Ca\(^{2+}\) entry is not inhibited (10). Furthermore, the effects of altered osmolarity are reversible, implying that cell function is not greatly perturbed. Such observations led to the chemiosmotic hypothesis for exocytosis, which proposed that swelling of the secretory vesicles provides the driving force for the fusion of the vesicular and cellular membranes (15). However, this hypothesis has been rejected because simultaneous optical and whole-cell capacitance measurements at mast cells revealed that fusion with the cell membrane actually precedes vesicular swelling (16).

Alterations in the amounts of catecholamine released from populations of cells or intact glands by extracellular solutions of different osmolarities could be due either to an alteration in exocytotic efficiency, i.e. an altered exocytotic frequency, or to an alteration of the amount secreted per exocytotic event. Indeed, an increased osmotic gradient between the cell interior and exterior promotes Ca\(^{2+}\) entry (17), promoting increased frequency of exocytotic events (18). To investigate the other possibility, we have examined the effects of osmotic pressure on catecholamine secretion at bovine adrenal medullary cells with amperometry because fura-2 fluorescence reveals an increase in intracellular divalent ions. Thus, this procedure provides an unique opportunity to temporally separate exocytotic secretion from entry of divalent ions.
EXPERIMENTAL PROCEDURES

Adrenal Medullary Cell Culture—Bovine adrenal medullary cells, enriched in epinephrine using a single-step Renografin gradient (27), were prepared from fresh tissue as described previously (28). Cells were plated on 12-mm-diameter glass coverslips (Carolina Biological Supply, Burlington, NC) contained in 35-mm-diameter culture dishes (Falcon 3001; Becton Dickinson, Lincoln Park, NJ) at a density of 6 × 10^4 cells per cover glass. Cells were incubated at 37 °C in a 5% CO_2 environment and used at room temperature between 2 and 5 days of culture.

Electrochemical Detection of Catecholamines—Carbon fiber microelectrodes for the detection of catecholamine release were prepared as described by Kawagoe et al. (29) using 5-μm-radius carbon fibers (Thornel P-55; Amoco Corp., Greenville, SC). A commercial patch-clamp instrument (Axopatch 200B; Axon Instruments, Foster City, CA) was used for amperometry. The instrument was operated in voltage clamp mode in the whole-cell (β = 1) configuration with a command potential of +0.650 V applied versus a locally constructed sodium-saturated calomel reference electrode. Electrodes were backfilled with 4 M NaCl and connected to the headstage input using a patch pipette holder with chlorided silver wire. The noise of this system is one-twentieth of that used in all of our prior work (30). Electrodes were calibrated with a flow-injection apparatus using 50 μM epinephrine.

Glass coverslips with adhering adrenal medullary cells were washed in isotonic (315 mosM) Krebs buffer (see Table I). To obtain rapid changes in the osmolarity of the media bathing the cells, the coverslips were placed in a perfusion chamber positioned on the stage of an inverted microscope (Axiovert 35; Zeiss, Thornwood, NY). Buffer was perfused at 2.5 ml/min, and new solutions were introduced with a valve placed close to the chamber. Amperometric measurements were performed with the carbon fiber microelectrode gently touching the cell membrane. Cells were stimulated to release by pressure ejection (Picospritzer; General Valve Corp., Fairfield, NJ) using 5 mM BaCl_2 for 5 s from a micropipette placed 25–30 μm away from the cell. Barium was used as a secretagogue because it does not require receptor activation or membrane depolarization, processes that can be affected by osmotic changes (31).

Data Analysis—Amperometric signals were low-pass filtered at 10 kHz (4-pole Bessel filter on Axopatch 200B) and recorded on 0.5-inch videotape (PCM-2 A/D VCR Adapter; Medical Systems Corp., Green- vale, NY). During analysis, current records were replayed through a 400-Hz low-pass filter and digitized to an IBM compatible personal computer at 1 ms/point (Cyberamp 320 and Axotape; Axon). Exocytotic events were located with locally written software, and the areas of individual current spikes (Q), their amplitudes (Q_max), and their widths at half-height (t_1/2) were determined as described previously (32). For evaluation of Q changes the quantity Q_1/2, which has a Gaussian distribution, was computed for each spike (33).

Intracellular Measurements of Divalent Ions—The fluorescent probe fura-2 was used as described elsewhere (34–35). Briefly, adrenal medullary cells were incubated in Krebs buffer containing 1 μM fura-2/AM (stock solution dissolved in 20% Pluronic F-127 in Me_2SO) and 0.1% bovine serum albumin for 30–40 min at room temperature. After loading, the cell plates were rinsed twice and refilled with the desired Krebs-Ringer buffer. The specimens were alternately excited at 340 and 380 nm through a 40× oil-immersion objective (Zeiss). The results were corrected for electrode autofluorescence and reflectance (34). The ratio of corrected fluorescence values (F_{340}/F_{380}) provided an estimate of concentration changes of intracellular divalent ions. This estimate can be due to changes in BaCl_2 or CaCl_2, and no attempts were made to distinguish between these two species.

Materials—The culture medium, Dulbecco's modified Eagle's/Ham's F-12 medium, was obtained from Life Technologies, Inc. Collagenase (Type I) for digestion of gland tissue was obtained from Worthington Biochemical. Renografin-60 was obtained from Squibb Diagnostics. Fura-2/AM, fura-2-free acid, and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma, and reagent-grade salts were dissolved with doubly distilled water to make solutions.

Solutions—Isotonic, hypertonic, and hypotonic solutions were prepared as described in Table I. With all bath and pipette solutions, the pH was adjusted to 7.40 with concentrated NaOH. Hypertonic solutions were made by addition of NaCl or sucrose to achieve the desired osmolarity. Solution osmolarities were checked with a freezing point depress- ion osmometer (Osmette A; Precision Systems, Inc., Natick, MA). For solutions with no CaCl_2, CaCl_2 was omitted with no MgCl_2 or calcium chelators added.

RESULTS

Morphological Changes of Cells Caused by Different Osmotic Solutions—The phase-contrast micrographs in Fig. 1 show the effect of different osmotic pressures on adrenal medullary cells. These experiments were conducted in a manner similar to the method used for vesicular release studies: cells were incubated in isotonic solution for 10 min, the perfusion buffer was changed to the osmotic solution of interest for another 10 min, and finally, isotonic conditions were restored. The micrographs shown were taken 2 min after solution changes.

Low osmotic strength (200 mosM), obtained by NaCl reduction (hypotonic, Table I), caused a rapid increase in cell volume. This change was immediately reversed upon return to isotonic conditions. In contrast, increased osmolarity (450–1000 mosM) caused cell shrinkage to occur. The changes seemed to be reversible except at 1000 mosM, at which some cells exhibited permanent alterations in their cytoplasmic appearance upon return to isotonic conditions.

Secretary Characteristics with Osmotic Pressure—Fig. 2 shows the effect of decreased and increased osmotic pressure on catecholamine release. In isotonic buffer before a solution change, secretion lasting several minutes was elicited by pressure ejection for 5 s of a 5 mM BaCl_2 solution. This result was obtained in CaCl_2-free solutions (Fig. 2, A and D) and also in solutions containing CaCl_2, as we have previously reported (26). The current spikes observed correspond to the exocytotic release of catecholamines from individual vesicles (19). When the osmolarity of the bathing media was decreased to 200 mosM (hypotonic, Table I), reexposure to BaCl_2 in the absence of CaCl_2 caused secretory spikes that were more frequent and of increased amplitude (Fig. 2B). When CaCl_2 was present, low osmotic pressure caused spontaneous release (data not shown),

1 The abbreviation used is: t_1/2, width at half-height.
as previously observed (18). When the cell was returned to isotonic medium, Ba$^{2+}$-induced spikes were similar (but not identical; Ref. 20) to those obtained before exposure to the decreased osmolarity (Fig. 2C).

In contrast, Ba$^{2+}$-induced secretion decreased in frequency and amplitude as the osmolarity was increased. Results from a single cell in Ca$^{2+}$-free media are shown in Fig. 2D–F, in which a cell originally in an isotonic medium was placed in a 750 mosM solution (hypertonic II, Table I). Similar effects were obtained when the osmolarity was elevated by adding sucrose instead of NaCl (Fig. 3) or with high-osmolarity media containing Ca$^{2+}$ (data not shown). Upon a return to isotonic conditions (Figs. 2F and 3C), every cell secreted similarly as before the osmotic change.

The effects of increased osmolarity were studied in detail at 630 mosM (hypertonic I, Table I). Each observed spike was characterized with respect to its average charge ($Q$), $t_{50}$, and maximal amplitude ($i_{\text{max}}$). The mean characteristics from a single cell are summarized in Table II and show a significant increase in $t_{50}$ and a significant decrease in $i_{\text{max}}$. A histogram of $t_{50}$ values (Fig. 4A) reveals a broader distribution and a greater proportion of wider spikes under hypertonic conditions. In addition, the data show a small but significant decrease in $Q$ (Fig. 4B) for secretion in the medium of higher osmolarity. In each of four cells examined in this way, $t_{50}$ increased significantly, and $i_{\text{max}}$ decreased significantly in response to the change from 315 to 630 mosM. In three of the four cells, a statistically significant decrease in $Q$ was observed.

**Secretion Induced in Very Hypertonic Media—**Spikes were initially induced with Ba$^{2+}$ in isotonic medium, and then the cells were exposed to solutions with an osmolarity of 970 mosM (Fig. 5A; hypertonic III, Table I). Spikes evoked by Ba$^{2+}$ were virtually abolished in both the absence (Fig. 5A) and presence of Ca$^{2+}$ (Fig. 6A). However, in both cases exposure to Ba$^{2+}$ evoked small current fluctuations (Fig. 5B, upper; Fig. 6B, upper) of similar amplitude to the prespike features or feet that precede some secretory events in isotonic media (21, 36). Such an event is shown in expanded form in Fig. 5A (lower inset). Because the amplifier noise in the measurements reported here is lower than that in our previous work, we were also able to observe similar amperometric fluctuations among the spikes that occurred during evoked secretion in isotonic media (Fig. 5A, upper inset). Such events have been described as flickering feet that lack accompanying spikes (37). Similar small changes in amperometric current after exposure to Ba$^{2+}$ were also observed in hypertonic media prepared with sucrose as the primary osmolyte (hypertonic IV, Table I).

When the cells were returned to an isotonic medium that was Ca$^{2+}$-free, the base line remained unchanged. However, a change from hypertonic medium with Ca$^{2+}$ to isotonic medium with Ca$^{2+}$ induced massive secretion that began about 1 min after the medium change and continued for many minutes (data not shown). Consistent with results obtained by Wakade et al. (17) with perfused adrenal glands, this phenomenon was only found to occur in the presence of external Ca$^{2+}$. Both with and without Ca$^{2+}$, subsequent exposure to Ba$^{2+}$-induced secretion in every case, but occasionally release was of lower frequency than before exposure to the very hypertonic medium.

### Table I

Composition and osmolarities of experimental solutions in which the amount of NaCl or sucrose was altered

| Extracellular solution | Composition | Osmolarity |
|------------------------|-------------|------------|
| Isotonic               | 150 NaCl, 5KCl, 1.2 MgCl$_2$, 5 glucose, 10 HEPES | 315 |
| Hypotonic              | 86 NaCl, 5KCl, 1.2 MgCl$_2$, 5 glucose, 10 HEPES | 200 |
| Hypertonic I           | 332 NaCl, 5KCl, 1.2 MgCl$_2$, 5 glucose, 10 HEPES | 630 |
| Hypertonic II          | 386 NaCl, 5KCl, 1.2 MgCl$_2$, 5 glucose, 10 HEPES | 750 |
| Hypertonic III         | 522 NaCl, 5KCl, 1.2 MgCl$_2$, 5 glucose, 10 HEPES | 970 |
| Hypertonic IV          | 435 sucrose, 150 NaCl, 5 KCl, 1.2 MgCl$_2$, 5 glucose, 10 HEPES | 850 |

### Figures

**FIG. 2. Effects of osmotic changes on vesicular catecholamine release from single cells.** Representative amperometric traces showing exocytotic release from individual cells as the osmolarity of the extracellular solution is changed. A single adrenal medullary cell was initially induced with Ba$^{2+}$ under (A) isotonic (315 mosM) conditions. The same cell was then exposed to (B) hypotonic conditions (200 mosM) and finally returned to (C) isotonic (315 mosM) conditions. Secretion evoked by Ba$^{2+}$ at another cell was monitored in the same manner at (D) 315 mosM, (E) 750 mosM, and (F) 315 mosM. Solutions (isotonic, hypotonic, and hypertonic II, Table I) were made with no external Ca$^{2+}$ and with NaCl as the osmotic agent.
Secretion Induced by Isotonic Ca\(^{2+}\)-free Medium—Although cells exposed to Ba\(^{2+}\) showed few spikes in very hypertonic media, it was found that a 15-s pressure ejection of isotonic solution onto the cells could evoke frequent spikes (Fig. 5B). In these experiments, a three-barrel ejection pipette was used containing isotonic buffer, 5 mM BaCl\(_2\) in isotonic buffer, and 5 mM BaCl\(_2\) in hypertonic buffer. Catecholamine release from single cells was first measured in isotonic solution, in which exposure to 5 mM isotonic Ba\(^{2+}\) induced release for several minutes. As expected, pressure ejection of isotonic buffer onto cells bathed in isotonic buffer did not cause amperometric current fluctuations. After exposure of the same cell to Ba\(^{2+}\) in a medium of 970 mosM and observation of the small current events, the cell was exposed to isotonic buffer. During the pressure ejection the spikes increased in frequency and reached a maximum at the end of the pressure ejection period (n = 15 cells). Similar results were obtained in the presence and absence of external Ca\(^{2+}\). Interestingly, spikes obtained from isotonic application in Ca\(^{2+}\)-free hypertonic buffer were somewhat smaller in amplitude (Fig. 6, A and B; note the difference in the current scales).

Role of Intracellular Divalent Cations—The role of intracellular divalent ions in these processes in hypertonic solutions (n = 6 cells) was examined with the fluorescent probe fura-2 (35). During application of Ba\(^{2+}\) in hypertonic media (970 mosM), fura-2 ratio measurements indicated an influx of divalent cations (note that fura-2 complexes both Ba\(^{2+}\) and Ca\(^{2+}\)). Similar to previous results under isotonic conditions (26, 31), this response was obtained in the presence and absence of extracellular Ca\(^{2+}\) (Fig. 6, A and B; note the difference in the current scales).

**TABLE II**

| Osmolarity (mosM) | Q (pC) | \(t_{1/2}\) (ms) | \(I_{\text{max}}\) (pA) | Spike frequency (Hz) |
|------------------|--------|----------------|------------------|---------------------|
| 315              | 2.26 ± 0.14 | 16.4 ± 0.8 | 170 ± 10 | 2.10^a          |
| 630              | 1.62 ± 0.15^b | 46.8 ± 4.4^b | 77 ± 11^b | 0.48^c          |

^a 504 current spikes over 240 s.
^b Means are significantly different (p ≤ 0.01).
^c 218 current spikes over 450 s.

Secretion Induced by Isotonic Ca\(^{2+}\)-free Medium—Although cells exposed to Ba\(^{2+}\) showed few spikes in very hypertonic media, it was found that a 15-s pressure ejection of isotonic solution onto the cells could evoke frequent spikes (Fig. 5B). In these experiments, a three-barrel ejection pipette was used containing isotonic buffer, 5 mM BaCl\(_2\) in isotonic buffer, and 5 mM BaCl\(_2\) in hypertonic buffer. Catecholamine release from single cells was first measured in isotonic solution, in which exposure to 5 mM isotonic Ba\(^{2+}\) induced release for several minutes. As expected, pressure ejection of isotonic buffer onto cells bathed in isotonic buffer did not cause amperometric current fluctuations. After exposure of the same cell to Ba\(^{2+}\) in a medium of 970 mosM and observation of the small current events, the cell was exposed to isotonic buffer. During the pressure ejection the spikes increased in frequency and reached a maximum at the end of the pressure ejection period (n = 15 cells). Similar results were obtained in the presence and absence of external Ca\(^{2+}\). Interestingly, spikes obtained from isotonic application in Ca\(^{2+}\)-free hypertonic buffer were somewhat smaller in amplitude (Fig. 6, A and B; note the difference in the current scales).

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served. However, inspection of the fluorescent recordings at the individual wavelengths showed that this was due to small differences that occurred in the decrease in fluorescence at both wavelengths. The decrease is due to a transient dye dilution as a result of cell expansion during exposure to the isotonic solution followed by its subsequent contraction.

**DISCUSSION**

Adrenal medullary cells respond as osmometers (10) that swell in hypotonic solutions and shrink in hypertonic media (Fig. 1). Despite the changes in cell volume, cells continue to secrete over a broad range of osmotic strengths. Prior results have demonstrated that under extreme hypertonic conditions, catecholamine secretion from adrenal medullary cells can be inhibited (9–12, 14). The results of the present study with amperometry support these findings and provide new insight into the origin by examining this effect at the level of individual vesicular events. The results show that the external osmolarity can have a dramatic effect on the time course of extrusion of the vesicular contents: in hypotonic solutions extrusion from the vesicles is more rapid than in isotonic medium (20), whereas hypertonic solutions slow the extrusion process until it virtually ceases at very high osmolarity.

It has long been recognized that the high concentration of catecholamines and other intravesicular components in adrenal medullary cells requires some form of internal association to reduce osmotic forces (3, 38). Experiments with amperometric detection of catecholamine secretion from individual vesicles show that the extrusion process is temporally prolonged even in isotonic medium (19, 32), consistent with a finite time required for dissociation of the intravesicular components (21). Even at 37 °C the mean half-width of secretory events exceeds 4 ms (25). The secretory spikes are found to change in shape with the pH and ionic composition of the extracellular media. Acidification of the external media to a pH of 5.5 makes extrusion of the vesicle contents more difficult (23), whereas spikes induced by Ba²⁺ are broader and shorter in the absence of external Ca²⁺ (26). Exposure of adrenal medullary cells to extracellular Zn²⁺, an ion that can further cross-link the vesicle contents, lowers the quantity of catecholamine released during secretory events (25). The findings reported here, that a
lowered osmotic gradient between the vesicle contents and the extracellular fluid also increases the time course of vesicular dissociation, are consistent with prior conclusions that the mechanism of storage of the vesicle contents is manifested in the kinetics of individual secretory events. Thus, the osmotic gradient present in isotonic medium immediately after cell-vesicle fusion promotes water entry through the fusion pore into the vesicular matrix, where it elicits the dissolution of the intravesicular contents (39).

Because secretion characteristics can vary from cell to cell, we minimized this effect by allowing each cell to serve as its own control. With the exception of the most hypertonic solutions, the osmotic changes did not permanently alter the cells because secretion was similar before and after osmotic stress. Furthermore, the observed changes are a direct consequence of solution osmolarity because similar results were obtained with NaCl and sucrose media. We used Ba$^{2+}$ as a secretagogue rather than one that interacts with cell receptors to avoid possible altered affinities of receptor states.

In solutions of intermediate hyperosmolarity (630 mosM) the spike frequency is reduced as well as the amount released in each spike. It is likely that the decreased amounts of secretion seen from populations of cells (1) are due to the lower spike frequency because it is decreased to a greater degree. However, at osmolarities comparable to the osmolarity calculated for the unassociated contents of the vesicles, spikes are virtually eliminated. Because occurrence of spikes only provides information on the last stage of exocytosis, the extrusion of the vesicle contents, we can only conclude that secretion has been arrested but cannot evaluate whether it is arrested at this stage or at a prior one. However, it seems that the extrusion process is the stage in a single exocytotic event that is the most sensitive to external solution osmolarity because cell-vesicle fusion is not inhibited by high osmolarity (16). Furthermore, most vesicular expansion seems to occur only after granule fusion (40), although a small enlargement may occur before fusion (41). Indeed, capacitance measurements in mast cells show that the time course of vesicle-cell fusion is little affected by the external solution osmolarity, even though the vesicular size is a function of external osmolarity (42).

In sufficiently hypertonic solutions, amperometric spikes are rarely observed upon exposure to Ba$^{2+}$, but small secretory events that resemble the prespike feature can be observed. The prespike feature, found before many secretory spikes in isotonic solution, has been attributed to catecholamine flux through the fusion pore formed in the initial stages of adrenal medullary cell-vesicle fusion (20, 36). As recently reported, these states can be observed without spikes and often flicker as would be expected for flux through a pore of fluctuating diameter (37). In very hypertonic medium, in which fura-2 measurements show that Ba$^{2+}$ entry into the cell still occurs, these features persist without spikes, suggesting that cell-vesicle fusion has occurred without subsequent extrusion of the cell contents. In other words, the absence of an osmotic gradient seems to have frozen the exocytotic process in a transient state that prevents secretion. This transient state has been observed in mast cells (24), and it appears under conditions in which partial secretory events (43) can be observed. Thus, by analogy, the intermediate state consists of a docked vesicle with a fusion pore open to the external media that releases a small portion of its contents without full dissolution of the entire contents from the granule matrix.

To test this hypothesis, we transiently exposed cells containing internal Ba$^{2+}$ in hypertonic solution to isotonic solution to restore the normal osmotic gradient between the vesicle contents and the solution. Transient restoration of an osmotic gradient caused a series of secretory spikes that are consistent with the hypothesis that fused vesicles with their contents intact are present at the cell surface. This effect was obtained with or without Ca$^{2+}$ in solution, and in both cases the occurrence of spikes was not accompanied by a change in the internal concentration of divalent ions. However, the data suggest thationic effects as well as osmotic effects are important for complete extrusion of the vesicle contents. This is evidenced by the fact that the presence of Ca$^{2+}$ in the external medium leads to spikes induced by isotonic solution that are of increased amplitude. This result is entirely consistent with our previous finding in isotonic medium that Ba$^{2+}$-induced spikes are of greater amplitude in Ca$^{2+}$-containing solutions (26). It seems that hydrated Ca$^{2+}$ can promote more rapid dissolution of the vesicle contents. Catecholamine release upon complete restoration of isotonic conditions after hypertonic treatment from hypertonic to isotonic conditions causes an increase in the rate of opening of voltage-activated Ca$^{2+}$ channels (17, 18). Our results with transient isotonic exposure differ in that secretion is not accompanied by an increase in internal Ca$^{2+}$ or Ba$^{2+}$. Indeed, these results represent the first time that entry of divalent cations and exocytotic secretion have been separated on a time scale of several minutes.

Taken together with previous findings, the results of this study clearly show that the associated state of the vesicle contents manifests itself during the extrusion of the vesicle contents after vesicle-cell fusion in adrenal medullary cells. In mast cells, in which the intravesicular association has significant ionic character (44), this has been quite clearly demonstrated because the vesicles are large enough for visual observation. Swelling of mast cell matrices increases ionic conductivity and allows the contents to be released (45). Our findings in this work show that an osmotic gradient that could swell the vesicle contents is required to obtain fully developed secretory spikes at adrenal medullary cells. Although pH and ionic gradients clearly must also play a role in dissociating the vesicle contents, the ease of manipulating osmolarity, coupled to the fact that it does not inhibit vesicle-cell fusion, makes this a particularly useful parameter to separate the individual stages during an exocytotic event. In addition, experiments with sucrose as the primary osmolyte clearly demonstrate that an ionic gradient is not sufficient to cause dissociation of the vesicle contents. The temporal separation of entry of divalent cations and secretion provides the opportunity to further investigate the individual steps in the complex phenomenon of exocytosis.

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