Mouse Chromaffin Cells Have Two Populations of Dense Core Vesicles

Chad P. Grabner,1 Steven D. Price,2 Anna Lysakowski,2 and Aaron P. Fox1
1Department of Neurobiology, Pharmacology, and Physiology, The University of Chicago; and 2Department of Anatomy and Cell Biology, University of Illinois, Chicago, Illinois

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Grabner, Chad P., Steven D. Price, Anna Lysakowski, and Aaron P. Fox. Mouse chromaffin cells have two populations of dense core vesicles. J Neurophysiol 94: 2093–2104, 2005. First published June 8, 2005; doi:10.1152/jn.00316.2005. The quantal hypothesis states that neurotransmitter is released in discrete packages, quanta, thought to represent the neurotransmitter content of individual vesicles. If true, then vesicle size should influence quantal size. Although chromaffin cells are generally thought to have a single population of secretory vesicles, our electron microscopy analysis suggested two populations as the size distribution was best described as the sum of two Gaussians. The average volume difference was fivefold. To test whether this difference in volume affected quantal size, neurotransmitter release from permeabilized cells exposed to 100 μM Ca2+ was measured with amperometry. Quantal content was bimodally distributed with both large and small events; the distribution of vesicle sizes predicted by amperometry was extremely similar to those measured with electron microscopy. In addition, each population of events exhibited distinct release kinetics. These results suggest that chromaffin cells have two populations of dense core vesicles (DCVs) with unique secretory properties and which may represent two distinct synthetic pathways for DCV biogenesis or alternatively they may represent different stages of biosynthesis.

INTRODUCTION

Numerous changes are known to take place at synapses, which are thought to represent the most plastic elements of the nervous system. New synapses are added and old ones are pruned even in adult brain. Changes in postsynaptic receptor numbers (Heynen et al. 2000; Malinow 2003; Malinow and Malenka 2002) or function (Lee et al. 2003) can change synaptic efficacy. Changes in secretion efficiency of synaptic vesicles has been described (Arancio et al. 1996; Stevens and Wang 1994; Tsien et al. 1996; Zucker and Regehr 2002). Under certain conditions, the presynaptic changes lead to an increase in transmitter secretion by elevating the probability of release at an individual synapse without concomitant changes in the amount of neurotransmitter released per vesicle (Malinow and Tsien 1990; Stevens and Wang 1994). Most studies assume that the transmitter quantal size is invariant; however, there is little direct evidence in support of this assumption at many synapses.

Clear differences in synaptic vesicle diameters have been observed in neurons. At Drosophila’s neuromuscular junction, two different glutamatergic motor neurons innervating the same muscle are distinguished by their vesicle sizes (mean diameters of 41 and 48 nm) (Karunanithi et al. 2002). Serotonergic Retzius neurons of leech release 5-HT from three different populations of vesicles, each having a unique vesicle size (Bruns et al. 2000). Two of the populations were dense core vesicles (DCVs) that are thought to store neuropeptides and 5-HT. Furthermore, the Drosophila and leech studies provided evidence that quantal size is a function of vesicle size. Other studies have suggested that vesicle neurotransmitter concentration can be modified (Kullmann and Nicoll 1992; Liao et al. 1992) or that only a fraction of the total vesicle content is released per fusion event (Elhamdani et al. 2001).

In the case of chromaffin cells, a model system for studying exocytosis, it is often assumed that a single population of DCVs supports regulated secretion. However, electron microscopic (EM) studies of bovine (Coupland 1968) and mouse chromaffin cells (Gorgas and Bock 1976) showed that DCV size does not strictly follow a single, normal distribution. In Coupland’s 1968 study, if one looks at the size distribution of noradrenaline vesicles (see Fig. 5 of that study), there are too many large-diameter vesicles to fit all the data with a single normal distribution. Physiological evidence for a heterogeneous population of DCVs is emerging. For instance, DCVs respond differentially to secretagogues as a function of the vesicles age (Duncan et al. 2003), and the size of vesicles that undergo regulated secretion are not uniformly distributed (Henkel et al. 2001). In PC12 cells, DCV biogenesis occurs in stages that are marked by changes in vesicle diameter (Tooze et al. 1991).

Our electron microscopic (EM) studies indicated that mouse chromaffin cells had two populations of DCVs of different size. The smaller-sized population had an average diameter of 179 nm, whereas the second group, large vesicles, was centered at 310 nm (a size ratio of 1.73). Amperometry was used to assay the neurotransmitter content of the vesicles. Catecholamine release was elicited by permeabilizing cells with digitonin (20 μM) followed by an exposure to 100 μM Ca2+. The amount of transmitter detected per individual release event (the quantal size) was measured as charge (Q). A useful convention for expressing differences in quantal size is to use Q1/3, as it is thought to reflect vesicle diameter (Bruns et al. 2000; Finnegan et al. 1996; Wightman et al. 1991). Our results showed that quantal sizes were bimodally distributed as small and large events with Q1/3 values of 0.40 and 0.69 pcC1/3, respectively (size ratio: 1.73), with amperometry and EM results showing a fivefold difference in the volumes of large and small vesicles. In addition, small amperometric events were kinetically distinct from large events. These results suggest that two separate pools of secretory vesicles give rise to events with different quantal size. The results also imply that under conditions of...
intense stimulation, as used in this study, vesicles were released in an all-or-nothing fashion.

**METHODS**

**Cell culture**

Animals were housed and handled as required by the Animal Resource Council (University of Chicago, IL). Cells were prepared similarly to previously published methods (Obukhov and Nowicky 2002). Briefly, adrenal glands were harvested from 5- to 8-wk-old male C57 BL/6j mice (The Jackson Laboratory, Bar Harbor, ME). The medulla was dissected from the cortex with a surgical scalpel and cut into two pieces. The tissue was digested for 35 min with 1 mg/ml Collagenase P (Roche Diagnostics, Indianapolis, IN) +25 µg/ml DNase (Sigma-Aldrich, St. Louis, MO), followed by a 15-min digestion in 0.03% Trypsin/EDTA (Invitrogen, Grand Island, NY) plus 50 µg/ml DNase. All digestions were carried out in calcium-free buffered saline at 37°C in a shaker water bath. Finally, the digested tissue was transferred to tissue culture media and mechanically dispersed by dipping the glass-fiber tip into a 14% hardener:resin mixture (w/w), preheated to 100°C (hardener, metaphenylenediamine, and resin, 225 Epon Resin; Millar-Stephenson Chemical, Morton Grove, IL). The tissue culture media and had the following composition: DMEM-F12K, 10% HS, 5% FBS and 1% of a 100× penicillin/streptomycin/glutamine cocktail (all media reagents were purchased from Invitrogen). EM and amperometry experiments were carried out 2 and 3 days post tissue harvest.

**Amperometry**

Carbon fiber electrodes were fabricated with 7-µm-diam carbon fibers (Fortafil Fibers, Knoxville, TN). The carbon fiber was threaded through a glass capillary pipette (Drummond Scientific, Broomall, PA) and pulled with a vertical glass pipette puller (Narashige, Tokyo, Japan). An epoxy seal between the carbon fiber and glass was created by dipping the glass-fiber tip into a 14% hardener:resin mixture (w/w), preheated to ~100°C (hardener, metaphenylenediamine, and resin, 225 Epon Resin; Millar-Stephenson Chemical, Morton Grove, IL). Electrodes were baked overnight at 66°C. The glass portion of the tip was painted with silicone elastomer (Sylgard, Dow Corning, Midland, MI) and then baked at 66°C for ≥1 h. On the day of the experiment, the carbon fiber was cut and visually inspected as described by Bruns et al. (2000). The electrode was pressed gently against the cell during the recording as the collection efficiency is thought to be highest under this recording configuration (Bruns et al. 2000; Schroeder et al. 1992). A newly cut surface or a new electrode was used for each cell to prevent fouling of the electrode. The electrode was backfilled with 3 M KCl. The electrode was held at +800 mV versus silver-chloride using an EPC-7 amplifier (HEKA Electronics, Lambrecht, Germany) to oxidize catecholamine transmitter. The electrode was mounted in a holder and then advanced until it gently touched the cell. The amperometric signal was low-pass filtered at 2 kHz (8-pole Bessel; Warner Instruments, Hamden, CT). A 16-bit A/D converter (National Instruments, Austin, TX) was interfaced with custom data acquisition software. The amperometric signal was acquired at 10 kHz and stored on a personal computer. The rms noise present in the acquired traces was typically under 1.0 pA as determined with the MiniAnalysis program (Synaptosoft, Decatur, GA). Records with rms noise >2 pA were not analyzed. Amperometric spike features, quantal size, and kinetic parameters were analyzed using the amperometric program in MiniAnalysis. The detection threshold for an event was set four to five times the baseline rms noise, and the spikes were automatically detected. Overlapping events were rejected, and they were considered overlapping if the initial spike had not returned to baseline before the next event occurred. Overlapping events were relatively rare. Rise-time values were measured over 10–90% of the spike’s maximal amplitude. The area under individual amperometric spikes is equal to the charge (Q) per release event, referred to as Q. The number of oxidized molecules (N) was calculated using the Faraday equation, N = Q/ne, with n = 2 electrons per oxidized molecule of transmitter, e is the elemental charge (1.603 ×10⁻¹⁹ coulomb). To make an approximate estimate of the total number of events released over the entire cell, the following assumptions were made. Release was assumed to occur uniformly over the cell (Wick et al. 1997), and the release rate measured at the electrode was multiplied by the ratio of cell surface area/electrode area. The electrode, gently pressed against the cell, was estimated to cover only 6.3% of the cell’s surface, and the average rate of 20 events/min detected at the electrode yields a global release rate of ~318 events/min. Amperometric data were measured in 32 cells.

**Recording solutions and stimulation protocols**

Recordings were made from adherent cells that were under constant perfusion (flow rate of 1.0 ml/min, and an approximate chamber volume of 150 µl). All recording solutions had the following standard composition (in mM): 145 NaCl, 2.0 KCl, 10 HEPES, and 1.0 MgCl₂. Ca²⁺-free solutions contained 100 µM EGTA. All solutions used during and after cell permeabilization contained 1.0 mM Na₃ATP. The solutions were adjusted to a final pH of 7.20 and an osmolarity of 300 ± 3 mosM/kg. Free-Ca²⁺ was estimated with WEBMAXCLITE (http://www.stanford.edu/~ccpton/maxcl.html). All experiments were performed at ambient temperature, ranging from 22°C to 26°C. Cells were stimulated repeatedly using the following protocol: 2 min in a Ca²⁺-free solution, permeabilized with 20 µM digitonin (Ca²⁺-free) for 10 s, stimulated 2 min with a solution containing free Ca²⁺ concentration of 100 µM, and washed for 1 min in Ca²⁺-free media before the cycle was repeated starting at step 2 (permeabilization). Cells were typically stimulated three to four cycles (and at most 6 cycles) or until the cell membrane changed from its initial, bright-field dark appearance to a granular texture (for details, see Jankowski et al. 1992). Quantal size from the first stimulation was compared it to later stimuli; nothing was changed. Digitonin was purchased from Calbiochem (La Jolla, CA). Cells stimulated with nicotine were exposed to 10 µM nicotine for 3 min in 2 mM Ca²⁺-free, followed by a 2-min wash without nicotine. Two additional stimulations repetitions were carried out.

**Electron microscopy**

After the cells were cultured for 48 h (described in Cell culture), they were prepared for EM. Coverslips were rinsed twice with 0.12 M cacodylate buffer (pH 7.4) to remove cell culture media and then fixed with 2% glutaraldehyde and 0.6% paraformaldehyde in 0.06 M cacodylate buffer pH 7.4 for 1 h. The coverslips were again rinsed three times and then postfixed for 1 h in a mixture of 1% osmium tetroxide in 0.06 M cacodylate buffer pH 7.4 containing 1.5% potassium ferricyanide, which was added immediately before use. The samples were washed three times, dehydrated in a graded series of EtOH (50–100%), with the 70% step containing 1% uranyl acetate (30 min), and finally dehydrated in propylene oxide. The samples were infiltrated with Durcupan (Fluka), and the coverslips were inverted over 15-mm round-well molds with the tissue side down and polymerized for 48 h at 60°C. After polymerization, the resin plug with cover slip was suspended in hydrofluoric acid for 10 min to dissolve the glass. Areas of interest were cut out with a jeweler’s saw, attached with SuperGlue to a blank Beem capsule stub, and sectioned parallel to the plane of the cover slip with a diamond knife. Sections with a silver interference color were collected on 1 × 2 mm Formvar-coated slot grids and counterstained with uranyl acetate and lead citrate. The sections were viewed in a Jeol 1220 electron microscope at 80 kV, and images were taken on EM film (Eastman Kodak, Rochester, NY) at ×12,000 magnification.
**Vesicle size measurements**

Digital images of EM micrographs were made and analyzed at a final magnification of $\times 100,000$ or $\times 200,000$. Random single sections from cells (11 cells total) that possessed multiple DCVs and well-fixed cellular constituents (mitochondria, nuclear material and plasma membrane) were selected for analysis. The criteria for DCVs that were included in the size distribution are as follows: the vesicle had to have an electron dense core and an intact, continuous vesicle membrane surrounding the dense core, and the membrane had to be approximately circular in geometry. All vesicles meeting these criteria were included in the analysis. The vesicle diameters were measured from 11 cells. The area of each vesicle was measured using Image-J software (National Institutes of Health, Bethesda, MD) or SigmaScan software (SPSS, Chicago, IL) (as described previously, Colliver et al. 2000). The two programs were compared and found to yield results that were essentially identical. Diameter was calculated from vesicle area [$\text{diameter} = 2^{\left(\text{Area}/\pi\right)^{0.5}}$]. Two investigators made independent measurements from the same single sections, and resulting vesicle size distributions were essentially identical. Corrected vesicle diameter was calculated using a previously published algorithm (Parsons et al. 1995). This algorithm is dependent on section thickness and the empirically measured vesicle diameter (referred to as apparent diameter). Section thickness was estimated to be 60 nm based on the silver empirically measured vesicle diameter (referred to as apparent diameter). Section thickness was estimated to be 60 nm based on the silver interference color of the sections (Sakai 1980). Apparent vesicle diameters were transformed to corrected diameters prior to statistical or graphical analysis of the data. Widest vesicle diameter was measured from serial sections using Image-J (National Institutes of Health, Bethesda, MA). Four to five sections through individual cells (3 cells in total) were aligned using cellular landmarks, such as mitochondria and large DCVs. Individual DCVs (meeting the morphological criteria described in the preceding text) were followed through multiple sections to determine which section captured the vesicle at its widest diameter. Small vesicles that were only obvious in a single section were considered to represent the vesicle captured at its greatest diameter.

Statistical analyses and plots were performed in Origin (Origin, Northampton, MA). To select the model that gave the best-fit to the size distributions, we applied the corrected Akaike Information Criterion (AICc) for small sample sizes ($n$) (Anderson 2002). This method selects the model that best represents the data while minimizing the number of parameters used in the model. The AICc equation is as follows:

$$AICc = \text{NLog}[SSR_n/N] + 2K_n + (K_n^2 + K_n)/(N - K_n - 1).$$

$K_n$ is the number of parameters in model $n$; $N$ is the number of binned data points, which is the same for each model; $SSR_n$ is the sum of the residuals resulting from the fit for model $n$, which was done in Origin. The model with the lowest AICc value is taken as the best fit (Anderson 2002), and the degree that one model better describes the data compared with a second model can be expressed numerically as a probability as follows: difference in AICc values for model 1 and 2: $\Delta AIC = AICc_1 - AICc_2$; probability of model 1 giving the best fit: $\text{Prob}_1 = 100 \times \exp^{-0.5(\Delta AIC)}/[1 + \exp^{-0.5(\Delta AIC)}]$; Probability of model 2 yielding the best fit: $\text{Prob}_2 = 100 - \text{Prob}_1$.

**RESULTS**

Electron microscopy studies reveal two populations of vesicles in individual cells

Previous studies using whole mouse adrenal glands identified two major chromaffin cell types, namely epinephrine- and norepinephrine-storing cells (Gorgas and Bock 1976; Kobayashi and Coupland 1977). These catecholamine transmitters react differently with aldehyde fixatives, which allows the two cell types to be distinguished by the appearance of their DCVs, the organelles that concentrate the transmitter (Coupland 1968; Kobayashi and Coupland 1993). Norepinephrine reacts with fixative aldehydes and osmium tetroxide (Coupland and Hopwood 1966a,b), which impart a core that is very electron dense and often surrounded by a halo of membrane (Coupland 1968). In contrast, epinephrine reacts poorly with fixatives, and as a result epinephrine-storing cells exhibit DCVs that are relatively less electron dense (Coupland 1968). The cells in our study generally fit the description of norepinephrine-storing cells that possess DCVs with electron dense cores, as described by Thureson-Klein et al. (1984), even though we did not select for this type of cell.

A quantitative analysis of DCV size revealed a striking heterogeneity within individual cells. Random sections from cells that possessed multiple DCVs and well-fixed cellular constituents (mitochondria, nuclear material, and plasma membrane) were selected for analysis (see Fig. 1A). The criteria for DCVs that were included in the size distribution can be found in METHODS. All vesicles meeting these criteria were included in the analysis. Typically the larger DCVs possessed an excess membrane giving a halo appearance around the vesicle’s core, and the cores of smaller vesicles often appeared to have a tightly associated membrane (see Fig. 1B) (Gorgas and Bock 1976). Under physiological conditions, catecholamines fill the intra-vesicular space (either in a freely diffusible state or bound to a protein matrix); therefore the entire area within the vesicle membrane was measured and then converted to a diameter (Colliver et al. 2000) and then transformed using an algorithm that corrects for sectioning errors (Parsons et al. 1995) (described in more detail in the following text). Typically, individual cells appeared to have a collection of smaller vesicles with diameters ranging from 80 to approximately 200 nm, and a second group of larger vesicles with diameters $>200$ nm (see Fig. 1C). Cells with a single Gaussian distribution of vesicle sizes were not observed.

**Verification of EM vesicle size corrections**

Vesicle size measured from single EM sections is known to result in an apparent diameter that is less than the correct diameter. In the past, a variety of corrections have been applied to adjust the data (Parsons et al. 1995). To verify the accuracy of the correction that we used in this study, serial sections were analyzed to determine the vesicle’s widest diameter. Figure 2A shows a region of a cell enriched in DCVs. The vesicles with large diameters span three to four sections (Fig. 2B), and smaller vesicles typically span one to three sections (Fig. 2C; sections are 60 nm in diameter). The results from three cells (four to five sections analyzed per cell) were pooled (both small and large vesicles were combined) and averaged, which yielded a widest diameter of 194 nm. Single-section results measured from the same sections produced an average apparent diameter of 164 nm (18% less than the widest diameter). Transforming the data from a single section using an algorithm that corrects for sectioning errors (Parsons et al. 1995) yielded a mean diameter of 202 nm (“corrected” diameter). The corrected and widest diameter results were in good agreement (4.2% difference).

Comparison of vesicle size distributions obtained using either widest diameter (serial reconstruction) or corrected diameter (individual sections) provided additional verification of
the correction algorithm. Figure 2, D–F, plots vesicle size distributions from individual cells. Figure 2D plots apparent vesicle diameters from an individual section, E plots the same data after correcting for sectioning artifacts, and F plots data from the same cells obtained using serial reconstructions (widest diameter). For all three cases, the data were best fit as the sum of two Gaussian distributions (described further below). Pooling data from three cells shows that the difference in widest diameter and corrected diameter was minor for both small (4.1%) and large vesicles (4.8%). The ratio of the number of large vesicles to small vesicles, provided by the Gaussian fits to the data, was 0.72 for the serial reconstruction technique (Fig. 2F) and 0.96 for the corrected diameter technique (E). Please note that we used five individual sections, from the same cell, to produce the corrected diameter data, while only the three interior sections were used to measure widest diameter. When we compared widest diameter and corrected diameter in the same section, the ratios of large/small vesicles were virtually identical (not shown). These findings demonstrate that the correction algorithm adequately accounts for sectioning errors associated with vesicle diameter, without distorting the size distribution.

Quantal size measured from individual chromaffin cells suggests two populations of vesicles

Amperometry was used to assay catecholamine release from mouse adrenal chromaffin cells. Release was stimulated by the application of 100 µM Ca\(^{2+}\) to digitonin permeabilized cells (Jankowski et al. 1992). Amperometric events were rare in the
absence of Ca\textsuperscript{2+}, even after digitonin permeabilization, which suggests that the secretory machinery functions properly in the presence of digitonin (Graham et al. 2002; Holz et al. 1989; Jankowski et al. 1992, 1993). Figure 3A shows a representative amperometric trace with multiple individual events occurring over a 2-min stimulation period. On average the electrode detected 20 events/min, which provides an estimated rate of release of ~318 events/min over the entire surface of the cell (the electrode samples ~6% of the cell surface, see METHODS for calculation). The amperometric events from Fig. 3A are shown on an expanded time scale in B. Each amperometric event, corresponding to an individual release event, has been aligned such that they all begin at the same time. The events differ considerably in amplitude and overall shape. The majority of events appear as a single spike that rises rapidly and falls at a slower rate; however, in some cells, a fraction of the events exhibit the small elevation from baseline prior to the spike known as a “foot,” which is thought to arise from neurotransmitter release from a partially open fusion pore (Chow et al. 1992).

Quantal size measurements from individual cells often do not appear as single Gaussian distributions. The integral of amperometric current over the time of an individual event yields the charge (\(Q\)), which is proportional to the total number of molecules oxidized (referred to as the quantal size). \(Q\) is related to vesicle volume if transmitter concentration is similar.
Vesicle size and quantal size measured from cell populations are best described as bimodally distributed

Combining vesicle size measurements from 11 cells resulted in a distribution that is best described as the sum of two Gaussians. Vesicle diameter was measured from single sections (11 sections, 1 per cell) and transformed to the corrected diameter, as described in the preceding text. The resulting distribution was fit best as a bimodal distribution with small and large vesicles centered at 179 and 310 nm, respectively (see Fig. 4A). Size distributions made from individual cells (see the Figs. 1C and 2E) or from multiple cells (Fig. 4A) share a similar bimodal distribution; this argues that the two populations of vesicles exist in the same cell.

Figure 4B plots the cumulative $Q^{1/3}$ distribution measured from 32 cells. The quantal size distribution is best fit as the sum of two Gaussians. The small and large $Q^{1/3}$ distributions are centered at 0.40 and 0.69 $pC^{1/3}$, respectively. Because individual cells appear to have both small and large events, the bimodal $Q^{1/3}$ distribution shown in Fig. 4B is likely to reflect heterogeneity common to individual cells (Fig. 3C).

for all vesicles and secretion results in complete emptying. Assuming this to be true, then $Q^{1/3}$ should reflect vesicle diameter (volume = diameter$^3$) (Bruns et al. 2000; Finnegan et al. 1996; Wightman 1991). The $Q^{1/3}$ distribution for a single cell does not appear as a single Gaussian distribution (Fig. 3C).

Rather the data are best described as the sum of two populations each one with its own Gaussian distribution. The first group of events is clustered at $\sim$0.4 $pC^{1/3}$. The second type of event has a broader distribution centered at $\sim$0.7 $pC^{1/3}$. Hence, neither vesicle diameter nor $Q^{1/3}$ distributions measured from individual cells appeared to be composed of a single uniform population of vesicles.

**Figure 3.** Quantitative analysis of quantal size reveals 2 populations of vesicles within individual cells. A: shown here is a representative 2-min amperometric trace with multiple events. Cells were permeabilized with digitonin (20 $\mu$M) and then exposed to Ca$^{2+}$ (100 $\mu$M). On average there were $\sim$20 events/min detected at the electrode. B: the amperometric events from A are shown on an expanded time scale. Each amperometric event has been aligned such that they all begin at the same time. The events differ considerably in amplitude and overall shape. C: the area under each of the amperometric events, like those shown in B, was integrated to obtain the quantal size ($Q$). Plotted in C is a frequency histogram for $Q^{1/3}$ collected from a single cell. The data are best fit as the sum of 2 Gaussian distributions. The first group of events is centered at 0.41 $pC^{1/3}$. The second group is centered at 0.74 $pC^{1/3}$.

**Figure 4.** Measurements from multiple cells revealed a strong correlation between vesicle size and quantal size. A: the corrected vesicle size distribution. The results represent data from 11 cells (1 section per cell, total of 2,390 vesicle profiles; mean: 227.0 nm), and the apparent vesicle diameters were transformed to corrected diameters using the algorithm of Parsons et al. (1995) (see Experimental procedures). The resulting distribution is best described as the sum of 2 Gaussians ($r^2 = 0.99$; single Gaussian: $r^2 = 0.93$; for additional comparison of models, see Table 2), which represent small and large vesicles centered at 179 and 310 nm, respectively. B: the cumulative $Q^{1/3}$ distribution measured from 27 cells (totaling 3,847 events; mean: 227.0 nm), and the apparent vesicle diameters were transformed to corrected diameters using the algorithm of Parsons et al. (1995) (see Experimental procedures). The resulting distribution is best described as the sum of 2 Gaussians ($r^2 = 0.99$; single Gaussian: $r^2 = 0.93$; for additional comparison of models, see Table 2), which represent small and large vesicles centered at 179 and 310 nm, respectively. B: the cumulative $Q^{1/3}$ distribution measured from 32 cells. The quantal size distribution is best fit as the sum of 2 Gaussians ($r^2 = 0.99$; single Gaussian: $r^2 = 0.93$; also see Table 2). The small and large $Q^{1/3}$ distributions are centered at 0.40 and 0.69 $pC^{1/3}$, respectively. The vesicle size and quantal size distributions appear very similar in overall shape, and the ratio of small and large distributions reflects a fivefold difference in volume and quantal size (see Table 1). The data used for each figure was pooled from multiple experiments. To ensure each experiment was not distinct from the total pooled values, data from each experiment was compared with the total pooled values using a 1-way ANOVA test. None of the individual experiments was found to be significantly different (alpha = 0.05) from the pooled data, and the average P value was 0.514.
The 3-Gaussian model was rejected by the fitting program (see METHODS) in are taken as the most likely models to fit the data (designated with bold font). presented in Fig. 4B.

**Akaike Information Criterion (AIC**<sub>c</sub>**)) and the corresponding probability is**

<table>
<thead>
<tr>
<th>Model</th>
<th>Diameter (nm)</th>
<th>Volume (10&lt;sup&gt;-18&lt;/sup&gt; L)</th>
<th>Concentration, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small vesicles</td>
<td>179 ± 61</td>
<td>3.00</td>
<td>0.11</td>
</tr>
<tr>
<td>Large vesicles</td>
<td>310 ± 110</td>
<td>15.60</td>
<td>0.11</td>
</tr>
<tr>
<td>Ratio of large to small vesicles</td>
<td>1.73</td>
<td>5.13</td>
<td>5.13</td>
</tr>
</tbody>
</table>

Values are means ± SD. *Q<sup>1/3</sup> values represent the mean (center) of the Gaussian distribution for small or large events (see Fig. 4B). Q was calculated by taking the cube of Q<sup>1/3</sup> for each distribution. †The number of molecules represents the amount of catecholamine transmitter per amperometric event (Q), which was calculated using the Faraday equation (see Experimental procedures). ‡Values represent corrected vesicle diameters measured at the center of their corresponding Gaussian distributions (see Fig. 4A). §The corrected vesicle diameter was used to estimate the vesicle volume with the following equation: volume = 4/3π(diameter/2)<sup>3</sup>.

The apparent bimodal nature of both vesicle size and Q<sup>1/3</sup> are in agreement (see Table 1). The size ratio of large and small vesicles is 1.73 (corrected diameter in nm: 310/179), which is identical to the ratio of the two quantal size populations, 1.73 (Q<sup>1/3</sup>: 0.69/0.40). The average ratio of quantal size and vesicle size translates to a fivefold difference in volume (volume <sup>1/3</sup>: 0.69/0.40). The average ratio of quantal size and vesicle size translates to a fivefold difference in volume (volume <sup>1/3</sup>: 0.69/0.40).

To test whether a bimodal Gaussian distribution is the best model for the secretion and EM distributions, a statistical approach was taken to select the appropriate model. One, two model for the secretion and EM distributions, a statistical approach was taken to select the appropriate model. One, two

**Calculating transmitter concentration**

The concentration of transmitter in each vesicle population was estimated from the vesicle size and quantal size data sets. The corrected vesicle diameter was used to calculate volume, and the mean charge was used to estimate the number of transmitter molecules (see Table 1). Assuming that the small quantal events arise from small vesicles, we calculate an intra-vesicular transmitter concentration of 0.11 M. Similarly, the large vesicles are estimated to have a transmitter concentration of 0.11 M. These concentrations are similar to previous estimates where both vesicle and quantal sizes were determined (Bruns et al. 2000). The results suggest that transmitter concentration remains constant while vesicle size changes, a conclusion similar to that found in other studies (Albillos et al. 1997; Bruns et al. 2000; Colliver et al. 2000).

**Distinct kinetic properties are consistent with two populations of vesicles**

Groups of amperometric events were averaged to better observe kinetic properties of small events. Amperometric events were binned according to size, in Fig. 5, and presented as ensemble averaged spikes (each ensemble was composed of ~50 individual amperometric events). The top inset (Fig. 5I<sub>1</sub>) highlights the rapid rising phase of the small events. Figure 5I<sub>2</sub> superimposes a small event group with a large event group, after scaling them to the same amplitude. The scaled small event group does not overlay exactly with the large event group and in particular the small event group has a slower falling phase.

Figure 6A plots the relationship between amperometric event amplitude and Q. The data can be described as the composite of two separate linear processes. The fits for small and large events were made to regions where overlap was minimal. Small events are fit to a line with a modest slope compared with the line used to fit large events. The intermediate region appears as a transition between the two types of vesicles. This

**TABLE 1. Electron microscopy and amperometry data**

<table>
<thead>
<tr>
<th>Amperometry*</th>
<th>Q&lt;sup&gt;1/3&lt;/sup&gt;</th>
<th>Q&lt;sup&gt;1/3&lt;/sup&gt;</th>
<th>Number of Molecules†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small events</td>
<td>0.40 ± 0.14</td>
<td>0.064</td>
<td>0.200</td>
</tr>
<tr>
<td>Large events</td>
<td>0.69 ± 0.24</td>
<td>0.329</td>
<td>1.025</td>
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<tr>
<td>Ratio of large to small events</td>
<td>1.73</td>
<td>5.13</td>
<td>5.13</td>
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Electron Microscopy

<table>
<thead>
<tr>
<th>Vesicle‡ Diameter (nm)</th>
<th>Vesicle Volume§ (10&lt;sup&gt;-18&lt;/sup&gt; L)</th>
<th>Transmitter Concentration, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small vesicles</td>
<td>179 ± 61</td>
<td>3.00</td>
</tr>
<tr>
<td>Large vesicles</td>
<td>310 ± 110</td>
<td>15.60</td>
</tr>
<tr>
<td>Ratio of large to small vesicles</td>
<td>1.73</td>
<td>5.20</td>
</tr>
</tbody>
</table>

Amperometry and electron microscopic data used to calculate corrected Akaike Information Criterion (AICc) and the corresponding probability is presented in Fig. 4B. The model with the lowest AICc and highest probability are taken as the most likely models to fit the data (designated with bold font). †The 3-Gaussian model was rejected by the fitting program (see METHODS) in the case of the amperometry data.
data show that small events grow more slowly in amplitude as quantal size increases when compared with large events.

Similarly, analysis of individual spikes reveals the rise time and half-width are separable into two phases (Fig. 6B). Both rise time and half-width increase in value and follow one another closely over the range where small events predominate, from 0.2 to 0.4 pC^{1/3}. Rise time and half-width decline slightly in the 0.6- to 0.85-pC^{1/3} range and then become more dispersed at larger values. Smaller events tend to have a slower rate of transmitter release relative to large events as seen in the rapid rise in amplitude for the large events. Data are presented as means ± SE.

Another possible concern is that the assigment of small events as an independent population is flawed due to the possibility that they arise from release sites far from the electrode. Under these conditions, the electrode would capture only a fraction of the total secreted transmitter. Previous theoretical and experimental descriptions of distant release events predict attenuation in quantal size with concurrent lengthening of rise-time and half-width values (Bruns et al. 2000; Haller et al. 1998; Schroeder et al. 1992). The rapid rise times of small events, shown in Fig. 5, are not consistent with release at a distant site. Furthermore, Fig. 6B shows that the smallest events, had the fastest rise times and half-widths of all the small events (also see Fig. 5I). Kinetic values grew with increasing quantal size over the range of small events (0.2–0.4 pC^{1/3}); this is the opposite relationship predicted for distant release events. Our data are consistent with the Bruns et al. (2000)’s model, which suggests that diffusional losses do not significantly alter size distributions. Therefore we believe that the small quantal events reported here were faithfully sampled with our recording configuration.

Previous studies found that DCVs in both norepinephrine- and epinephrine-storing cells exhibited broad size distributions (Gorgas and Bock 1976; Kobayashi and Coupland 1977). Gorgas and Bock (1976) reported that a cell type, which resembled norepinephrine-storing chromaffin cells (also see

\[ \text{FIG. 6. Kinetic properties are distinct for small and large quantal sizes. A:} \]

\[ \text{the relationship between the amplitude and} \ Q \ \text{of individual events. The data are best fit by a composite of 2 separate linear processes. The fits for small and large events were made using data in regions where overlap was minimal. Small events could be fit with a line that exhibits a modest slope compared with that used to fit large events. B:} \]

\[ \text{analysis of individual spikes reveals the rise time} \ (\bullet) \ \text{and half-width} \ (\square) \ \text{are separable into 2 components. Both rise time and half-width increase in value and follow one another closely over the range where small events predominate, from 0.2 to 0.4 pC}^{1/3}. \ \text{Rise time and half-width decline slightly in the 0.6- to 0.85-pC}^{1/3} \ \text{range and then become more dispersed at larger values. Smaller events tend to have a slower rate of transmitter release relative to large events as seen in the rapid rise in amplitude for the large events. Data are presented as means ± SE.} \]
A recent study provided evidence for two types of vesicles in rat adrenal chromaffin cells; in one type, the vesicular membrane fit tightly around the dense cores, whereas in the other, the vesicular membrane was loosely fitting, leaving an electron-lucent halo (Pothos et al. 2002). Although, we did not attempt to categorize the vesicles into two groups based on their appearance as done by Pothos et al. (2002), large vesicles tended to have halos. Their study also reported that there was an increased number of vesicles with a halo after a 40-min stimulation (Pothos et al. 2002). The length of the stimulation used in that study makes it difficult to interpret whether the different types of vesicles are lost at different rates, or if some of the vesicles were newly formed. It is important to note that the rate of recycling for some DCVs has been reported to occur in <10 min (Bauer et al. 2004a,b), and de novo, TGN-derived, DCV synthesis has been suggested to occur in ~30 min in PC-12 cells (Tooze et al. 1991).

Previous amperometric studies in either murine or bovine chromaffin cells posited the existence of a single, broad distribution of $Q^{1/3}$ values. Murine chromaffin cells were reported to have mean charge and mean amplitude values similar to our own (Colliver et al. 2000; Sorensen et al. 2003); however, these studies did not present the data as a distribution. Bovine chromaffin cells also have broad $Q^{1/3}$ distributions (Albillos et al. 1997; Glavinovic et al. 1998; Wightman et al. 1995). Therefore the data presented in this study are not qualitatively different from these earlier studies. We fit $Q^{1/3}$ with a bimodal distribution, even though these earlier studies did not, because our statistical analysis indicated that the sum of two Gaussians yielded a significantly better fit than did a single Gaussian, and the addition of a third Gaussian did not improve the fit (Table 2). Because bimodal $Q^{1/3}$ distributions were observed in individual cells as well as ensembles of cells, the results cannot be explained as separate distributions originating in different types of chromaffin cells. Our data are consistent with a recent study by Tang et al. (2005), who also concluded that rat adrenal chromaffin cells possess multiple populations of vesicles.

The data in this manuscript show a tight correlation between vesicle size (as measured by EM with appropriate corrections) and $Q^{1/3}$. The simplest explanation for the tight correlation between vesicle sizes as measured with EM compared with amperometry is that the stimuli used in this study results in complete emptying of vesicles. Other studies have also found a close relationship between quantal size and vesicle size in cells that possess small and large secretory vesicles (Bruns et al. 2000; Chen et al. 1995). Bruns et al. (2000), using amperometric and EM methods, distinguished three different vesicle populations in leech neurons. Due to a segregated cellular distribution of the three kinds of vesicles, they were able to pair morphological and secretion data to convincingly show a linear relationship between vesicle size and quantal size. Karunanithi et al. (2002) showed that the two types of nerve terminals that form the Drosophila neuromuscular junction were distinguish-
able by their vesicle sizes, which had mean diameters of 41 and 48 nm. This difference in vesicle diameter leads to a $\Delta \approx 70\%$ increase in vesicle volume and a corresponding increase in quantal size (Karunanithi et al. 2002). These same authors also showed that some individual synaptic boutons exhibited more than one class of quantal event (Wong et al. 1999). Proteins that regulate vesicle formation may change vesicle size (for review, see Murthy and De Camilli 2003). Alterations in the expression levels of adaptor proteins, which are known to be involved in vesicle formation, can modify vesicle size (Fergestad et al. 1999; Karunanithi et al. 2002; Morgan et al. 1999; Nonet et al. 1999; Zhang et al. 1998), which gives rise to a change in quantal size (Fergestad et al. 1999; Karunanithi et al. 2002; Zhang et al. 1998). Please note that incomplete emptying of the vesicles would introduce errors into our calculations of vesicle size and neurotransmitter concentration, using amperometric data. It would not change the observation that chromaffin cells possess two populations of vesicles.

Amperometric events from the two vesicle populations exhibited distinct kinetic properties. The amperometric spike’s amplitude for small quantal events increased slowly with increasing quantal size, whereas large events exhibited a much larger increase in amplitude as a function of quantal size. These unique kinetics may reflect differences in rates of fusion pore dilation or possibly different rates of transmitter escape from the DCV’s protein matrix (for review of each possibility, Travis and Wightman 1998).

One could ask why have two populations of vesicles if they both store the same neurotransmitter? If the large vesicles are less fusogenic, then weak stimulation would preferentially release small vesicles, whereas strong stimulation would release both large and small vesicles. In this way cells would have tight control of low levels of release, as each vesicle that fused with the plasma membrane only releases a small amount of transmitter, but strong stimulation is able to release large amounts of catecholamines for rapid responses to stress or danger etc. A similar mechanism has been observed in parotid salivary cells where distinct vesicles are believed to mediate basal and stimulated secretion (Huang et al. 2001). Similarly, Aplysia bag cells release different peptide products from distinct vesicle classes (Sossin et al. 1990).

Whether neurotransmitter is released via full-fusion or a kiss-and-run mechanism and whether vesicles empty completely, or not, are still controversial topics. Cell-attached capacitance studies have functionally defined full-fusion as a patch of vesicle membrane stably incorporated into the plasma membrane for seconds; in a fraction of these events, it was possible to measure a conductance for the fusion pore prior to final dilation (Albillos et al. 1997; Klyachko and Jackson 2002; Tabares et al. 2003). On the other hand, kiss-and-run events were defined as reversible changes in membrane capacitance; such events occurred much less frequently (Albillos et al. 1997; Klyachko and Jackson 2002). Other techniques, though, provide strong evidence for kiss-and-run (Taraska et al. 2003). A cell-attached capacitance-amperometry study was able to shift release between full-fusion and kiss-and-run by varying the Ca$^{2+}$ concentration. In this study, Ales et al. (1999) were able to demonstrate that under recording conditions that favored kiss-and-run, the quantal size and vesicle size maintained a linear relationship just as it had under conditions that favored full-fusion (Ales et al. 1999). These results argued that vesicles emptied completely.

Although examples of complete release of transmitter have been proposed based on EM and amperometry results (Bruns et al. 2000; Colliver et al. 2000), other amperometry studies have yielded results that can be interpreted as partial emptying of vesicles. In dopamine neurons, flickers in the fusion pore result in the partial release of vesicular dopamine (Staal et al. 2004). The amount of secretion is variable in the porocytosis model as well (Kriebel et al. 2005). Elhamdani et al. (2001) recorded amperometric events while applying different patterns of electrical stimulation to chromaffin cells; they found that quantal size increased as a function of increased intracellular Ca$^{2+}$. The strongest stimulation conditions were thought to reflect complete emptying of vesicles (Elhamdani et al. 2001). In our study, we used a relatively intense stimulus to elicit catecholamine release, which would be consistent with the complete emptying of the DCVs found in the chromaffin cells. Our data suggest that under these release conditions, the amperometric events are truly quantal and represent the neurotransmitter content of individual vesicles.

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