

Time Course of Release of Catecholamines from Individual Vesicles during Exocytosis at Adrenal Medullary Cells

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ABSTRACT The time course of extrusion of the vesicular contents during exocytosis has been examined at adrenal medullary cells with carbon-fiber microelectrodes. Two electrochemical techniques were used: cyclic voltammetry and amperometry. Spikes obtained by amperometry had a faster time course than those measured by cyclic voltammetry, consistent with the different concentration profiles established by each technique. However, the experimental data obtained with both techniques were temporally broadened with respect to dispersion of an instantaneous point source by diffusion. Measurements with the electrode firmly pressed against the cell surface established that the temporal broadening is a result of a rate-limiting kinetic step associated with extrusion of the vesicular contents at the cell surface. The data do not support a rate-limiting process due to restricted efflux from a small pore. When combined with previous results, the data suggest that the rate-limiting step for chemical secretion from adrenal medullary cells during exocytosis is the dissociation of catecholamines from the vesicular matrix at the surface of the cell.

INTRODUCTION

Electrochemical measurements with microelectrodes at isolated adrenal medullary cells provide direct chemical evidence for the quantal nature of exocytosis in real time (Leszczyszyn et al., 1990; Wightman et al., 1991). The adrenal medullary cell was selected for these studies because secretion at these cells has been extensively characterized (Livett, 1984; Burgoyne, 1991; Carmichael and Stoddard, 1993). Amperometric spikes measured with electrodes fabricated from carbon fibers have been shown to be due to the oxidation of the catecholamines contained within a single vesicle (Wightman et al., 1991). These measurements have excellent temporal resolution and provide a means to probe the kinetic processes associated with the extrusion of amines into the extracellular space (Chow et al., 1992; Jankowski et al., 1993; Alvarez de Toledo et al., 1993) and its diffusion away from the cell (Kawagoe et al., 1991; Schroeder et al., 1992). Fast-scan cyclic voltammetry at individual adrenal cells lacks the time resolution of amperometry, but allows the detected species to be identified as a catecholamine (Leszczyszyn et al., 1990, 1991), and can be used to observe changes in the identity of the secreted catecholamines (epinephrine or norepinephrine) during secretion (Ciolkowski et al., 1992; Pihel et al., 1994).

In prior work we have characterized the temporal characteristics of individual catecholamine packets detected by amperometry during exocytosis (Schroeder et al., 1992; Jankowski et al., 1993, 1994). We compared the spike shape with that predicted for diffusional dispersion of an instan-

taneous point source released at random locations on the cell surface as a function of distance of the electrode from the release site. With the electrode 5 μm from the cell surface, good agreement was found. The shape of the spikes (similar to an exponentially modified Gaussian) correlated well with that predicted for diffusional dispersion, and the range of widths at half-height ($t_{1/2}$) of the individual spikes was consistent with that expected for a random distribution of release sites across the cell surface (Schroeder et al., 1992). However, amperometric measurements with the electrode 1 μm from the cell surface do not agree with this model (Jankowski et al., 1993). Although the shape of the individual spikes is consistent with diffusional dispersion, fewer narrow spikes are observed than predicted for an instantaneous point source. In addition, if the amperometric data are purposely over low-pass filtered, low amplitude spikes are revealed which are much broader than predicted for diffusional dispersion of an instantaneous point source originating at a release site at the most distant portion of the cell surface (unpublished results). In more recent work the assumption of randomly distributed release sites has been shown to be incorrect as well, because discrete zones on the cell surface which support exocytosis were revealed with the use of dual electrodes with micron dimensions (Schroeder et al., 1994).

Thus, prior results suggest that the simplest view of exocytosis, instantaneous release from the cell surface followed by diffusion of the catecholamines away from the cell, is incorrect at adrenal medullary cells. Rather, there appears to be a rate-determining step in the secretion process which is slower than the diffusional rate of mass transport of catecholamines. This could be associated with the fusion of the vesicular and cellular membranes or with the dissociation of the vesicular contents either at the cell surface or, possibly, at the sensor surface, if the contents are released in an intact form. In this work, we address these questions by examining vesicular release with carbon-fiber microelectrodes with two different electrochemical techniques: cyclic voltammetry

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and amperometry. Both techniques have sufficient time resolution to resolve individual exocytotic events. Cyclic voltammetry is particularly well suited for this investigation since it does not generate as steep a concentration gradient as amperometry. This is because it is a transient technique and does not consume the catecholamines. Thus, cyclic voltammetry should not affect the exocytotic process as much as amperometry. The combined results from measurements at different distances from the cell surface reveal that the rate-limiting step in exocytotic release from adrenal medullary cells is the extrusion and/or the dissociation of the vesicular contents at the surface of the cell.

MATERIALS AND METHODS

Single-cell secretion experiments

Bovine adrenal chromaffin cells were isolated from fresh tissue, enriched in epinephrine producing cells, and maintained in culture at a density of 6×10^5 cells per 35-mm diameter plate as previously described (Leszczyszyn et al., 1991; Ciolkowski et al., 1992). Experiments were performed between days 4 and 8 of culture. For secretion experiments, the culture medium was replaced with a balanced salt solution containing (in mM) NaCl (150), KCl (4.2), NaH_2PO_4 (1.0), glucose (11.2), MgCl_2 (0.7), CaCl_2 (2.0), and HEPES (40), adjusted to pH 7.4 with sodium hydroxide. The cell culture plates were placed on the stage of an inverted microscope (Axiovert 35, Zeiss, Thornwood, NY) and the carbon-fiber microelectrode was positioned adjacent to a single cell with a piezoelectric driver (PCS-250 patch clamp driver, Burleigh Instruments, Fishers, NY) as previously described (Schroeder et al., 1992). The uncertainty in the relative position of the electrode from the cell surface is estimated to be 0.5 μm . Cells were induced to secrete by local application of agents through a pressure ejection pipette. The agents used were 20 μM digitonin (Jankowski et al., 1993), 2 mM Ba^{2+} (Schroeder et al., 1994), or 60 mM KCl (prepared in buffer with the concentration of NaCl lowered proportionately).

Reagents

All chemicals were used as obtained from commercial sources. The culture media, consisting of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium, was obtained from GIBCO Laboratories (Grand Island, NY). Digitonin was acquired from Fluka (Ronkonkoma, NY). Collagenase (type I) for digestion of gland tissue was obtained from Worthington Biochemicals (Freehold, NJ). Renografin-60 was from Squibb Diagnostics (New Brunswick, NJ). All other chemicals were purchased from Sigma (St. Louis, MO). Solutions were prepared with double-distilled water.

Electrochemistry

Microelectrodes were fabricated from carbon fibers (Thornel P-55, Amoco Corp., Greenville, SC; nominal radius, 5 μm) sealed in glass capillaries (Kawagoe et al., 1993). The electrodes were polished at a 45° angle on a micropipette beveller (model BV-10, Sutter Instruments, Novato, CA) and the elliptical tips were immersed in 2-propanol until ready for use. Electrodes of smaller diameter were made by flame-etching the carbon fibers (Kawagoe et al., 1991; Strein and Ewing, 1992) and insulating the sides of the resulting cone with poly(oxyphenylene) (Schroeder et al., 1994). Calibrations were performed before and after experiments using a flow-injection apparatus (Howell et al., 1986). A sodium-saturated calomel electrode (SSCE) was used as a reference electrode in all experiments.

The electrochemical measurements used an EI-400 potentiostat (Ensmann Instrumentation, Bloomington, IN). For amperometry the applied potential at the carbon-fiber electrode was 650 mV vs. SSCE and the output was digitized with a VCR adapter (model PCM-2, Medical Systems Corp.,

Greenvale, NY) and recorded on videotape. For cyclic voltammetry the output from the potentiostat was interfaced (Labmaster, Scientific Solutions, Solon, OH) to an IBM-compatible computer and acquired with locally written software. The current was measured during the application of a triangular potential wave form to the working electrode (-0.5 V to $+1.0$ V vs. SSCE) at a scan rate of 800 V/s. Successive voltammograms were generated at 16.7-ms intervals to allow for relaxation of the electroinduced concentration gradient between scans. The sample-and-hold circuitry of the potentiostat was set to monitor the current from successive voltammograms at the peak potential for the oxidation of catecholamines (0.75 V vs. SSCE), and its output was also digitized and stored on videotape. The sample-and-hold circuitry was modified to provide a low-pass cutoff frequency of 32 Hz.

Data analysis

Data were transferred from videotape to computer using commercially available hardware and software (Cyberamp 320, Fetchex, and Axotape, Axon Instruments, Foster City, CA). Locally written software was used to determine individual exocytotic events and to extract maximal amplitudes, widths at half-height, and areas. Only signals with amplitudes greater than five times the root-mean-square noise were accepted as individual events. For cyclic voltammetric measurements, the current amplitudes were converted to concentrations based on post-calibrations. Because the diffusion layer generated during the rapid potential scan is small (<1 μm), calibrations obtained in solution can be used to evaluate the measurements at cells. The detection limit for an individual event measured by cyclic voltammetry was ~ 3 μM . Amperometric measurements cannot be converted to concentrations because the diffusion layer distance in solution is much larger than the cell-electrode spacing used in the experiments.

Digital simulation

A three-dimensional random-walk simulation was used to evaluate the diffusional dispersion of an instantaneous point source of molecules which originates on the surface of a hemisphere with an adjacent disk-shaped sensor (Schroeder et al., 1992). The geometry used in the simulation is shown in Fig. 1 A. The distance between the electrode and the pole of the cell was divided into a minimum of 10 spatial intervals (length, x) for the simulation, and time (t) in the simulation was related to this interval by $x^2 = 2Dt$ (Berg, 1983), where D is the diffusion coefficient. Nine release sites were evaluated along the cell surface spaced at 1- μm increments in the axial plane. The spacing of the sites divides the hemisphere into equal surface area segments. In this way, if release occurs at random locations on the cell surface, the simulations provide a prediction of the range of spike amplitudes and widths at half-height as a result of diffusion from the release sites to the sensor surface. Each point source was modeled as the release of 3.5×10^6 molecules (Schroeder et al., 1992) on the hemisphere surface at the specified location.

To simulate the results sampled by cyclic voltammetry, the electrode surface was treated as a reflecting plane, and the average number of molecules immediately adjacent to the electrode surface was computed after each iteration. This value was converted to a concentration by computing the effective volume defined by the electrode radius and the size of the random-walk step. To simulate the amperometric experiment, each molecule which reached the electrode surface was removed from the experiment. This flux of molecules at the electrode surface was then converted to a current.

To evaluate the effects of diffusional dispersion on surface events other than an instantaneous point source, the surface functions were convoluted (Engstrom et al., 1988) with the curves generated by the random-walk simulations using the Fourier transform approach (Bracewell, 1986). The surface function and the diffusional dispersion (the impulse response function determined by the simulation) were each converted to the frequency domain, multiplied, and the response was given as the inverse Fourier transform. The Fourier transform package used was 87FFT (Ver. 2.03, MicroWay, Kingston, MA).

RESULTS

Quantal detection of catecholamine release by cyclic voltammetry

Release was monitored simultaneously by cyclic voltammetry and amperometry with two electrodes placed adjacent to a cell. Secretion was induced by exposure of individual cells to 20 μM digitonin in the presence of 2 mM Ca^{2+} , conditions previously shown to induce exocytosis for several minutes (Jankowski et al., 1993). For measurements at three cells, the average spike frequency during the first 120 s of release was 0.92 Hz at the amperometric electrode and 0.90 Hz at the voltammetric electrode. Thus, these two methods appear to monitor the same exocytotic events, although the detected percentage of the total number of exocytotic events is not known. Representative traces recorded by cyclic voltammetry are shown in Fig. 2. Concentration changes in the form of sharp spikes are observed with the electrode positioned 1 or 5 μm from the top of the cell (Fig. 2, A and B, respectively). The individual cyclic voltammograms shown in each trace are from a single spike and verify that the species detected are catecholamines (Wightman et al., 1991).

Voltammetric spike characteristics

The shape of the spikes was compared to that predicted by the simulation of instantaneous release and diffusional dispersion. For this comparison the concentration was normalized to its maximum value ($[\text{CA}]_{\text{max}}$) and the time axis to its width at half-height ($t_{1/2}$), a procedure which allows simulated spikes to be overlaid irrespective of the distance over which they diffuse (Schroeder et al., 1992). At both cell-electrode spacings the shape of the experimental spikes was similar to the theoretical curve (Fig. 3 shows two examples and the average correlation coefficients are given in Table 1). Some spikes deviate from this shape in that they exhibit a pre-spike feature (Fig. 3 B), a feature also observed by amperometry (Chow et al., 1992; Jankowski et al., 1993). The cyclic voltammograms recorded during these pre-spike features clearly identify that they are due to catecholamines.

TABLE 1 Characteristics of spikes detected by cyclic voltammetry at cells permeabilized with 20 μM digitonin in the presence of 2.0 mM Ca^{2+} in pH 7.4 buffer at room temperature

Characteristic	Cell-electrode spacing	
	1 μm	5 μm
$t_{1/2}$ (ms)	63 (80 ± 3)	69 (83 ± 2)
$[\text{CA}]_{\text{max}}$ (μM)	12.3 (18.0 ± 0.8)	9.9 (13.4 ± 0.4)
Correlation coefficient*	0.831	0.808
Number of spikes	529	809
Number of cells	8	9

Data are reported as medians because the data distributions are not symmetrical. Means \pm SEM are also reported (in parentheses) for comparison to prior work.

*Correlation of normalized experimental spike shape with normalized simulated spike shape.

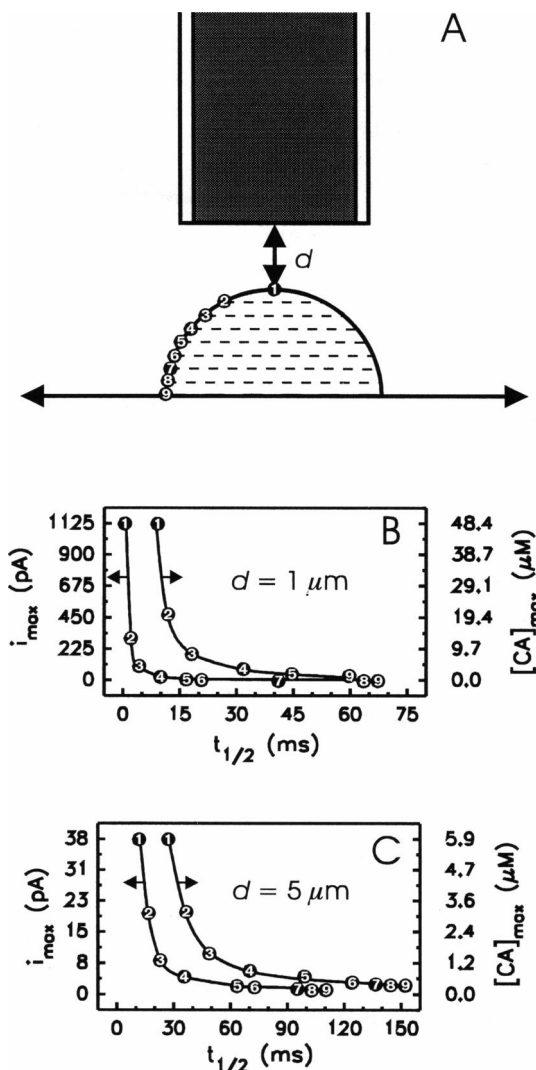


FIGURE 1 Geometry and results of the random-walk simulations. (A) The cell is represented by a hemisphere with 8- μm radius (Winkler and Westhead, 1980) located on an infinite reflecting plane. The electrode is represented by a disk with a 6- μm radius located symmetrically above the pole of the cell at a distance d . The electrode is surrounded by a 1- μm ring of insulation. The release sites are given by the numbered solid circles and occur at 1- μm increments in the axial direction. B and C give the spike characteristics predicted from the random-walk simulations for an electrode located 1 and 5 μm from the cell, respectively. Exocytotic release was assumed to be an instantaneous point source from a vesicle with a 156-nm radius (Coupland, 1968) which contained 0.36 M catecholamine (Schroeder et al., 1992). The diffusion coefficient for catecholamines used was $6 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$ (Gerhardt and Adams, 1982). The numbers on the points in B and C indicate their sites of origination on the cell surface (points 6–8 in B were not run for voltammetry). The calculations for cyclic voltammetry show the maximal concentration ($[\text{CA}]_{\text{max}}$) and the width at half-height ($t_{1/2}$). For amperometry, the maximum current (i_{max}) is shown with its associated $t_{1/2}$ value. The arrows on the curves indicate which axis applies for the respective curves.

To quantitatively characterize the measured spikes, the $t_{1/2}$ and $[\text{CA}]_{\text{max}}$ values of each spike were determined. A broad range of values for each parameter was found at each cell-electrode spacing. Table 1 contains a summary of the experimentally determined values of $[\text{CA}]_{\text{max}}$ and $t_{1/2}$ (the

FIGURE 2 Cyclic voltammetric response measured at the catechol oxidation wave (750 mV vs. SSCE) in successive scans from individual cells in response to 20 μ M digitonin (exposure for 8 s) in the presence of 2 mM Ca^{2+} . The concentration axis was obtained from electrode calibration. (A) 1- μ m cell-electrode spacing. (B) 5- μ m cell-electrode spacing. In each trace, the stimulus application is shown by the horizontal bar below the trace. The background-subtracted voltammograms were obtained from the points designated by asterisks.

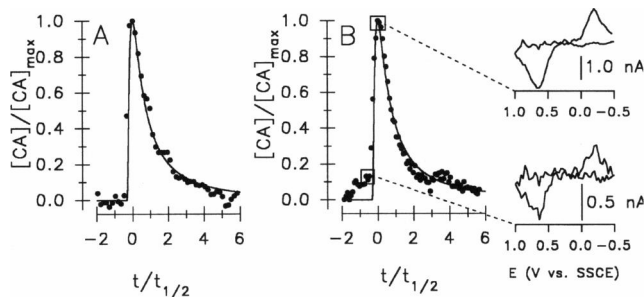
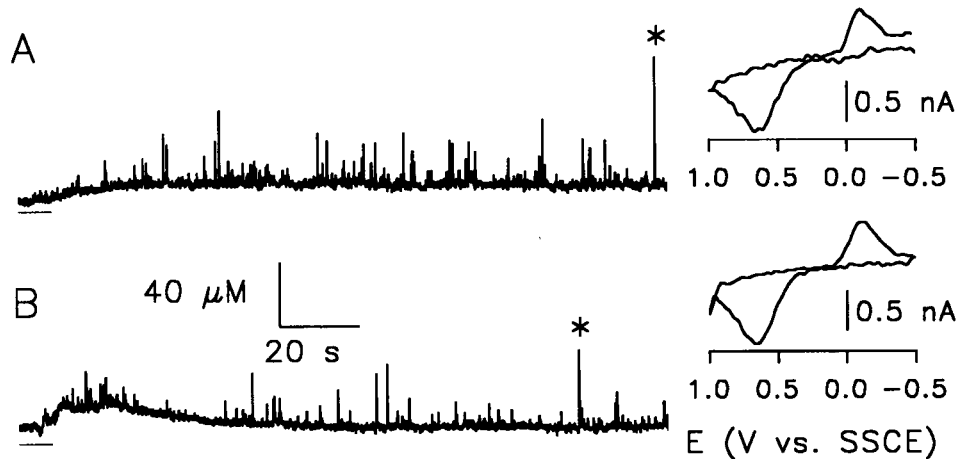


FIGURE 3 Normalized spikes detected by cyclic voltammetry. Each data point is the concentration determined from a single cyclic voltammogram recorded at the time indicated, and the solid lines are the curves obtained by digital simulation of instantaneous point-source release and diffusional transport to the electrode. (A) Spike recorded with a 5- μ m cell-electrode spacing. Spike characteristics: $[\text{CA}]_{\text{max}} = 22.4 \mu\text{M}$; $t_{1/2} = 62 \text{ ms}$; $R = 0.978$. (B) Spike recorded with a 1- μ m cell-electrode spacing. Spike characteristics: $[\text{CA}]_{\text{max}} = 40.8 \mu\text{M}$; $t_{1/2} = 177 \text{ ms}$; $R = 0.975$. Background-subtracted voltammograms were obtained by averaging the three voltammograms obtained at the times indicated by the points enclosed by the associated boxes.

individual values are plotted logarithmically in Fig. 4). For measurements with a 5- μ m cell-electrode spacing, $\sim 93\%$ of the spikes have $t_{1/2}$ values within the range predicted by simulation of diffusional dispersion from an instantaneous point source located at different regions on the cell surface (27 to 147 ms, Fig. 1). In contrast, the spikes obtained at a cell-electrode spacing of 1 μ m are not consistent with the diffusional predictions. The $t_{1/2}$ distribution is little changed from that obtained with a 5- μ m cell-electrode spacing. Approximately 45% of the $t_{1/2}$ values are greater than the largest value predicted by the instantaneous point-source model (60 ms for an exocytotic event which occurs at the base of the cell). Furthermore, the median concentration is similar at each cell-electrode spacing (Table 1) whereas the model predicts an eightfold decrease when the spacing is increased from 1 to 5 μ m.

The broad range of $[\text{CA}]_{\text{max}}$ values clearly indicates a large degree of biological variability in the individual exocytotic events (Fig. 4). Superimposed on the logarithmic plot of the data measured 1 μ m from the cell surface are the predictions

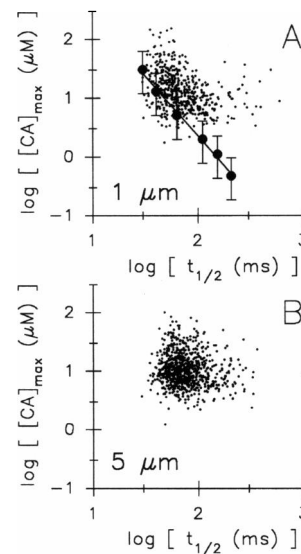


FIGURE 4 Logarithmic plot of data obtained by cyclic voltammetry from individual spikes. (A) 1- μ m cell-electrode spacing. Solid circles are digital simulation predictions assuming a diffusion coefficient of catecholamines of $1.8 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (see text); least-squares linear fit (slope = -2.0) to the circles is shown by the lower solid line. See text for explanation of error bars. Data from 8 cells. (B) 5- μ m cell-electrode spacing. Data from 9 cells.

from the instantaneous point-source model with standard deviations placed on the simulated data points which are taken from the reported deviations in the radii of the granules (Coupland, 1968). The position of the theoretical line on this Fig. (4 A) has been adjusted to fit the data by lowering the apparent diffusion coefficient by a factor of 3.4 from the value in free solution ($6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, Gerhardt and Adams, 1982). From this plot it appears that the full distribution of spikes with wide $t_{1/2}$ values is not detected because of the signal-to-noise limit ($\approx 3 \mu\text{M}$). Nevertheless, the distribution appears to be appropriately sampled for $t_{1/2}$ values $< 150 \text{ ms}$. The poor agreement between the experimental data and the model are apparent in the differences in linear least-squares slopes of the data (slope of -2 for the diffusional model (Fig. 4 A) and -0.8 for experimental spikes with $t_{1/2} < 150 \text{ ms}$ (not shown)).

Amperometric detection of spikes

As we have previously shown, the spike characteristics measured by amperometry with digitonin permeabilization are also inconsistent with diffusional dispersion of an instantaneous point source originating at random locations on the cell surface (Jankowski et al., 1993). However, this does not unequivocally prove the instantaneous point-source model is inappropriate, because the predicted spike characteristics depend upon the location of the exocytotic site on the cell surface which is unknown. To remove this uncertainty, measurements were made in the amperometric mode with the electrode 1 μm from the cell surface and then repeated at the same cell with the electrode moved until the electrode surface was in complete contact with the cell as confirmed by physical deformation of the cell. This action did not in itself induce secretion even though a potential of 0.650 V was applied to the electrode (at some cells the cyclic voltammetry wave form did induce exocytosis with the electrode touching the cell (data not shown)). In the latter position, the detected catecholamines can only originate from the region of the cell surface directly under the electrode. The secretagogue used was 60 mM K^+ , which causes secretion for 25–45 s after exposure to a 3-s ejection.

The spike characteristics from these measurements are shown in logarithmic form in Fig. 5. Superimposed on the data in Fig. 5 A (1- μm cell-electrode spacing) is the theoretical prediction from the instantaneous point-source model. The slope of the best-fit line to the experimental data (-0.411 , $R = 0.308$ (not shown)) is significantly different than the theoretical slope (-1.449). The median characteristics are summarized in Table 2. Because the data in the two columns come from the same cells, the differences in characteristics are only a function of the cell-electrode spacing. With the electrode touching the cell the median spike area increased by 15%, the median peak current increased by 89%, and the median $t_{1/2}$ value decreased by 16% (Fig. 5 C, Table 2). While these are significant differences, their changes in magnitude, especially of the $t_{1/2}$ value, are small.

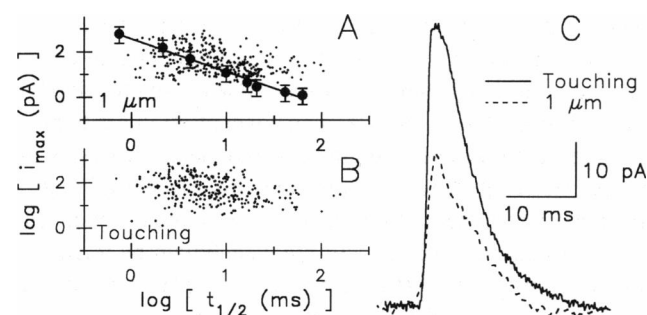


FIGURE 5 Amperometric data obtained at individual cells during exocytosis induced by 60 mM K^+ (3-s exposure). (A, B) Logarithmic plot of results from individual spikes. (A) 1- μm cell-electrode spacing. Solid circles are digital simulation predictions assuming a diffusion coefficient of catecholamines of $6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$; least-squares linear fit to the circles is also shown (slope = -1.449). See text for explanation of error bars. (B) Electrode in physical contact with the surface of the cell. (C) Single spikes with the median spike amplitude and half-width values for each data set.

TABLE 2 Characteristics of spikes measured at cells exposed to 60 mM K^+ with the microelectrode 1 μm away or touching the cell

Characteristic	Cell-electrode spacing	
	1 μm	Touching
Q (pC)	0.47 (0.84 ± 0.06)	0.54^{\dagger} (1.10 ± 0.09)
$t_{1/2}$ (ms)	7.4 (11.9 ± 0.8)	6.2^{\ddagger} (11.2 ± 1.0)
i_{\max} (pA)	27 (82 ± 7)	51^* (108 ± 10)
Number of spikes	334	284
Number of cells	7	7

Amperometric data were collected at 1 ms/pf and low-pass filtered at 400 Hz. Data are reported as medians because the data distributions are not symmetrical. Means \pm SEM are also reported (in parentheses) for comparison to prior work. All data was collected from the same seven cells.

*Values are significantly greater than amperometry 1 μm away (Mann-Whitney test, $p < 0.001$).

† Values are significantly greater than amperometry 1 μm away (Mann-Whitney test, $p < 0.02$).

‡ Values are significantly less than amperometry 1 μm away (Mann-Whitney test, $p < 0.05$).

If the concentration in each vesicle is assumed to be uniform (Wightman et al., 1991), then the amount which reaches the electrode will be a function of the volume, or radius cubed, of the vesicle in which it was originally contained. Thus, a histogram of the cube root of the integrated current of each amperometric spike (Q, units of charge, related by Faraday's law to the number of moles of oxidized catecholamines) should have a Gaussian shape. This is the case for the spikes measured with the electrode touching the cell (Fig. 6 A) as well as for the more remote location (data not shown). The percent standard deviation of the curve in Fig. 6 (36%) is consistent with the reported standard deviation (Coupland, 1968) of the vesicular radii (32%).

Comparison of amperometric spikes at 1- μm and 5- μm spacing

Simultaneous measurements were made with two adjacent electrodes of 1- μm radius positioned 1 μm from the cell surface to monitor release induced by 2 mM Ba^{2+} (Fig. 7, A and B). During the prolonged release, one of the electrodes was retracted to 5 μm from the cell (Fig. 7 A, second portion of trace). Consistent with our prior report (Jankowski et al., 1993), an approximately fivefold decrease in spike amplitude was seen (compare Fig. 1, B and C), whereas the model for an instantaneous point source predicts a more than 20-fold decrease in amplitude for the further distance. To examine whether this is consistent with a temporally broadened surface process and diffusional dispersion, the events observed 1 μm from the cell (Fig. 7 B, second portion of trace) were convoluted with the point-source response for an event 5 μm from the cell surface (Schroeder et al., 1992). The relative decrease in amplitudes of the convoluted response is in close agreement with the measured response (compare first portion of trace (1- μm spacing) in Fig. 7 B and convolution (convoluted 5- μm spacing) in Fig. 7 C with traces in Fig. 7 A, where the electrode was physically moved from 1 μm to 5 μm).

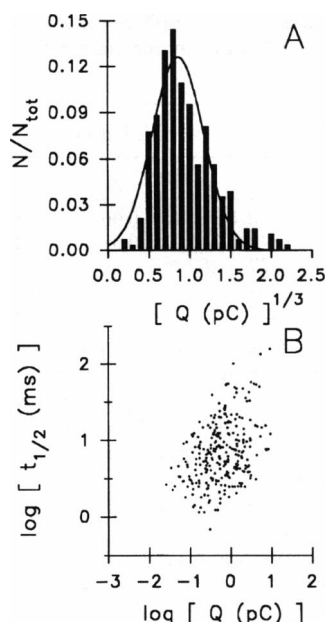


FIGURE 6 (A) Histogram of the cube root of the total charge of each amperometric spike (Q) for the experiments with the electrode touching the cell. The solid line is the best fit of a Gaussian distribution (mean = $0.86 \text{ pC}^{1/3}$, corresponding to a charge of 0.64 pC with standard deviation = $0.31 \text{ pC}^{1/3}$) to the data as determined by nonlinear regression. (B) Correlation of $t_{1/2}$ values with Q values obtained by amperometry with the electrode touching the cell.

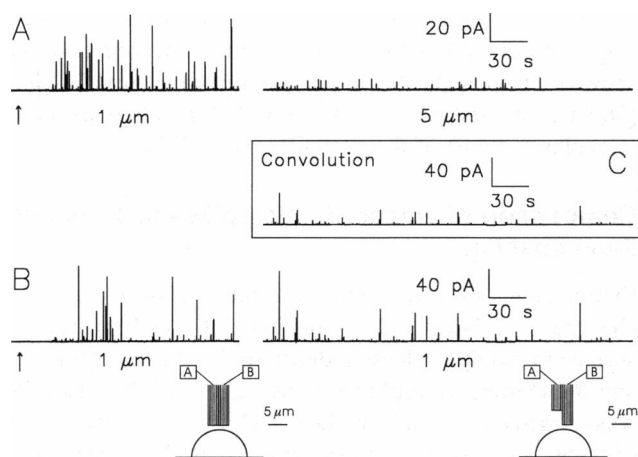


FIGURE 7 Comparison of simultaneous amperometric recordings from two adjacent electrodes during exocytosis induced by 2 mM Ba^{2+} (stimulus application indicated by the arrow). Figures below the current traces indicate relative placement of electrodes A and B corresponding to trace A and trace B. First portion of traces: current at each electrode positioned $1 \mu\text{m}$ from the cell surface. In the second portion of each trace the electrode in trace A was retracted to $5 \mu\text{m}$ from the cell surface while the electrode in trace B was left at its original position. Trace C (inset) is the result of convolution of the response in the second portion of trace B with the impulse response function for diffusion over $5 \mu\text{m}$ (Engstrom et al., 1988; Schroeder et al., 1992).

DISCUSSION

Carbon-fiber microelectrodes placed adjacent to individual bovine adrenal medullary cells enable the quantal release of

catecholamines to be monitored as individual, time-resolved events (Wightman et al., 1991). Prior work has shown that secretion from individual vesicles measured by amperometry appears to be slower than predicted for instantaneous release of the vesicular contents followed by diffusional dispersion before reaching the detecting electrode (Jankowski et al., 1993). In the present work, this view is supported by measurements with a different electrochemical technique, cyclic voltammetry. Furthermore, amperometric measurements with the electrodes touching the electrode surface unequivocally show that the temporally restricted process occurs at the cell surface. Taken together, the data demonstrate that the process of exocytosis at adrenal medullary cells is rate limited by the extrusion of catecholamines from the vesicle. Thus, for the first time it is established that there is a rate-determining step in secretion that occurs after formation of the fusion pore (Monck and Fernandez, 1994) between the vesicle interior and the plasma membrane.

Cyclic voltammetry allows the released substances to be identified, and thus, allows the pre-spike feature to be identified as a change in catecholamine concentration (Fig. 3 B). This feature is only present on a small percentage ($<20\%$, Jankowski et al., 1993) of the spikes, but appears to be a marker of the initial stages of the vesicular extrusion process (Chow et al., 1992; Alvarez de Toledo et al., 1993). This technique also is advantageous with respect to amperometry, because the amplitude of the oxidation current is directly proportional to the spatially averaged concentration at the electrode surface, whereas amperometry provides a signal proportional to the flux of catecholamines. The chief disadvantage of cyclic voltammetry is the reduced temporal resolution. For example, spikes with $t_{1/2}$ values of 66.7 ms will only have five points across each half-width with the repetition value used in this work which can lead to underestimation of the maximal concentration by as much as 7% and concomitant overestimation of the half-width by 9% . In contrast, amperometry with carbon-fiber electrodes can be used to monitor submillisecond changes (Pihel et al., 1994).

The major difference between the two techniques, however, is their respective concentration profiles (Bard and Faulkner, 1980). In amperometric measurements, all of the catecholamines which reach the electrode are oxidized to the quinone form. The result is a steep concentration gradient of the catecholamines between the cell and electrode during an exocytotic event. We have shown that this concentration gradient can force release to occur from cells under conditions where vesicle fusion can occur but catecholamine release is normally precluded (for example, in $\text{pH } 5.5$ media; Jankowski et al., 1993). In contrast, during a cyclic voltammogram the catecholamines are transiently oxidized and then rapidly returned to their initial state. Thus, the concentration profile of the catecholamines between the cell and the electrode, and its associated temporal changes, are similar to those which would exist at any adjacent surface, including an opposing membrane in a synapse. This difference leads to the larger temporal half-width of the spikes predicted for

cyclic voltammetry by the instantaneous point-source diffusional model when compared to amperometry (Fig. 1, *B* and *C*). Experimentally this prediction is confirmed: $t_{1/2}$ values from catecholamine spikes determined with cyclic voltammetry are greater than those found with amperometric measurements.

While spikes measured with cyclic voltammetry (1- μm cell-electrode spacing) have the exponentially modified Gaussian shape expected for diffusional dispersion (Fig. 3), 45% of the spikes have $t_{1/2}$ values which are greater than expected for diffusional dispersion of an instantaneous release event at the base of the cell. Similar results have been reported for amperometric measurements with this cell-electrode spacing (Jankowski et al., 1993) and are confirmed in the present report with K^+ stimulation. Disagreement of the instantaneous point-source model and the data is also seen when the slopes from linear regression of the data and the model are compared (Figs. 4 *A* and 5 *A*). Indeed, the wide range of the measured values suggest that the rate-determining process is stochastic and that these attempts at linearizing the data are inappropriate. Note that the lower temporal sampling frequency with cyclic voltammetry is likely to skew the observed half-widths to larger values for the measurement with 1- μm spacing. However, the distribution of $t_{1/2}$ values is little changed with the electrode 5 μm from the cell surface, and only a modest decrease in $[\text{CA}]_{\text{max}}$ is seen relative to the measurements with 1- μm spacing.

The uncertainty in the above analysis is that the location of the sampled release sites on the cell surface is not clearly defined. This would be the only source of variability in the measured spikes if each originated as an instantaneous point source. This uncertainty is removed when the electrode touches the cell because release sites which are not under the electrode are precluded from contributing to the measured response. In addition, the close proximity of the electrode and cell prohibit diffusional dispersion. The disadvantage of this approach is that the characteristics of the spikes, and possibly, the exocytotic process could be altered by the physical presence of the electrode or its associated electric field. However, we find that the minor changes in spike half-widths with the electrode touching the cell can be completely accounted by the lack of diffusional broadening. The electric field of the electrode is dissipated over the double-layer dimensions (a few nanometers), which may be the reason that it does not induce release. Thus, the key feature revealed by this experiment is that the spikes still have a significant time course, and one which is little different from that measured 1 μm away. Therefore, the rate-limiting step of release from these vesicles must be due to the extrusion of the vesicular contents.

The broad range of measured values makes it difficult to evaluate a specific mechanism to explain these results. However, two hypotheses are consistent with modern views of exocytosis. First, consider the intravesicular matrix of bovine adrenal medullary cells which is known to be a complex chemical milieu. Its dissociation, which may be due to a pH and Ca^{2+} induced conformational change of chromogranin A

(Yoo and Lewis, 1992; Jankowski et al., 1994) the major soluble protein component of the vesicular matrix (Winkler and Westhead, 1980; O'Connor and Frigon, 1984), may be rate limiting. Such precedent has been established in exocytosis of larger vesicles which are visible by optical microscopy. Upon exocytosis of mucin from goblet cells, swelling of the vesicular contents is observed (Verdugo, 1990). Swelling of the matrix of isolated mast cell vesicles has also been reported under the influence of electric fields which undoubtedly exist during cell-vesicle fusion (Nanavati and Fernandez, 1993). Also consistent with this hypothesis are our observations that the spike shape is dependent on the extracellular environment (Jankowski et al., 1993, 1994).

A second hypothesis to explain the results is that the size and/or the rate of opening of the vesicular fusion pore restricts the rate at which material exits the vesicle (Alvarez de Toledo et al., 1993; Almers, 1989). According to this model the restricted dimension of the fusion pore limits the rate of catecholamine efflux in the same way as an ion channel restricts transport of ions. This model predicts that the surface concentration during a single exocytotic event should decrease in a manner which is exponential with time, consistent with the shape of the measured spikes as an exponentially modified Gaussian. However, the low correlation between the measured maximal concentration and $t_{1/2}$ (Fig. 4, *A* and *B*), as well as the low correlation between the measured amperometric maximal current from each vesicle and $t_{1/2}$ (Fig. 5, *A* and *B*), suggest this is not the case. Further evidence against this hypothesis comes from the logarithmic plot of $t_{1/2}$ against the total charge of each spike (Fig. 6 *B*). The fusion pore hypothesis predicts that the time course of secretion will be proportional to the volume of the vesicle. Since the distribution of Q values is consistent with the distribution of volumes (Fig. 6 *A*), the plot in Fig. 6 *B* is predicted to have a slope of 1 (Eq. 4 in Almers et al., 1989). Instead, the slope by linear regression is 0.3 ($R = 0.405$, not shown). Secretion from mast cells has also been shown to be inconsistent with this model (Alvarez de Toledo et al., 1993). In either case, the observed slow release may account for part of the latency observed in release with patch-clamp stimulation (Chow et al., 1992).

Having experimentally established the shape of vesicular release at the cell surface, the results obtained at more remote locations of the electrode can be evaluated. The median of the $t_{1/2}$ values obtained with the electrode 1 μm from the cell surface is 1.2 ms greater than the median obtained with the electrode touching the cell. Convolution of the diffusional based model with a surface event with the shape shown in Fig. 5 predicts a similar increase (0.8 ms) for diffusion across 1 μm . In addition, this convolution predicts the decrease in amplitude and the slight increase in width observed when the electrode is positioned further (5 μm as opposed to 1 μm) from the cell surface (Fig. 7). The range of $t_{1/2}$ values obtained by cyclic voltammetric measurements 5 μm from the cell are consistent with that predicted for diffusional dispersion alone, as are the amperometric data (Schroeder et al.,

1992). This is because the temporal broadening caused by diffusional dispersion masks the temporal nature of the surface process at this more remote distance. Thus, a slow event at the surface followed by diffusional dispersion accounts for all of the observed differences between the measurements at cell-electrode spacings of 1 μm and 5 μm . Therefore, an alternate possibility for the observed slow release, ejection of the intact, associated vesicular matrix followed by dissociation at the electrode surface, is not likely.

In summary, the results of this work show that exocytotic secretion of catecholamines from bovine adrenal medullary cells is a kinetically controlled process occurring at the cell surface. Additional dispersion of the catecholamines does occur as they diffuse away from the cell surface, but it is not the major factor influencing the temporal width of the spikes. Because of this, measurements made 1 μm from the cell have virtually the same shape as those at the cell surface, and the actual surface event should be able to be reconstructed by deconvolution. The slow time course of serotonin release from mast cells (Alvarez de Toledo et al., 1993) suggests this may be a common feature of release from dense-core vesicles. In addition, analysis of the data does not support an extrusion process which is limited through a fusion pore. Rather, the sensitivity of the spike shape to the extracellular environment (Jankowski et al., 1993, 1994) suggests that the dissociation of the vesicular matrix at the cell surface is the rate-determining step.

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