Temporally Resolved, Independent Stages of Individual Exocytotic Secretion Events

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ABSTRACT The stages of the complex events involved in exocytotic secretion after vesicle-cell membrane fusion have been examined at the level of individual vesicles. Catecholamine flux from single bovine adrenal medullary cells was measured with carbon-fiber microelectrodes firmly touching the cell surface. The data reveal that secretion during exocytotic events has three distinct stages: a small increase in catecholamine flux, a rapid, but not instantaneous, rise to a maximum, followed by an exponential decrease in the flux. These stages are interpreted in the following ways. The initial stage corresponds to catecholamine secretion through a fusion pore. The rate of pore expansion appears to control the rise time of the flux to its maximum value. The final exponential stage is consistent with chemical dissociation of the intravesicular matrix or gel.

INTRODUCTION

Exocytosis is a mechanism by which many cells, including neurons, release chemical messengers into the extracellular environment. It entails fusion of the secretory vesicle and plasma membranes, which is followed by release of vesicular contents (Monck and Fernández, 1992, 1994; Jan and Südhof, 1993). The use of whole-cell capacitance techniques and optical microscopy have made it possible to investigate in considerable detail the sequential steps of exocytosis in cells with large vesicles such as beige mouse mast cells (Zimmerberg et al., 1987; Monck et al., 1991). After cell stimulation to induce exocytosis, the cell capacitance increases stepwise, indicating vesicle-cell membrane fusion events. There is a time delay between the capacitance change and visual observation of the extrusion of the vesicle matrix into the extracellular space. During this delay patch-clamp recordings reveal a finite conductance that is attributed to the continued existence of the initially formed fusion pore (Alvarez de Toledo et al., 1993). The duration of the delay is exponentially distributed, indicative of a stochastic process (Monck et al., 1991). Subsequently, the conductance rapidly increases, indicating complete exposure of the vesicular contents to the extracellular environment. Simultaneous amperometric measurements with carbon-fiber microelectrodes show that a steady-state flux of serotonin occurs during the time of finite conductance that is followed by the major portion of release in the form of a concentration spike (Alvarez de Toledo et al., 1993). Because the initial flux from mast cells tracks the temporal characteristics of the conductance, it appears to result from diffusion of the intravesicular contents through the pore to the external solution. The contents of the dense core vesicles of the mast cell are tightly associated before fusion (Uvnäs and Åborg, 1984; Nanavati and Fernández, 1993), and thus the delay before full release could reflect the time required to initiate expansion and dissociation of the matrix. Alternatively, it could reflect the time between fusion pore formation and its full opening.

Carbon-fiber electrodes allow secretion from individual vesicles to be examined at cells with vesicles smaller than those conveniently measurable with capacitance or optical techniques. Exocytotic vesicular release has been examined in chromaffin cells (Leszczyszyn et al., 1990), PC12 cells (Chen et al., 1994), β-pancreatic cells (Kennedy et al., 1993), and dopamine invertebrate cell bodies (Ewing et al., 1992), and similarities with exocytosis at mast cells are being discovered. For example, this technique has revealed that some vesicular catecholamine secretion events in chromaffin cells are preceded by a small, transient, steady-state flux of catecholamines (Chow et al., 1992; Jankowski et al., 1994; Wightman et al., 1995), sometimes termed the "foot." The subsequent, main release phase has a flux that increases rapidly and then decreases in a more gradual manner (Jankowski et al., 1994). The extrusion of the contents is remarkably slow, with a mean width at half-height exceeding 5 ms. This slow rate is not due to diffusional dispersion in the extracellular fluid, because it is found with the electrode in contact with the cell surface (Wightman et al., 1995). Rather, it is a consequence of surface-associated events at the vesicular level. In a previous paper in this journal we hypothesized that this slow secretion could be due to either rate-limiting dissociation of the vesicular matrix or rate-limiting opening of the fusion pore (Wightman et al., 1995).

The subject of this paper is a more detailed examination of the shape of the secretory current spikes and the factors that affect them. We evaluate the amplitude of the flux

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during the foot and find it to be consistent with a small fusion pore. The main portion of the flux is found to have a more complex shape than that predicted by mathematical models that consider diffusion from the vesicle interior to be rate limiting. These models fail to predict the gradual increase in flux that is readily apparent in the data. Rather, we find that the spikes can be described as the convolution of an exponential and a gaussian curve. The time constants of the two deconvoluted curves are poorly correlated, suggesting that these rate-limiting steps are independent and governed by different mechanisms. The later, exponential portion of the curve is consistent with diffusional control of secretion at later times of the secretory event. The gaussian portion of the curve may reflect a rate-limiting opening of the fusion pore. The time constants of the curves are found to be decreased in solutions of low osmolarity, which causes an increase in membrane tension. Taken together, the data provide a new view of the steps associated with catecholamine efflux from chromaffin cells during exocytosis and show that both steps we previously considered may be rate limiting in the secretion from individual vesicles.

MATERIALS AND METHODS

Three-dimensional random-walk simulations

Simulations employing a three-dimensional random-walk algorithm (Schroeder et al., 1992) were used to evaluate the rate of escape of particles from a vesicle in which diffusion is spatially restricted. The vesicle was modeled as a sphere (radius of 150 nm), which was inserted into an inert reflecting plane so that particles could only depart from the upper hemispherical portion. The flux across this surface was determined as a function of time. The time increment employed was 0.833 μs, and the spatial step size was adjusted with respect to the diffusion coefficient for the spatial location of the particle. Fifty thousand particles were employed in the simulation.

Electrode construction and electrochemistry

Flame-etched carbon-fiber microelectrodes with tip radii of approximately 1 μm were constructed as described elsewhere (Strein and Ewing, 1992; Schroeder et al., 1994). Estimates of the active tip radius were made electrochemically with solutions containing known concentrations of electroactive species. If the electrochemically determined radii differed from those obtained by optical microscopy, the polymer insulation was not intact and the electrodes were discarded. Experiments in solutions of varied osmotic pressure employed 6-μm-radius glass-encased, carbon-fiber microelectrodes (Wightman et al., 1991).

Single-cell secretion experiments

Primary cultures of bovine adrenal medullary cells were prepared as described elsewhere (Leszczyszyn et al., 1990). Experiments were performed at room temperature (23.0 $\pm$ 0.1°C) between days 4 and 10 of culture. For the experiments that employed Ba^{2+} as the secretagogue, the culture medium was replaced with a solution containing (in mM): NaCl (154), KCl (4.2), $MgCl_2$ (0.7), glucose (11.2), and HEPES (10) brought to pH 7.4 with NaOH. Sodium phosphate was not employed, to prevent precipitation of $Ba_3(PO_4)_2$.

For the experiments that involved changing the osmotic pressure, cells were placed in a superfusion chamber on the microscope stage to allow rapid changes of the bathing solution. In this case, the incubation buffer contained (in mM): NaCl (150), KCl (5), $MgCl_2$ (1.2), $CaCl_2$ (2), glucose (5), and HEPES (10) brought to pH 7.4 by addition of NaOH. The osmolality was decreased to 200 mOsm by NaCl reduction to 86 mM. The osmolality of the micropipette solution was the same as that in the incubation buffer.

Secretagogues were applied to the cells with pressure ejection (Picospritzer; General Valve Corp., Fairfield, NJ) from micropipettes (~10-μm outside tip diameter) positioned 40–50 μm from the cell surface. Typical ejection pressures were 5–10 psi.

Analysis of spikes

Electrochemical data were digitally recorded and then were replayed through a fourth-order, low-pass (400 Hz) filter (CyberAmp 320; Axon Instruments, Foster City, CA) digitized at a rate of 1 ms/point via a commercially available software package (Axotape; Axon Instruments, Foster City, CA) and stored on the hard drive of a PC-compatible computer (Gateway 2000 4DX2/66, North Sioux City, SD). Locally written software employed a notch filter to remove residual 60 Hz noise. The digitization rate and filter frequency were selected so that distortion of the temporal characteristics did not occur.

Software was also used to locate spikes and extract their characteristics: spike maximum, half-width, area (units of charge), half-rise time, maximum current time, onset time, and ending time (Schroeder et al., 1992). A signal was designated as a spike if the amplitude was greater than 5 times the value of the rms noise measured over a baseline of 1 s. The presence of prespike features was identified by visual inspection of each spike and is therefore more subjective.

An exponentially modified gaussian (EMG) function was fit to each spike. The parameters describing the gaussian portion, σ, and the exponential portion, τ, were estimated from the second and third statistical moments of the experimental curves using a modified protocol to calculate the higher-order statistical moments (Yau, 1977). The mathematics for curve fitting of this equation have been extensively developed because it is frequently used to describe peaks in liquid and gas chromatography.

Reagents

The culture medium, Dulbecco's modified Eagle's medium/Ham's F12 medium, was obtained from Gibco Laboratories (Grand Island, NY). Collagenase (Type I) for digestion of glands was obtained from Worthington Chemicals (Freehold, NJ). Renografin-60 was purchased from Squibb Diagnostics (New Brunswick, NJ). All other chemicals were reagent grade from Sigma (St. Louis, MO), and solutions were prepared with doubly distilled water.

THEORY

Previous measurements of the time course of catecholamine release from individual vesicles at chromaffin cells have established that the events are temporally finite (Wightman et al., 1995). In this section we consider three models that could lead to the observed temporal profile. Diffusion in the extracellular space is ignored in all cases because the models describe results obtained with the electrode in contact with the cell surface. However, diffusion in the vesicular matrix is considered. In mast cells the intravesicular protein matrix expands upon exposure to the extracellular fluid (Zimmerberg et al., 1987; Curran and Brodwick, 1991) and in the presence of applied voltages (Nanavati and Fernández, 1993). The vesicular protein mucin in goblet cells also expands after exocytosis (Verdugo, 1990). In chromaffin cells the contents of the vesicle are also tightly associated

(Uvnäs and Åborg, 1984), although the chemical nature of the intravesicular association is not fully understood (Daniels et al., 1978). Thus, we consider a rate-determining swelling of the vesicular matrix as well.

Diffusion from a complex matrix

The random-walk algorithm was employed to simulate release of a uniformly distributed species from a sphere with a slower diffusion coefficient than that for the species in free solution (Fig. 1 *A*). This situation is equivalent to diffusion in a complex matrix and is a possible controlling mechanism for the release of catecholamine from within chromaffin granules. The flux of particles out of the hemispherical surface was used to model the flux of catecholamine from the exocytosed vesicle at the cell surface. The surface flux rises instantaneously to a maximum and then decays gradually in an exponential fashion to the original baseline. Because a uniform distribution of particles within the sphere necessitates that the largest number of particles are in the volume of the outer shell of the sphere, the largest flux of material occurs at the initial time.

Expanding gel matrix

Upon exocytosis, the intravesicular matrix is exposed to the extracellular environment and the influx of ions and water allows dissociation of the vesicle contents that, in mast cells, is accompanied by swelling of the matrix (Zimmerberg et al., 1987). The rate of expansion of this matrix could control the release of bound catecholamine. The kinetics of swelling spherical gels (Tanaka and Fillmore, 1979) can be described by a displacement vector $u(r, t)$, which represents the displacement of any point in the gel matrix from its final equilibrium location after the gel is fully swollen. This vector is described by

$$u(r, t) = -6\Delta a_o \sum_{n=1}^{\infty} \frac{(-1)^n}{n\pi}\left[\frac{X_n \cos X_n - \sin X_n}{X_n^2}\right] \times \exp\left(\frac{-n^2 t}{\tau}\right), \quad (1)$$

where

$$X_n \equiv n\pi\left(\frac{r}{a}\right) \quad (2)$$

and

$$\tau \equiv \frac{a^2}{D}. \quad (3)$$

The final radius of the spherical gel matrix is a, Δa_0 denotes the total increase in the radius of the sphere, r is the radial coordinate of the gel matrix, and D is the diffusion coefficient of the gel matrix ($D = E/f$, where E is the longitudinal bulk modulus of the network and f is the coefficient of friction between the polymer network comprising the gel and the gel fluid (Tanaka and Fillmore, 1979)). Thus, τ is the characteristic swelling time constant of the gel matrix.

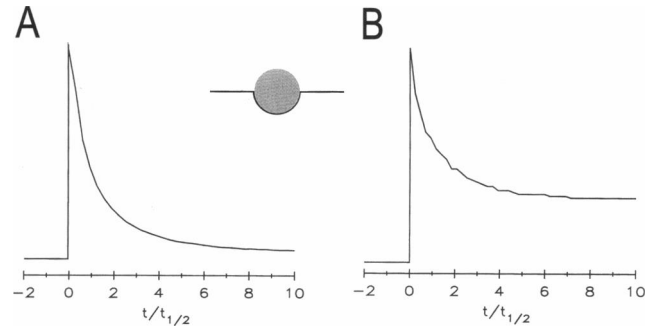

FIGURE 1 Flux of catecholamine from a complex matrix could slow diffusion out of the matrix and control the time course of exocytotic secretion from the vesicle. The cartoon in the center of the figure represents the simulation geometry. (*A*) Flux from a hemispherical surface of a species contained within the sphere calculated by random-walk simulation in which movement is limited only by diffusion. (*B*) Temporal course of the flux from a spherical surface that is rate limited by the expansion of a matrix. The curve takes a greater time to reach a baseline than the curve in *A*.

Assuming that the bound catecholamine within the protein matrix is not releasable until the matrix has fully swollen, the flux of bound species from the matrix will be controlled by the rate of expansion of the matrix. The partial derivative of $u(r, t)$ with respect to r is given by

$$\frac{\partial u(r, t)}{\partial r} = -6\Delta a_o \sum_{n=1}^{\infty} \frac{(-1)^n}{a}\left[\frac{2 \sin X_n - 2X_n \cos X_n - X_n^2 \sin X_n}{X_n^3}\right] \times \exp\left(\frac{-n^2 t}{\tau}\right). \quad (4)$$

Evaluation at $\partial u(r, t)/\partial r = 0$ gives the location of maximum swelling as a function of time. For the assumption given above, the time course of this relationship describes the cube root of the flux of species from the matrix because concentration is proportional to the expanded volume. The normalized curve is characterized by an instantaneous rise to the maximum amplitude and followed by a multiexponential decay that is very slow to reach the baseline (Fig. 1 *B*). In this case, the decaying region is determined by τ, which dictates the percentage expansion of the gel matrix. The protein matrix within chromaffin granules is a multiply charged species (Uvnäs and Åborg, 1984) and might be more appropriately modeled as a polyelectrolyte gel (Grimshaw et al., 1990). However, this was not attempted because it is dependent on a large number of unknown parameters.

Compound rate-limiting steps

The models above, in which diffusion of catecholamines through the vesicular matrix is rate limiting, predict an

initial instantaneous rise in concentration of the extruded species. In contrast, the experimental spikes exhibit a more gradual rise to the maximum than either of the models described above predict. Thus, these theoretical models give an incomplete description of the measured curves. An alternative description of the data is that more than one step is rate limiting. In the case of kinetic control by two steps, the observed response will be the convolution of the individual mechanisms.

In prior work we have shown the spikes can be characterized by an exponentially modified gaussian (EMG) curve (Jankowski et al., 1993; Wightman et al., 1995). This curve is composed of a gaussian with a standard deviation given σ and an exponential characterized by a time constant τ. The EMG curve, $i(t)$, is defined by the convolution integral:

$$i(t) = \frac{i_{max}\sigma}{\tau\sqrt{2\pi}} \times \int_0^\infty \exp\left[\frac{-(t - t_r - z)^2}{2\sigma^2}\right]\exp\left[\frac{-z}{\tau}\right] dz, \quad (5)$$

where i_{max} is the spike amplitude, t_r is the center of gravity of the gaussian, and z is a dummy variable of integration (Grushka, 1972). The exponential portion of this equation is consistent with the first diffusion model considered above. The convolution of this function with a gaussian leads to a curve shape consistent with those of the measured spikes and indicates that a second rate-determining process is also affecting the shape of the observed spikes.

RESULTS

Spikes from single cells induced by Ba^{2+}

Transient exposure of single chromaffin cells to 2 mM Ba^{2+}, which mimics many of the actions of Ca^{2+} (Jankowski et al., 1994; Pryzwara et al., 1993; von Ruden et al., 1993), induces long-lasting release of catecholamines. The flux of catecholamines during individual release events was measured amperometrically with a flame-etched carbon-fiber microelectrode gently pressed against the surface of the cell. Release events were observed as sharp current spikes (Fig. 2), which, when examined on an expanded time scale, exhibit a rapid but not instantaneous rise to the maximum followed by a more gradual decay to the baseline. Thus, as reported previously (Jankowski et al., 1994), the shape of the spikes resembles the EMG function, a shape that is more complex than suggested for the two models that consider diffusion from the vesicle to be rate limiting. We have previously shown that the microelectrodes used in this work can respond to changes in flux on a submillisecond time scale (Pihel et al., 1994).

In the following analysis 223 spikes from one cell are considered, but measurements at other cells gave similar results. As illustrated by the representative spikes in Fig. 3 *A*, none of the spikes exhibited an instantaneous rise to the maximum as predicted by the first two models considered above. Therefore, the spikes were fit to the EMG function (Eq. 5) to determine whether a two-step mechanism better

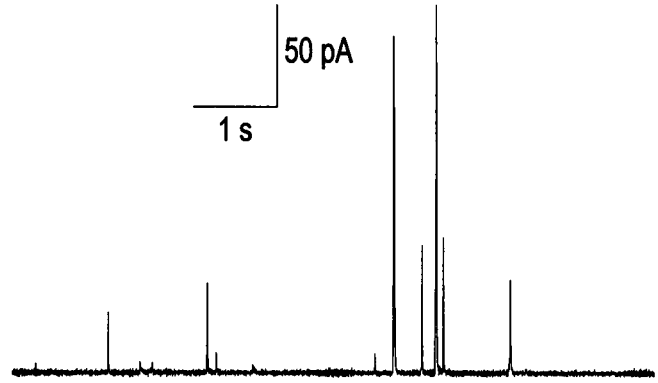

FIGURE 2 Single secretory events recorded at the surface of a chromaffin cell. Amperometric detection of exocytotic events induced by pressure ejection of 2 mM Ba^{2+} for 5 s.

describes the main portion of the spike. Values of σ, the standard deviation of the gaussian, and τ, the time constant of the exponential decay, were obtained (Fig. 3). The fit of this model to each spike was quite good, with an average correlation coefficient of 0.932. For this cell the mean value of σ was 2.1 ± 0.1 ms and τ was 9.7 ± 0.8 ms.

If the same kinetic event determines the magnitude of both σ and τ, their values would be expected to be strongly correlated. They are correlated (Fig. 3 *B*, correlation coefficient = 0.667), but the correlation is not very strong. Plots of σ and τ versus the integrated area of the spike (Fig. 4, *A* and *B*), which is proportional to the total quantity of catecholamine released (Wightman et al., 1991), also showed considerable scatter. The phenomenon characterized by the parameter σ appears stochastic (Ross, 1983) because it follows an exponential probability density function (Fig. 5).

The values of σ and τ allow calculation of the skew of the spikes, a statistical term that describes the similarity of the curves to a gaussian shape. Skew values of unity indicate a gaussian shape, whereas the shape for an exponential has a skew value of 2.0 (Grushka, 1972). For these spikes the skew varies from 1.2 to 1.9, with 32% of the spikes having a skew value less than 1.7. The broad range in skew values shows that the spikes exhibit considerable differences in

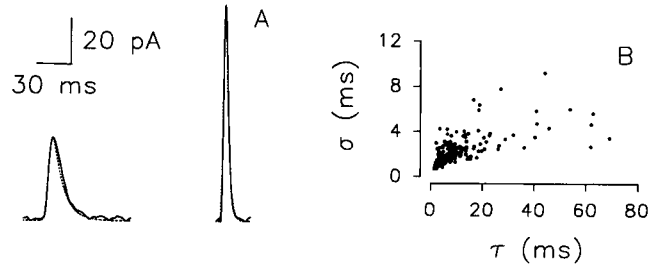

FIGURE 3 Characterization of release kinetics. (*A*) Representative current spikes obtained from experiments as described in Fig. 1. Superimposed on each trace is an EMG function with parameters σ and τ calculated from the associated statistical moments of the spike (Yau, 1977). (*B*) Graph of σ versus τ for 223 spikes measured at a single cell; linear regression of these data gives a correlation coefficient of 0.667.

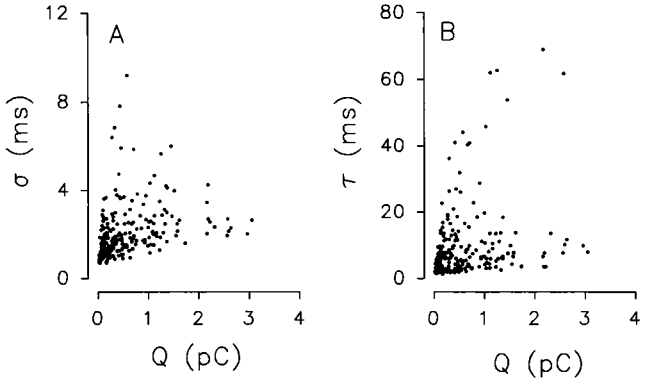

FIGURE 4 The kinetic parameters σ and τ show little correlation with the integrated current (Q) of the spike. (A) Plot of σ versus Q. (B) Plot of τ versus Q. The same 223 spikes presented in Fig. 3 B were analyzed.

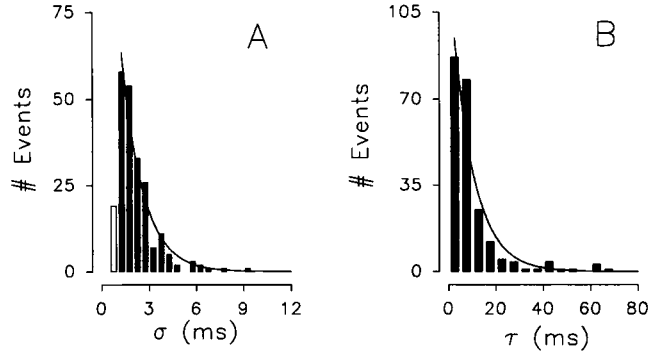

FIGURE 5 Distributions of the parameters σ (A) and τ (B) from the data presented in Fig. 3. Superimposed on each of the histograms is a best-fit exponential probability density function (PDF). The time constants of the exponential PDFs are 1.3 ms and 9.0 ms for σ and τ, respectively. The events in the white bars in A are distorted by the analog filtering (low-pass cutoff of 400 Hz) and were not included in the fit. In A data conform to an exponential distribution as determined by a χ^2 test ($\gamma^2 = 20.5$, df = 37). In B the observed data are significantly different from an exponential distribution ($p < 0.005$, $\gamma^2 = 106$, df = 21).

shape from one another when examined under these physiological conditions. In contrast, more than 95% of skew values were greater than 1.7 for spikes from cells in an extracellular medium of pH 8.2 (Jankowski et al., 1994), a condition selected to promote more complete dissociation of the vesicle contents.

Prespike features

Preceding some spikes (~27%) a small steady-state flux was found to occur (Chow et al., 1992). The spikes that exhibited a prespike feature were indistinguishable from the remaining spikes with respect to area under the spike, peak current, and width of the spike at half-height. The amperometric current (i) during the prespike feature was related to the catecholamine flux (f) by the relationship

$$f = \frac{i}{nF}, \tag{6}$$

where n is the number of equivalents of electrons in the oxidation (2 equivalents/mol for catecholamines) and F is Faraday's constant (96,485 coulombs/equivalent). A mean flux of 34,000 ± 2,000 molecules/ms was obtained (measured for 67 prespike features with a mean duration of 8.4 ± 0.4 ms, errors given as SEM).

An alternative electrochemical technique, cyclic voltammetry, was employed to determine the extracellular catecholamine concentration during the prespike feature (Wightman et al., 1995). With the sensor 1 μm from the cell surface, a concentration of 4.5 ± 0.5 μM was measured (prespike features from 16 spikes measured in 8 cells). Previous theoretical analysis has shown that the concentration measured in this way is related to the original concentration at the cell surface diluted by the volume of solution located between the cell surface and the electrode (Wightman et al., 1995). Assuming the source of the concentration has the mean spherical volume of a chromaffin vesicle (r = 170 nm; Coupland, 1968), the surface concentration (and that in the vesicle) is computed to be 34 mM.

Spikes from cells in low osmolarity solutions

The kinetics of catecholamine exocytosis were also investigated in solutions of low osmolality. Cells are stable and release can be induced in hyposmolal solutions (Hampton and Holz, 1983), whereas hyperosmolal solutions inhibit exocytosis (Hampton and Holz, 1983; Zimmerberg and Whitaker, 1985). Consistent with prior work, cells were observed visually to swell when the external solution was changed from an isosmotic medium (315 mOsm) to 200 mOsm. Release was measured in response to Ba^{2+} exposure. The lowered osmotic pressure increased the "quantal" size (charge) of the measured spikes (Fig. 6, Table 1). This

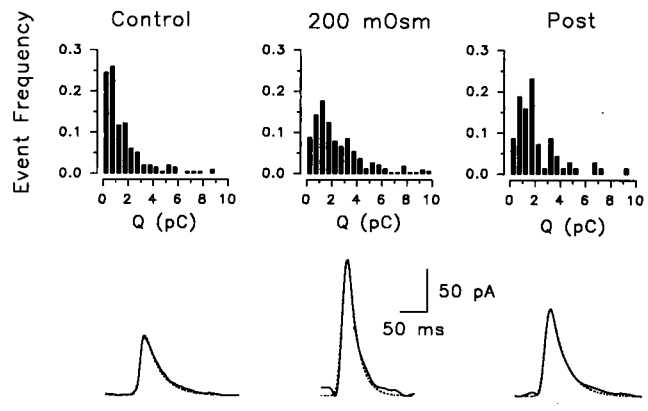

FIGURE 6 Osmotic changes alter quantal size and time course of release. The lower three spikes, fitted with an EMG function, are representative spikes obtained from cells bathed in each osmolality solution. The upper three graphs are the corresponding Q histograms for the data. Pooled data from six cells.

suggests an increased extent of dissociation of catecholamine from the vesicular matrix in hyposmotic media. Whether this occurs before or after vesicle-cell membrane fusion cannot be discerned, but the effect was partially reversed when the cell was returned to isosmotic conditions (Table 1).

Analysis of the spikes obtained in hyposmolal solutions with the EMG function revealed that the lowered osmotic pressure significantly decreased both τ and σ (Table 1). The changes in τ and σ were fully reversible because they were not significantly different from the original values when returned to isosmotic solution (Table 1).

DISCUSSION

The analysis of spike shape given above reveals that vesicular release from bovine adrenal medullary cells exhibits at least three sequential stages after vesicle-cell membrane fusion (Fig. 7). The first stage, the foot, is taken to be the formation of a transient fusion pore, by analogy to results in mast cells. Deconvolution of the main body of the spike with the EMG function reveals it is characterized by two time constants. The initial, gaussian portion of the curve, characterized by σ, is followed by an exponential decay, characterized by τ. The exponential nature of the decreasing flux during the latter portion of the spike is that predicted for a rate-limiting diffusion of catecholamine from the vesicular matrix but is not consistent with a rate-limiting swelling of the matrix. The process characterized by σ thus lies between the formation of the fusion pore and the full exposure of the vesicle contents to the extracellular space.

The catecholamine flux during the foot is frequently steady state but is only observed in a minority of vesicular events. Presumably, the fusion pore is short lived in the spikes where it is not observed and thus is merged with the main body of the spike. The duration of this transient state is unrelated to the subsequent steps, because the time course of spikes that exhibit this feature is indistinguishable from those that do not. The combined amperometric and cyclic voltammetric data allow an estimation of the diameter of the pore. In the case where mass transport from the pore mouth is rate limiting and the intravesicular concentration remains

TABLE 1 Mean spike characteristics measured at cells exposed to 2 mM Ba^{2+} in extracellular buffer of 315 mOsm control, 200 mOsm, and 315 mOsm post-hyperosmotic change

Extracellular buffer	Control	200 mOsm	Post
Q (pC)	1.7 ± 0.15	2.9 ± 0.21*	2.1 ± 0.22*
σ (ms)	4.6 ± 0.32	3.4 ± 0.22*	5.1 ± 0.61
τ (ms)	34 ± 2.5	23 ± 1.8	30 ± 3.6

Means are reported ± SEM. Statistical significance was tested using the Mann-Whitney test for nonparametric data. Data is pooled from six cells. *Means are significantly different ($p \leq 0.001$).

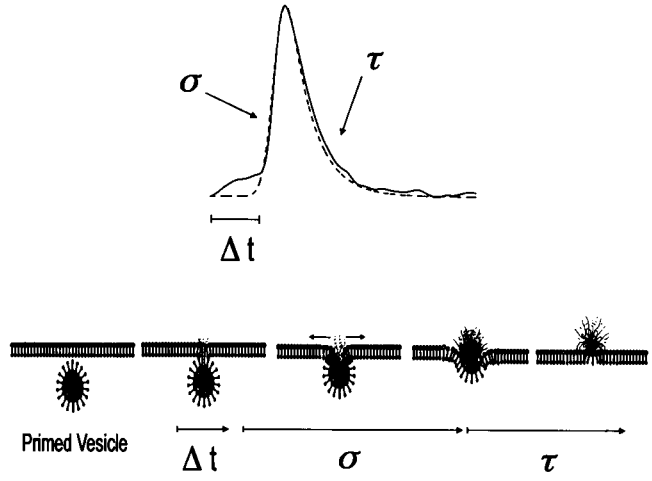

FIGURE 7 A single secretory event with an EMG function superimposed. Cartoon: Proposed stages in exocytosis. In response to stimulation, a vesicle fuses with the plasma membrane, resulting in a pore connecting the vesicular contents to the external medium. Flux of free intravesicular catecholamine is almost steady-state at this time because of restriction by the pore diameter. The duration of this stage is given by Δt. Next, the fusion pore expands, which leads to a gaussian flux from the vesicle characterized by a time constant σ. The dissociation rate of catecholamine from the protein matrix retards the release and is reflected in the parameter τ.

constant, the flux is related to the radius r of the disk-shaped pore by

$$f = 4rDC, \tag{7}$$

where C in this case is the concentration of extruded substance at the exterior surface of the pore and D is the diffusion coefficient (Crank, 1975). With the apparent concentration (C = 34 mM calculated from the cyclic voltammetry results) and the free solution diffusion coefficient ($D = 6 \times 10^{-6}$ cm^2 s^{-1}; Gerhardt and Adams, 1982) for catecholamines, a pore radius of 0.5 ± 0.1 nm is obtained from the measured flux. This calculated radius is in remarkable agreement with that found for the initial fusion pore at mast cells and is similar to the radius of ionic channels (Monck and Fernández, 1992, 1994). Note that this estimate is a lower limit; if diffusion of catecholamines adjacent to the cell is restricted (leading to a lower value of D), the pore radius will be proportionately larger.

The catecholamine concentration during the foot estimated by cyclic voltammetry is 10 times lower than the vesicular catecholamine concentration (Schroeder et al., 1992; Winkler and Westhead, 1980). Thus, only a portion of the vesicular catecholamine is free for release at this stage. The steady-state flux preceding the main secretion flux in mast cells is also of lower concentration than expected from the vesicular concentration (Alvarez de Toledo et al., 1993), and this is attributed to association with the vesicular matrix of heparin sulfate. In chromaffin cells association of the majority of the vesicular catecholamine can occur with chromogranin A and other intravesicular components

(Daniels et al., 1978; Yoo, 1994). During the time where the foot is apparent (the delay characterized by Δt in Fig. 7) the intravesicular matrix is exposed to the extracellular fluid. Water can enter the vesicle during this phase, hydrating the matrix and eventually causing it to swell, as observed during exocytosis in goblet cells (Verdugo, 1990) and in beige mouse mast cells (Zimmerberg et al., 1987).

In previous work we have shown that the spike shape is similar to that predicted by release from a point source followed by diffusion from a remote site through the extracellular fluid to the amperometric sensor (Schroeder et al., 1992; Jankowski et al., 1993). However, this interpretation can be excluded for these results because the EMG spike shape is retained when the sensor is touching the cell and no significant extracellular space exists for diffusion before detection (Wightman et al., 1995). The spike shape predicted by the extracellular diffusion model is an exponentially modified gaussian, in agreement with the data. However, that model predicts that the relative shape should remain unchanged (i.e., that σ and τ would be exactly correlated) irrespective of the distance of diffusion or value of the diffusion coefficient. In fact, we find that the rate of the initial, gaussian process characterized by σ is poorly correlated with τ, further negating the extracellular diffusion model. The lack of correlation strongly implies that two rate-limiting processes occur during the main body of the spike.

Thus, the time characterized by σ appears to be due to a separate kinetic step that is temporally located between the initially formed fusion pore, where the majority of catecholamines are tightly associated with the matrix, and the final stage, where the matrix is dissociated and release of catecholamines has fully developed. This gaussian portion of the spike may reflect the time for the fusion pore to expand from its initial dimensions to a larger structure that allows the maximal release. Pore expansion during the time characterized by σ could be driven by the tension of the cell membrane. Alternatively, it could be driven by expansion of the vesicular matrix caused by diffusion of extracellular ions into the vesicular space and accompanying hydration. Indeed, we have shown that alteration of the ionic composition of the extracellular fluid can dramatically alter the spike duration in this work and elsewhere (Jankowski et al., 1993, 1994). However, microscopic observations of exocytosis that suggest pore expansion can occur independently of swelling of the matrix (Monck et al., 1991; Terakawa et al., 1994) are consistent with the former view.

As a further test of the model given in Fig. 7, we investigated release in hypoosmotic solutions. The value of τ decreased, consistent with a more rapid swelling of the vesicular matrix, resulting in a more rapid efflux of catecholamines. Indeed, the strong association of the vesicular contents provides a way to lower the osmotic pressure in intact vesicles (Daniels et al., 1978; Yoo, 1994) and provides a driving force for release upon vesicle-cell fusion that would be increased in hypoosmotic solutions. However, this condition also causes a greater membrane tension because of cell swelling. Thus, the decreased value of σ could result from either effect. However, for both parameters, the stress causes reversible changes because they return to their original values in isosmotic solutions. Remarkably, a significant change is seen in the amount of catecholamine released in medium of lowered osmolality. This suggests that under physiological conditions exocytosis results in only partial release of vesicular contents, a concept for which previous evidence exists (Alvarez de Toledo et al., 1993; Jankowski et al., 1993, 1994).

Because the nature of fusion proteins and lipidic membranes seems to be conserved (White, 1992; Barinaga, 1993), observations of these processes in chromaffin cells may be relevant to those in neurons. The sequential events should be more rapid for smaller neuronal vesicles, which will have greater lipidic tension but lack the dense core (Monck and Fernández, 1992, 1994). In fact, initial pore formation (Spruce et al., 1990; Khanin et al., 1994) or pore expansion could control the rate of transmitter arrival at the postsynaptic membrane because diffusion across the narrow synaptic cleft is almost instantaneous (Eccles and Jaeger, 1958). However, neuronal vesicles contain proteoglycan (Jan and Südhof, 1993; Scranton et al., 1993), and this could play a role similar to that of the proteinaceous matrix that limits the rate of dissociation of the chemical messengers in the present work. Thus, this process could dictate the duration of neurotransmitter occupancy in the synaptic cleft.

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