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Temporally resolved catecholamine spikes correspond to single vesicle release from individual chromaffin cells

(exocytosis/voltammetry)

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ABSTRACT **Secretion of catecholamines from single bovine chromaffin cells in culture was elicited by brief pressure ejections from a micropipette containing nicotine, carbamoylcholine, or potassium ions or by mechanical stimulation. Release was monitored electrochemically with a carbon-fiber microelectrode placed adjacent to the cell. Cyclic voltammetry was used to identify secreted species, whereas constant potential amperometry was used for improved temporal resolution (millisecond range) of catecholamine detection. During secretion, brief current spikes were observed, which were shown to be due to detection of catecholamines by electrooxidation. The spikes have the physical characteristics of multimolecular packets of catecholamines released at random times and locations from the surface of the single cell. The half-width of the spikes was found to increase with an increase in cell–electrode spacing. The properties of the catecholamine spikes correlate well with expectations based on secretion from individual storage vesicles. Spikes do not occur in the absence of Ca^{2+} in the buffer, and the majority of spikes are found to be distributed between 0.2 and 2 picocoulombs, corresponding to 1–10 attomoles of catecholamine detected. The frequency of the spikes increases with the intensity of the stimulus, but the average quantity of catecholamine in each spike is independent of the stimulus. Thus, these measurements represent time-resolved observation of quantal secretion of catecholamines and provide direct evidence for the exocytotic hypothesis.**

The process of exocytosis is common to many different types of cells and is central to our current understanding of the process of chemical intercellular communication. The concept of exocytosis evolved from a combined body of anatomical, biochemical, and physiological evidence (for reviews, see refs. 1–4), from which the following picture has emerged. Substances that are involved in chemical communication between cells are frequently stored in vesicles. These vesicles fuse with the cell membrane in the presence of an appropriate stimulus, and their multimolecular contents are extruded (2, 3). Thus, the minimal amount of chemical secretion should depend on the volume of the vesicle and the concentration of its contents.

Consistent with the exocytosis hypothesis is the quantized nature of neurotransmitter release (5). At the neuromuscular junction, excitatory postsynaptic potentials are observed to be quantized and appear to be the consequence of single vesicular events (5, 6). Measurements of minute capacitance changes have been related to the fusion of secretory vesicles with the plasma membrane (7, 8). However, until recently, the central consequence of the exocytotic hypothesis, the secretion of chemical substances in discrete packets, had not been observed by direct chemical means. In a preliminary note we have presented results that suggest this phenomena can be measured directly at catecholamine-containing cells with a voltammetric microelectrode placed adjacent to a single cell (9).

The results described in this work were obtained with adrenal medullary chromaffin cells. These were selected because they contain secretory vesicles that store the catecholamine hormones epinephrine and norepinephrine. Their neuroectodermal origin and biochemical and functional similarities with true sympathetic neurons have made adrenal chromaffin cells an excellent model for studies of neurotransmitter biosynthesis, metabolism, and secretion (3, 10). They can be maintained in culture where they exhibit most of the properties of the cells in the intact gland (11, 12). We have shown that catecholamine secretion from individual chromaffin cells detected with carbon-fiber electrodes is pharmacologically indistinct from that found in multiple cell preparations (13). Secretion during chemical stimulation results in individual catecholamine spikes that at high stimulation intensities fuse into a broad concentration envelope (9, 13). In this paper we examine in detail some of the properties of individual spikes of secreted catecholamines from such cultured cells and demonstrate that they correspond to the predictions of the theory of exocytosis.

EXPERIMENTAL PROCEDURES

Electrodes and Voltammetric Procedures. Working electrodes were constructed from carbon fibers (5-μm radius, Thornell P-55; Amoco, Greenville, SC) sealed in glass capillaries (14). The sensing tip of the electrode was polished at a 45° angle on a micropipette beveler (Sutter Instruments, Novata, CA) to give an elliptical surface. No other physical treatment of the electrode surface was used. The reference electrode was a sodium-saturated calomel electrode.

Cyclic voltammograms were obtained with a potentiostat (EI-400; Ensman Instrumentation, Bloomington, IN) under computer control (15). In the amperometric mode, the applied potential was 0.65 V vs. the sodium-saturated calomel electrode and the signal was digitized at 44.1 kHz (PCM-2; Medical Systems, Great Neck, NY) and stored on videocassette recorder tape. During playback the data were filtered with a 24 decibel per octave roll-off filter (model 3750; Krohn Hite, Avon, MA).

Single Chromaffin Cell Secretion Experiments. Primary cultures of bovine adrenal medullary chromaffin cells were prepared and maintained as described (11–13). The purified cells were plated at a low density [6×10^5 cells per 35-mm plate (Becton Dickinson)]. Experiments were performed at room temperature (23.0 ± 0.1°C) between days 4 and 8 of

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culture. Immediately prior to the experiments, the culture medium was removed and the cells were washed and then maintained in a balanced salt solution containing 150 mM NaCl, 4.2 mM KCl, 1.0 mM NaH_2PO_4, 11.2 mM glucose, 0.7 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes, adjusted to pH 7.4. $MgCl_2$ was substituted for Ca^{2+} in Ca^{2+}-free solutions.

Experiments were conducted on the stage of an inverted microscope as described (13). The working electrode was positioned adjacent to a cell (16) with a hydraulic micropositioner (model 640; Kopf Instruments, Tujunga, CA). The electrode was manipulated so that it lightly touched a cell and then was retracted to the desired position. Chemical stimulation of a cell was accomplished with a pressure-ejection system (Picospritzer; General Valve, Fairfield, NJ) connected to a triple-barrel micropipette. Mechanical stimulation of individual cells was accomplished by bringing a second carbon-fiber electrode into nondestructive but forceful contact with the cell and immediately retracting it.

Data Treatment. Electrodes were calibrated with catecholamine standards after each set of experiments. Reported values are given with their SEM. Significance testing employed a two-sided Student's *t*-test.

Current spikes measured in the amperometric mode were integrated with respect to time to determine the amount of charge [units of picocoulombs (pC)]. To accomplish this, the recorded signals were placed in computer memory, and a smoothed (0.05-Hz filter) record was generated. This was subtracted from the unfiltered data to remove the secretion envelope. Spike maxima were found by examination of zero-crossings in the time derivative of the subtracted record, generated with a nine-point smooth. Minima on either side of the spike were found in the same way. Spikes were considered significant if the amplitude in the subtracted record was five times larger than the rms value of the noise determined before the cell was stimulated. The charge in each spike is related to the number of moles electrolyzed by Faraday's law.

Materials. Dulbecco's modified Eagle's medium and Ham's F12 medium were from GIBCO. All other chemicals were obtained from Sigma.

RESULTS

Catecholamine Release from Single Cells. Cyclic voltammograms have been used to show that the substance detected during stimulated release at individual adrenal chromaffin cells is a catecholamine (13). The upper left portion of Fig. 1 shows such a voltammogram obtained with a microelectrode placed adjacent to a cell during exposure to 100 μM nicotine. The shape is identical to cyclic voltammograms of catecholamine standards (13).

Examination of the current from successive voltammograms allows identification of the released substance(s) with 100-msec resolution. The top and middle traces of Fig. 1 were constructed from successive voltammograms using the average current from the region where catecholamines are oxidized and rereduced, respectively. In each trace the response is a broad envelope on which are superimposed sharp spikes. As expected, the amplitude is smaller for the reduction trace because of diffusional and chemical losses of the quinone form. However, the simultaneous occurrence of spikes on both traces demonstrates that these are due to concentration changes of catecholamines. The bottom trace, which is constructed from the same voltammograms, but from a potential range where catecholamines are electroinactive, does not show either the envelope or the spikes. As we have previously reported, similar spikes are also observed during release induced by K^+ (60 mM), carbamoylcholine (1 mM), and lower concentrations of nicotine (13). In all cases,

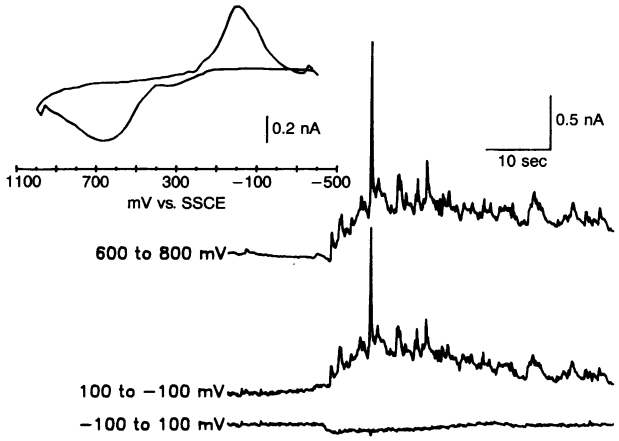

FIG. 1. Voltammetric data (scan rate, 200 $V \cdot s^{-1}$; repetition rate, 10 Hz) obtained with a carbon-fiber electrode during exposure of a single cell to 100 μM nicotine (3-sec pressure ejection). The subtracted voltammogram obtained following nicotine exposure is shown in the upper left portion of the figure. The voltammogram was obtained by subtraction of the average of the 50 cyclic voltammograms collected immediately after the maximal secretion response from the average of 50 voltammograms recorded immediately prior to stimulus application. The traces show average currents obtained from successive voltammograms. The potential regions examined are 600 to 800 mV on the positive scan (top trace), 100 to −100 mV on the reverse scan (middle trace), and −100 to 100 mV on the initial positive scan (bottom trace). Current traces are plotted in the direction in which concentration changes will occur. SSCE, sodium-saturated calomel electrode.

cyclic voltammetry has been used to confirm that the spikes are rapid concentration changes of catecholamines.

Ca^{2+} Dependence of Release. Two amperometric recordings made with a carbon-fiber microelectrode placed adjacent to a single cell in Ca^{2+}-free buffer are shown in Fig. 2. Prior to and during pressure ejection, a noise-free baseline was obtained in both cases, and this remained unchanged unless Ca^{2+} was present. [During pressure ejection, convection removes species from the electrode causing the observed delay in spike generation (13).] After application of nicotine with Ca^{2+} present, the current rose in a broad envelope on which were superimposed sharp spikes. The total area of the increase in current measured in response to nicotine exposure in the absence of Ca^{2+} was 1.4% ± 0.7% of the response for the same cells exposed to nicotine in the presence of 2 mM Ca^{2+} (n = 6 cells for each case). In addition, four individual cells were exposed to 60 mM K^+ in the absence of extracel-

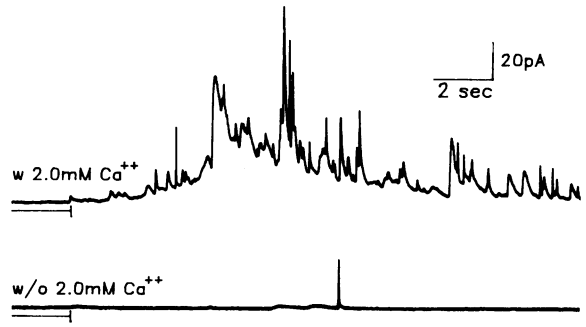

FIG. 2. Amperometric response to 100 μM nicotine in the presence and absence of Ca^{2+}. Cells were maintained in a Ca^{2+}-free balanced salt solution. The upper trace represents response of the cell to a 9-sec application of 100 μM nicotine with 2.0 mM Ca^{2+}; the lower trace represents a subsequent response of the same cell to a 9-sec application of 100 μM nicotine without Ca^{2+}. The bar indicates the final 2 sec of nicotine exposure.

lular Ca^{2+}, and the response was similarly attenuated when compared to the response with Ca^{2+} present.

Spikes are infrequently observed in the absence of a secretagogue, or, as seen in Fig. 2, in the absence of Ca^{2+}. Typical baseline behavior is seen in Fig. 1 during the time before exposure to nicotine.

Spike Frequency and Distribution of Sizes as a Function of Secretagogues. In previous work it has been shown that a 3-sec pressure ejection of 100 μM nicotine and 1 mM carbamoylcholine both lead to release of catecholamines characterized by broad envelopes that have a maximal concentration of greater than 10 μM (13). In contrast, the total amount released from single cells by 10 μM nicotine or 60 mM KCl is significantly less. The area of the measured release induced by 10 μM nicotine is 23% ± 4% (n = 4) of that induced by 100 μM nicotine, whereas the response induced by 60 mM KCl is 22% ± 11% (n = 21) of the 100 μM nicotine-induced response. To test whether the occurrence of spikes also is lower with these stimuli, the frequency of the spikes measured in the amperometric mode was determined. The spike frequencies measured during exposure to 10 μM nicotine and 60 mM K^+ (0.5 ± 0.2 Hz and 0.6 ± 0.2 Hz, respectively) are significantly different from the frequency observed during exposure to 100 μM nicotine (1.2 ± 0.2 Hz) at the 95% confidence level. This is consistent with the reduced area of the secretion response. The frequency of spike occurrence was 0.7 ± 0.2 Hz during exposure to carbamoylcholine (1 mM).

The histograms in Fig. 3 illustrate the distribution of charge measured in the amperometric mode for individual spikes during exposure of cells to various secretagogues. Note that for each secretagogue a skewed distribution of spike areas is observed, but in each case the majority have an area less than 2 pC. The average spike area was 1.19 ± 0.09 pC with 60 mM K^+, 0.98 ± 0.13 pC with 1 mM carbamoylcholine, 1.04 ± 0.16 pC with 10 μM nicotine, and 1.19 ± 0.10 pC with 100 μM nicotine.

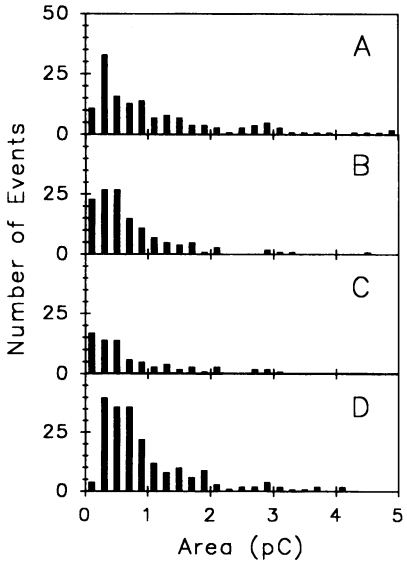

FIG. 3. Frequency distribution of individual spike areas determined for various secretagogues. The spikes were measured over a 30-sec segment beginning at the half-rise time. The bins are divided into 0.2-pC intervals. (*A*) Spike distribution during exposure to 10 3-sec, 60 mM K^+ exposures (136 spikes; the balanced salt solution in the ejection pipette contained 90 mM Na^+ and 60 mM K^+). (*B*) Spike distribution during 7 exposures to 1 mM carbamoylcholine stimulations (137 spikes). (*C*) Spike distribution during 10 exposures to 10 μM nicotine (80 spikes). (*D*) Spike distribution during 10 exposures to 100 μM nicotine (211 spikes).

Spike Characteristics as a Function of Cell–Electrode Separation. If the spikes of catecholamine concentration reach the electrode by a diffusional process, then they should become temporally narrowed when the electrode is placed closer to the cell. The electrode was positioned at 1 or 5 μm from the cell surface, and the cell was stimulated with 100 μM nicotine. The spikes measured at the more distant location were, on average, lower in amplitude and temporally broadened, consistent with a diffusion-controlled process. The net result is a comparable area of spikes measured at each location. Histograms of the half-widths of the spikes measured at the two locations are given in Fig. 4.

Spikes During Mechanical Stimulation. Catecholamine release from individual cells during mechanical stimulation also is in the form of spikes. When a cell is touched with an electrode, the initial response is a burst of spikes that superimpose to form an envelope. After this initial discharge, the spike frequency diminishes, and single concentration spikes occur on a flat baseline. The response continues for an extended period of time (Fig. 5). Cyclic voltammograms showed that catecholamines are responsible for the observed spikes and that the occurrence of spikes is Ca^{2+} dependent (data not shown). Fig. 5 *Inset* shows a time-resolved spike that has an area of 5.8 pC, which is much larger than the average spike area. The smooth rise and fall of the spike seen at this high temporal resolution suggests that the spike is a single event, not the summation of several individual spikes.

A histogram of the areas of spikes observed during mechanical stimulation of nine cells is shown in Fig. 6. The histogram was constructed from spikes that occurred during the period where the baseline was flat. Spikes were discarded if they were not temporally resolved from adjacent spikes. The mean frequency of the spikes in the collection period was 0.32 Hz. The average area was 1.03 ± 0.08 pC, and the range of spike areas was from 0.1 pC to 9.5 pC.

DISCUSSION

The measurements described here exploit the sensitivity of the microelectrodes, as well as their spatial resolution and rapid temporal response, to *directly* monitor and *time resolve* catecholamine secretion events at the single-cell level. In the cyclic voltammetric mode, the released substances can be chemically defined, whereas greater temporal resolution can be obtained in the amperometric mode. The average external radius of a vesicle in adrenal chromaffin cells is <200 nm (17),

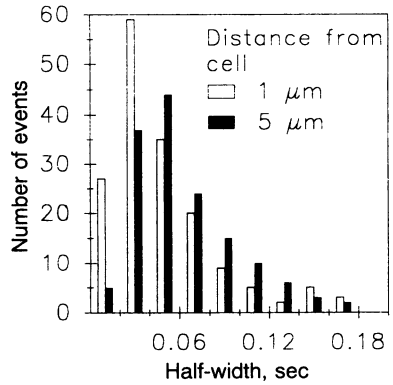

FIG. 4. Distribution of spike half-widths as a function of the electrode distance from the cell. Spikes were obtained in response to a 3-sec exposure to 100 μM nicotine with the electrodes 1 μm (n = 152 spikes) and 4–5 μm (n = 152 spikes) away from the cell surface (four cells for each). Half-widths are grouped in 20-msec bins. The half-width is defined as the width of the peak at half its maximum amplitude.

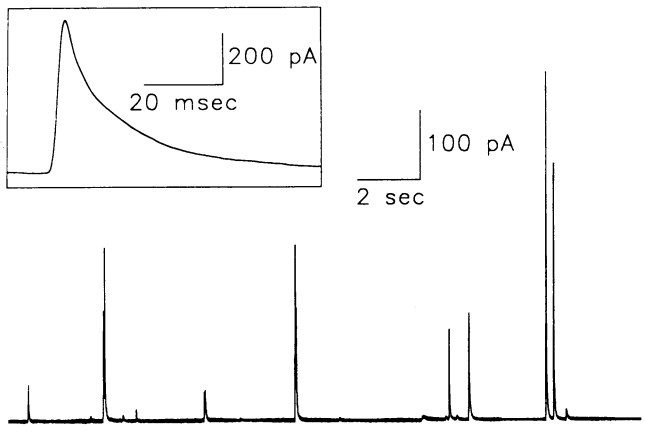

FIG. 5. Response to physical stimulation, with the electrode 1 μm from the cell, collected at 200 Hz and filtered at 100 Hz. (*Inset*) Single concentration spike from the given response, collected at 50 kHz and filtered at 6 kHz.

which is much smaller than the size of the electrodes used in this work. Sites of exocytosis on the cell surface that could lead to the measured catecholamine spikes therefore would be expected to be spatially localized with respect to the sampling electrode. Thus, the concentration spikes appear to be a consequence of fusion of the storage vesicles, release of the vesicle contents, and subsequent diffusion of catecholamines to the electrode and are totally consistent with the hypothesis of exocytosis as a mode for cellular secretion.

The following points, which are based on physical and chemical aspects of the measurements described in this work, summarize the evidence for this conclusion:

(*i*) Previous experiments with cyclic voltammetry revealed that spikes are concentration pulses of catecholamines (9, 13). Examination of data such as those shown in Fig. 1 shows that every spike is due to a concentration pulse of catecholamines.

(*ii*) The occurrence of chemical spikes is most prevalent during stimulations (i.e., under conditions where release is known to occur). However, stimulation is not sufficient to cause release; Ca^{2+} must be present in the external medium (Fig. 2), consistent with the requirement of Ca^{2+} for exocytosis (3, 10, 18); and blockade of nicotinic receptors or Ca^{2+} channels can inhibit spikes (13).

(*iii*) Instantaneous release of vesicle contents after fusion with the cell membrane should lead to discrete packets of catecholamines, which diffuse away from the cell surface. Consistent with diffusional mass transport, the spikes have a greater half-width when the electrode is placed further away from the cell surface; however, the average spike area remains similar (16).

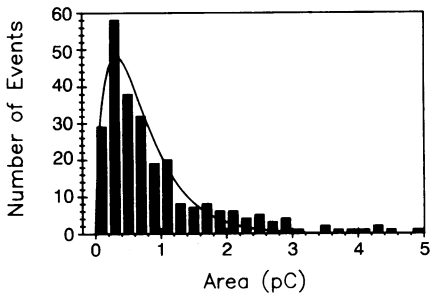

FIG. 6. Distribution of spike areas obtained in response to mechanical stimulation with the electrode 1–2 μm away from the cell surface (n = 265 spikes, 8 spikes with areas >5 pC are not shown; nine different cells). Areas are grouped in 0.2-pC bins. The solid line generated from the probability density function $f_Q(Q)$ is superimposed on the histogram (see text for explanation).

(*iv*) The average quantity per spike, ≈1 pC, is not a function of the stimulation (chemical or mechanical) or its intensity. This is an expected condition for an exocytotic release process.

(*v*) In contrast to the behavior of the spike areas, the spike frequency is a function of the intensity of the stimulus. Thus, low concentrations of nicotine lead to a low frequency of spikes and a smaller secretion envelope than that observed with a higher concentration.

(*vi*) Assuming a two-electron oxidation process, the quantity of charge in the average spike corresponds to detection of 5 attomoles. This value is in the range of the average calculated vesicular content of catecholamines (19).

In addition to these points, we have shown in prior work that measurements with two electrodes placed on opposite sides of a single cell result in spikes that are noncoincident (13). Secretion appears to occur from all parts of the exposed surface of the cells in culture, consistent with previous immunohistochemical evidence (20, 21). A localized, stochastic process such as vesicle fusion with the cell membrane is expected to exhibit this behavior.

Taken together, the evidence is overwhelming that the spikes measured during stimulated secretion of individual chromaffin cells represent the temporal resolution of packets of catecholamines resulting from vesicular exocytosis. Therefore, the shape of histograms such as those shown in Figs. 3 and 6 should provide insight into the nature of the exocytotic process. The histograms have a skewed distribution, with some spikes that are much larger than the average value. We have considered several mechanisms that could lead to such a distribution. One possibility is that the larger concentration spikes are a result of simultaneous membrane fusion of multiple vesicles that occur adjacent to the electrode. This interpretation seems unlikely, especially for the data from mechanical stimulation, because the spikes were all baseline resolved, and clearly overlapping spikes were discarded from the analysis. Furthermore, the time-resolved measurements of the large spikes do not reveal composite events. Because of diffusional distortion, spikes temporally separated by <2 msec would appear merged as one. However, the low frequency of spike occurrence during mechanical stimulation makes the probability of such small interspike intervals very unlikely if the exocytotic process is stochastic.

Other possibilities for the observed distribution include vesicle–vesicle fusion inside the cell prior to exocytosis, referred to as compound exocytosis, fusion to the plasma membrane of vesicles with a large range of concentrations, or fusion of vesicles with uniform catecholamine concentration but a large range of radii. Confirmation of these or other possibilities requires a knowledge of the distribution of vesicle size and catecholamine content. Although the concentration distribution is unavailable, the distribution of vesicle radii in adrenal chromaffin cells has been measured and is approximately Gaussian (17). With the assumptions that the vesicles are spherical and have a constant concentration of catecholamine, the observed spike areas in units of charge (Q) can be directly related to the vesicle radii by Faraday's law:

$$Q = \frac{4}{3}\pi nFCr^3,$$

where C is the vesicular concentration of catecholamines, F is Faraday's constant, and n is the number of electrons involved in the oxidation of the catecholamines (two electrons are required to form the *o*-quinone). The probability

density function (22) for the distribution of spike areas based on these relationships can be shown to be:

$$f_Q(Q) = \frac{1}{3\sigma\left[\left(\frac{\pi}{2}\right)^{1/2} + \left(\frac{\pi}{2}\left[1 - \exp\left(\frac{-\mu^2}{2\sigma^2}\right)\right]\right)^{1/2}\right]} \left(\frac{4}{3}\pi nFCQ^2\right)^{-1/3} \exp\left(-\frac{\left[\left(\frac{Q}{\frac{4}{3}\pi nFC}\right)^{1/3} - \mu\right]^2}{2\sigma^2}\right),$$

where μ and σ^2 are the mean and variance of the vesicle radii. The solid line in Fig. 6 is a nonlinear, least-squares fit of this probability density function with $\mu = 156$ nm and $\sigma = 42$ nm (both values taken from ref. 17 for epinephrine-containing vesicles). This fit gives an estimated vesicular concentration of 0.19 M and a correlation coefficient of 0.968. The vesicular concentration agrees with that determined from the average concentration per cell [determined by analysis of plate contents (13)] and using an estimate of 30,000 vesicles per cell (21). If the concentration value is calculated based on the internal vesicular volume rather than the total volume (a membrane thickness of 10 nm was assumed) and a water content of 68% (23), the calculated catecholamine concentration would be 0.33 M, which is in accord with the estimate of 0.55 M by Hillarp (24).

The function fits well to the data obtained during mechanical stimulation. This data set is most appropriate for analysis since the spikes are baseline resolved. Note that a similar model has been used to evaluate the variability in quantal size of miniature excitatory postsynaptic currents measured at acetylcholine synapses in hippocampal tissue (6). However, in the present work, calculation of the histogram has no undefined parameters.

Compound exocytosis should lead to discrete, multiple peaks in the histograms. Although such patterns may be present in the data, quantitative interpretation requires consideration of the limitations of the measurements. For example, some spike areas may be underestimated because not all of the vesicle contents passed by the detection surface. Errors could arise in the estimation of the area of spikes that are superimposed on the secretion envelope as with 100 μM nicotine. Note that the observed spike frequency only reflects the number of events adjacent to the electrode, not the total that occur, because the electrode only samples from a portion of the cell surface.

The demonstration presented here of catecholamine secretion in the form of sharp spikes is evidence that quantal secretion also can occur from non-neurite-bearing endocrine cells. Though the quantal hypothesis for neurotransmitter release (5) is widely accepted, it has been extensively documented only at cholinergic synapses using the postsynaptic potential as a biological detector. More recently, micropipettes with attached outside-out patches of acetylcholine postsynaptic membranes have been used to monitor the secretion of acetylcholine molecules from detached motor nerve terminals and growth cones in culture (25, 26). Viveros *et al.* (24) gave evidence for the release of the total soluble contents of catecholamine storage vesicles during stimulation of the rabbit adrenal medulla *in vivo*, thus demonstrating that secretion must arise from the multimolecular content of individual vesicles. However, to our knowledge, the data presented here provide the first temporally resolved measurements of chemical secretion, which is that expected for quantal release.

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