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Heterogeneity of Catecholamine-Containing Vesicles in PC12 Cells

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Vesicular catecholamine release has been measured amperometrically from undifferentiated rat PC12 cells using carbon fiber microelectrodes. During superfusion with high K^+ saline, vesicular release was detected from ~50% of 200 cells investigated. On repeated stimulation the releasable pool of vesicles is rapidly depleted, while vesicle contents remains constant. Vesicular catecholamine release is not restored within 1 h after depletion of the releasable pool. Although the distribution of the cube root of vesicle contents of many cells is apparently Gaussian, maximum likelihood analysis of single cell data demonstrates double Gaussian distributions with median vesicle contents of 141 and 293 zeptomole. It is concluded that the releasable pool of vesicles in PC12 cells is heterogeneous. In the presence of L-DOPA mean vesicle contents increases, but cessation of release cannot be prevented, indicating that the number of releasable vesicles in PC12 cells is limited by a slow rate of vesicle cycling. © 2000 Academic Press

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The exocytosis of neurotransmitter-filled vesicles plays a major role in intercellular communication and involves intracellular fusion of the vesicle and plasma membranes, the formation of a fusion pore, and the subsequent release of vesicle contents. The actual release of the contents of a neurotransmitter vesicle takes a few milliseconds only (1). Exocytosis is preceded by slower processes, which proceed on a seconds to minutes time scale and include vesicle filling, translocation, docking, and priming, and is followed by the endocytosis of empty vesicles. The ensemble of these processes is known as the vesicle cycle (for review see Ref. 2).

PC12 cells, derived from a rat adrenal gland phaeochromocytoma, are commonly used as an *in vitro* model for both neurosecretory and neuronal cells. PC12 cells express a variety of receptors and ligand-gated ion channels, as well as T-, L-, N-, and P/Q-type Ca^{2+} channels (3, 4). Dopamine and norepinephrine are synthesized (5) and are presumably stored in large dense-core vesicles (6), many of which are in the close vicinity of the plasma membrane (7).

Real-time amperometric detection of catecholamines using carbon fiber microelectrodes (8) has revealed vesicular catecholamine release from PC12 cells on exposure to elevated K^+ concentrations in the presence of extracellular Ca^{2+} (9, 10) with an average vesicle contents of ~200 zeptomole (9, 11–14). Distributions of vesicle contents are skewed, but cube root-transformed data show an apparently normal distribution (10–13, 15, 16). Under the assumption that the vesicular catecholamine concentration is constant (17), these results may suggest that catecholamines are released from a homogenous population of vesicles. These conditions appear to hold for catecholamine release from chromaffin cells (Ref. 18, but see also Ref. 19). However, it is uncertain whether the same conditions apply to PC12 cells, since morphometric evidence on vesicle size (20) is not conclusive and intravesicular catecholamine concentration in PC12 cells is unknown. Here we demonstrate that catecholamines are released from a heterogeneous population of vesicles in PC12 cells.

MATERIALS AND METHODS

Cell culture. PC12 cells (CRL-1721; ATCC, Manassas VA) and PC12 cells from the Hubrecht Laboratory (Utrecht, The Netherlands) were grown in RPMI-1640 medium (Gibco, Grand Island NY) at 37°C in a 5% CO_2 atmosphere. The culture medium was supplemented with 5% fetal calf serum (ICN, Costa Mesa CA), 10% heat-inactivated horse serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all from Gibco). Cells were seeded in 25 cm^2 flasks at a density of $5 \cdot 10^5$ cells/flask and subcultured in 35 mm diameter dishes at a density of $5 \cdot 10^4$ cells/dish. Cell culture flasks and dishes (Nunc Inc., Naperville IL) were coated with 5 $\mu g/cm^2$ poly-L-lysine (Sigma, St. Louis MO). The culture medium was refreshed every 2–3 days. Experiments were performed 3–8 days after subculture.

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Experimental protocol. Carbon fiber microelectrodes were pulled from borosilicate glass capillaries (GC150; Clark Electromedical Instruments, Pangbourne Reading, UK) containing a 10 μm ∅ carbon fiber (Thornel, P-55W-2K, Amoco Corp. Greenville SC). The glass-carbon fiber junction was insulated with Sylgard elastomer (Dow Corning 182) and the carbon fiber was trimmed close to the glass junction using a scalpel blade. Pipettes were back-filled with 3 M KCl and tested for rapid and stable responses using saline solution containing 500 μM norepinephrine. Before experiments cells were washed with saline solution containing (in mM) 125 NaCl, 5.5 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 20 HEPES, 24 glucose, and 36.5 sucrose at pH 7.3. The carbon fiber was polarized to 750 mV and was placed gently on the membrane surface of a PC12 cell under continuous superfusion with saline through one barrel of a theta superfusion pipette (Clarke TGC150; pipette tip ∅ 80 μm) at a rate of ~150 μl/min. In general, exocytosis was evoked by superfusion with high K^+ saline (K^+ elevated to 125 mM and Na^+ reduced to 5.5 mM). Following each stimulus the cell was allowed to recover for at least 5 min, during which saline was superfused. For specific experiments other drugs were added to the saline immediately before the experiment. All experiments were performed at room temperature (21–23°C).

Data recording and analysis. Amperometric current was recorded using an EPC-7 patch clamp (List Electronic, Darmstadt, Germany), filtered at 2 kHz (8 pole Bessel filter), sampled at 4 kHz, and stored on disk for off-line analysis. Current transients associated with vesicular catecholamine release were identified and analyzed using custom-designed Labview routines (National Instruments, Austin, Texas). The peak amplitude and the 50–90% rise time of events were determined from the original signal. The original signal was digitally filtered (low-pass 100 Hz, 2nd order Bessel filter) for integration of the detected events. All events and their integrals were inspected visually to remove overlapping events and artifacts in the amperometric data (< 5% of the total number of events detected). Vesicle contents was calculated from the total charge (Q) transferred during the event according to Q/nF, where $n = 2$ electrons for the oxidation of one catecholamine molecule (21) and F is Faraday's constant (96,485 C/mole). In general, this method allowed for the detection of quanta ≥ 15 zeptomole of catecholamines.

Chemicals. NaCl, KCl, and HEPES were obtained from Merck (Whitehouse Station NJ), $MgCl_2$ and $CaCl_2$ from BDH Laboratory Supplies (Poole Dorset, UK). All other chemicals were obtained from Sigma. Saline solutions were prepared with Milli-Q (Millipore, Bedford MA) distilled water. Stock solutions of 1 mM ionomycin in dimethylsulfoxide (DMSO) and of L-DOPA in distilled water were kept at −20°C and thawed before the experiment. DMSO alone (0.5% v/v) did not affect release.

Statistical analysis. In order to relate vesicle contents to vesicle volume (17) distributions of the cube root of vesicle contents were constructed and analyzed using custom-designed Matlab routines (The Math Works Inc., Natick, MA). Cube root-transformed data were tested for normality using the Kolmogorov-Smirnov test. Maximum-likelihood estimates of the parameters of Gaussian distributions were calculated using the Matlab simplex optimization routine. A correction was made for events that were too small to be detected according to the method described by Colquhoun and Sigworth (22). The goodness-of-fit of a double versus a single Gaussian distribution was tested by the log likelihood-ratio test (22). Results were compared with Student's *t*-test and tests for correlations were performed using Spearman's rank correlation test. All values are reported as mean ± SD.

RESULTS

During superfusion of PC12 cells with saline the frequency of quantal catecholamine release, recorded with the 10 μm diameter carbon fiber microelectrodes, was very low (< 1/min). Following a switch of the superfusion to high K^+ saline quantal events were recorded from ~50% of the cells ($n = 200$). Exocytosis was strongly linked to depolarization, as the first events were usually detected within the first second after switching the superfusion to high K^+ saline and exocytosis stopped within seconds after switching back to normal saline (Fig. 1A). The amperometric events displayed typical characteristics of quantal catecholamine release. The evoked current transients had a rapid rising phase with 50–90% rise times that were generally < 2.5 ms and a relatively slow exponential decay. The release of single vesicle contents was generally complete within 10–50 ms. Occasionally, foot signals, which are assumed to represent early transmitter leakage during formation of the fusion pore (8), could be resolved (Fig. 1B). The distribution of cube root of vesicle contents obtained from data pooled from 18 cells with mean vesicle contents in the range of 118–315 zeptomole is apparently Gaussian (Fig. 1C) with a mean ± SD of 5.67 ± 1.16 zeptomole$^{1/3}$, corresponding to a median value of 182 zeptomole. This confirms previously reported results suggesting that

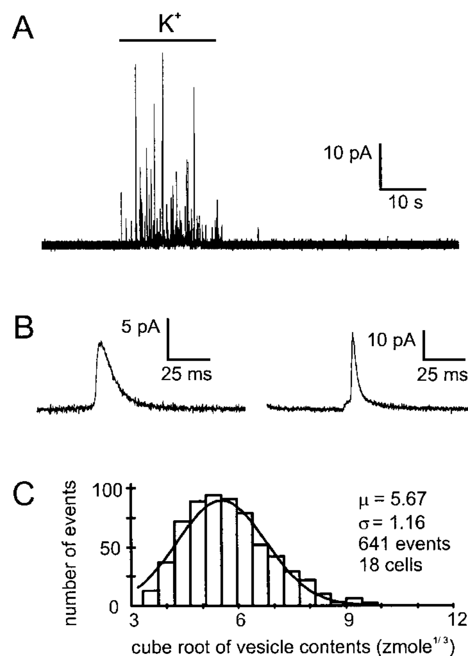

FIG. 1. (A) Amperometric recording from a PC12 cell showing vesicular catecholamine release during superfusion with high K^+ saline. The bar on top indicates the period of superfusion with high K^+ saline. (B) Two examples of amperometric current transients on an expanded time scale. The transients represent single vesicle release without (left trace: 50–90% rise time = 1.9 ms; vesicle contents = 850 zeptomole; taken from the recording in A) and with a so-called foot signal (right trace: 50–90% rise time = 0.5 ms; vesicle contents = 750 zeptomole; recorded from another PC12 cell). (C). Gaussian distribution of the cube root of vesicle contents obtained from 18 cells. The solid line is a Gaussian function with mean (μ) and variance (σ) obtained by the Kolmogorov-Smirnov test ($P > 0.10$).

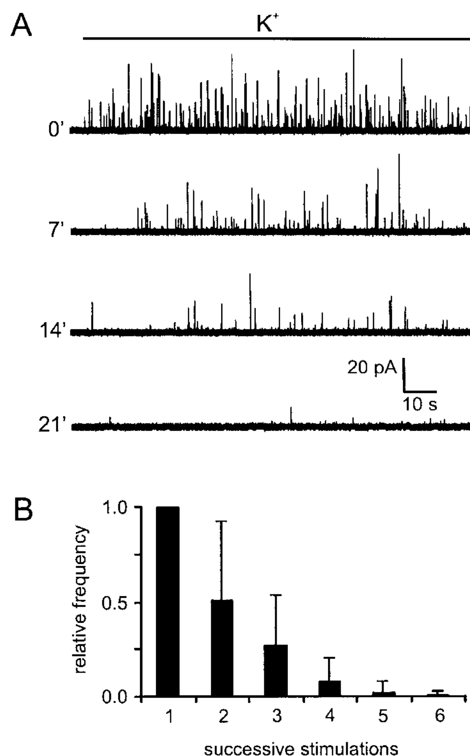

FIG. 2. Depletion of releasable vesicles during repeated depolarizing stimuli. (A) Amperometric traces obtained from a single PC12 cell during repeated 2 min depolarizations alternated by 5 min recovery periods. The bar on top indicates the period of superfusion with high K^+ saline. (B) Relation between the exocytotic frequency and the number of successive stimulations. The exocytotic frequency is expressed as the fraction of the value obtained during the first response. Each bar represents mean ± SD of 12 cells.

catecholamines are released from a homogenous population of vesicles in PC12 cells (10–13, 15, 16).

Factors limiting vesicular release. The number of quanta that could be released from single PC12 cells by stimulation with high K^+ saline was limited and varied markedly with total numbers ranging between 1 and 603 (mean = 53; median = 25; n = 117 cells). In only ~14% of the cells > 100 amperometric events could be detected. The vesicle contents also differed strongly between cells with mean values in the range of 90–600 zeptomole (n = 72 cells).

Since few cells released large numbers of vesicles, efforts were made to characterize the factors underlying this apparent limitation. The number of quanta released by repeated depolarizations decreased on each subsequent stimulus, despite a 5–10 min recovery interval between successive depolarizations (Fig. 2A). The average decrease of the number of exocytotic events evoked by repeated stimulation in 12 cells is illustrated in Fig. 2B. It is readily seen that the number of events decreases by approximately 50% on each subsequent stimulus, resulting in the depletion of releasable vesicles after up to six stimuli. Reappearance of catecholamine release did not occur within the time span of experiments. When cells were repeatedly depolarized until exocytosis stopped completely, recovery of the exocytotic response was not observed within 1 h, during which cells were superfused with saline solution (n = 3). Control experiments showed that the superfusion itself had no effect on exocytosis. Cells superfused for 1 h with saline still responded with exocytosis when stimulated with high K^+ saline (n = 3; results not shown). Limited numbers of events, which were comparable to those recorded from cells repeatedly stimulated with high K^+ saline, were also recorded from cells which were stimulated with ionomycin (n = 10) as well as from cells that were repeatedly stimulated with 200 μM ATP (n = 6). After depletion of releasable vesicles by repeated depolarizations, superfusion with saline containing 1–5 μM ionomycin was unable to evoke additional release in 5 cells, and in a 6th cell ionomycin evoked a marginal response only. This indicates that depletion of the depolarization-sensitive pool of vesicles parallels depletion of the Ca^{2+}-sensitive pool (results not shown).

Cessation of release could also be caused by depletion of catecholamines following exocytosis, resulting in less efficient vesicle filling and in an increase in the number of quanta below the detection limit. Although vesicle contents differed considerably between cells, there appeared to be no relation between vesicle contents and event number of the quanta released. Testing for trends did not reveal a statistical significant relation between vesicle contents and event number for 5 cells from which > 300 events were recorded (Spearman's rank correlation test, $P > 0.8$). Since vesicle contents remains unaffected until depletion of all releasable vesicles, the intracellular concentration of catecholamines appears not to be a limiting factor for the number of releasable quanta.

To further elucidate the role of the intracellular catecholamine concentration, cells were superfused with saline containing 100 μM L-DOPA in between high K^+ stimuli, which were repeated every 15 min. Within the first 15 min period of superfusion with L-DOPA mean vesicle contents significantly increased (t-test, $P < 0.01$) and remained increased for at least 60 min when L-DOPA was superfused in between depolarizations. The kinetics of the increase in vesicle contents are described by a single exponential function with a time constant of 21 min (Fig. 3A). In addition to the increase in mean vesicle contents, L-DOPA initially caused a threefold increase in the mean number of quanta released by the depolarizing stimulus (Fig. 3B; t-test, $P < 0.05$). However, after the initial increase the number of quanta decreased with successive stimulations and releasable vesicles were depleted in a way comparable to that observed in control cells (Fig. 3B). Since the apparent charging time constant of vesicles is 21 min (Fig. 3A), these results rule out the possibility

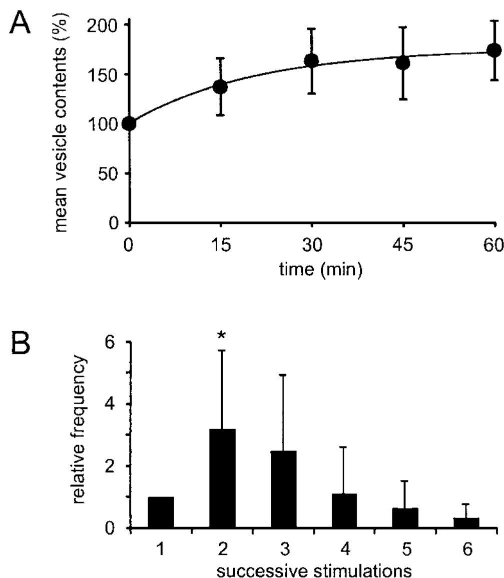

FIG. 3. (A) Relation between mean vesicle contents and the duration of superfusion with saline containing 100 μM L-DOPA. Release was evoked by superfusion with high K^+ saline for 15 s, shortly after L-DOPA was removed. During the 15 min intervals of superfusion with L-DOPA-containing saline, the carbon fiber electrode voltage clamp was switched off. Each point represents mean ± SD ($n = 8$) and at all time points the mean vesicle contents was increased significantly as compared to control (*t*-test, $P < 0.01$). The drawn line is an exponential curve fitted to the data with a maximum increase amounting to 76% and an exponential time constant of increase of 21 min. (B) Relation between the exocytotic frequency and the number of successive stimulations with high K^+ saline for 15 s at 10–15 min intervals, during which 100 μM L-DOPA-containing saline was superfused. The exocytotic frequency is expressed as the fraction of the value obtained during the first response, which was evoked before exposure to L-DOPA. Each bar represents mean ± SD ($n = 8$). During the first stimulus after L-DOPA superfusion the frequency of events was significantly higher than the control value (*t*-test, $P < 0.05$).

that a reduction in the availability of intracellular catecholamines causes the cessation of vesicular release in PC12 cells. Therefore, it is concluded that the depletion of the releasable vesicle pool is due to a very slow rate of vesicle cycling.

Single-cell distributions of vesicle contents. Although it has been common practice before, the pooling of data to obtain distributions of vesicle contents containing large numbers of events appears incorrect because of an up to sevenfold difference in mean vesicle contents between cells. Therefore, only those cells from which more than 300 events were recorded during high K^+ saline stimuli ($n = 5$) were used for a detailed analysis of single cell distributions of vesicle contents.

Statistical analysis demonstrates that the distributions of the cube root of vesicle contents in 4 of the 5 cells (Fig. 4) cannot be described by a single Gaussian function (Kolmogorov-Smirnov test, $P < 0.05$). Likelihood analysis showed that fitting the four distributions by double Gaussian functions resulted in a significant improvement of the goodness-of-fit as compared to a single Gaussian (log likelihood-ratio test, $P < 0.005$). Post-hoc testing of the binned data presented in Fig. 4 by the χ^2 goodness-of-fit test for the same four cells also showed significant deviations from single Gaussian distributions ($P < 0.05$), whereas the fits by double Gaussian functions could not be rejected ($P = 0.21 - 0.74$). The double Gaussian distribution of the cube root of vesicle contents indicates the presence of distinct populations of large and small quanta with a mean of 5.20 ± 0.39 zeptomole$^{1/3}$ and 6.64 ± 0.58 zeptomole$^{1/3}$ ($n = 4$; *t*-test, $P < 0.01$). The fitted mean values correspond to median vesicle contents of 141

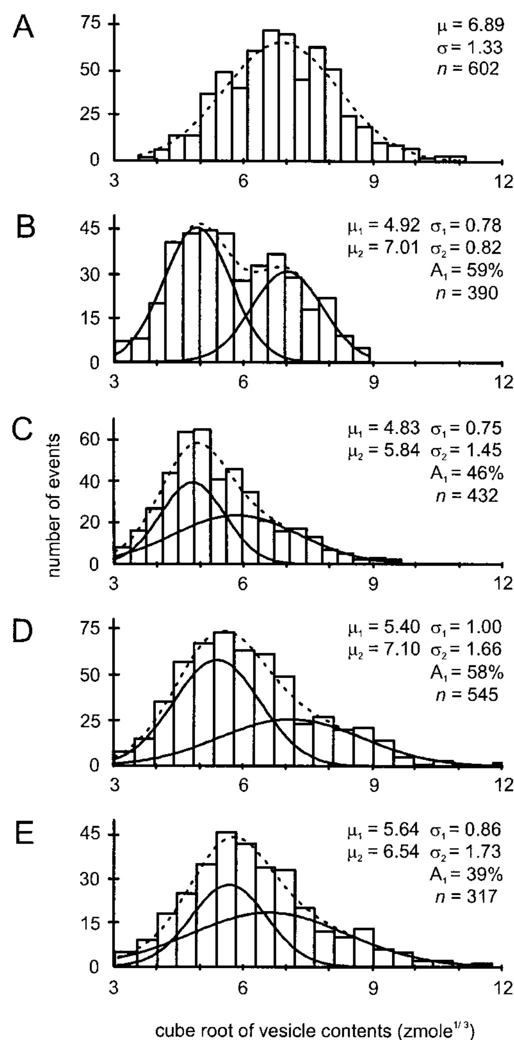

FIG. 4. Single cell distributions of the cube root of the vesicle contents of 5 PC12 cells from which > 300 events were recorded during repeated depolarizing stimuli with high K^+ saline. The distributions are fitted by double Gaussian functions for 4 of the 5 cells (B–E) and by a single Gaussian function for the cell in (A). The dashed lines represent the sum of the Gaussian distributions (solid lines) with the maximum likelihood estimates of mean (μ) and variance (σ) indicated in each panel. The estimated number of small events (A_1) is indicated as a percentage of the total number of events (n).

and 293 zeptomole, respectively. The number of large events, as a fraction of the total number of events, is 0.50 ± 0.09 for the cells presented in Fig. 4B–4E. The distribution in Fig. 4A, which was fitted by a single Gaussian with a mean value of 6.89 zeptomole$^{1/3}$, corresponding to a median value of 327 zeptomole, may be composed of large events mainly.

Since the cells used for analyzing the distribution of vesicle contents released many more vesicles than found on average, it is possible that the quanta detected in these experiments originate from multiple sites. Therefore, the possibility that the heterogeneity in the distributions in Fig. 4 originates from different distances between multiple sites of catecholamine release and the carbon fiber surface should be ruled out. It has been pointed out before that event rise time increases steeply with the distance between the site of release and the electrode surface (8) and that the amount of charge detected with a large flat electrode surface remains constant for distances up to 5 μm (23). Analysis of the events used for the distributions in Fig. 4 showed that all events had 50–90% rise times < 2.5 ms. If the small events originate from distant release sites, they should have slow rise times and a negative correlation between rise time and vesicle contents would be expected. Conversely, for the data presented in Fig. 4A, 4C, 4D, and 4E rise time and vesicle contents showed a weak positive correlation (Spearman's rank correlation test, $R_S = 0.12, 0.11, 0.21, 0.47$; $P < 0.05$), and a negative correlation was apparent only for the data of the cell presented in Fig. 4B ($R_S = -0.21$; $P < 0.01$). Therefore, the results demonstrate that catecholamines are released from a heterogeneous pool of vesicles in PC12 cells.

DISCUSSION

Availability of catecholamines and vesicle cycling. PC12 cells release only a limited number of catecholamine-containing vesicles. Although previous amperometric studies have not specifically addressed the question of how many vesicles can be released from single PC12 cells, all results published thus far show a similar limitation of vesicular catecholamine release (10, 11, 13, 14). From the increase of membrane capacitance evoked by rapid photolysis of caged Ca^{2+}, undifferentiated PC12 cells have been estimated to contain a readily releasable pool of 1000 large dense-core vesicles (24). Since the carbon fibers used in the present experiments (10 μm $\varnothing$) will cover up to ~20% of the cell surface, the release of 1000 vesicles from the cell would result in the detection of no more than 200 events. The present results show that, despite the competence of PC12 cells for exo- and endocytosis (24) and despite the demonstrated expression of many of the proteins essential for the different steps of the vesicle cycle (reviewed in Ref. 25), the releasable pool of vesicles is rapidly depleted by exocytotic stimuli, e.g., high K^+ saline, ionomycin, and ATP. If the availability of catecholamines would be a limiting factor, vesicle contents would be expected to decrease before cessation of release, since quantal size is reduced by inhibition of tyrosine hydroxylase (14). However, vesicle contents remains constant over time until complete cessation of release and the cessation of release cannot be prevented by increasing the availability of catecholamines by continuous superfusion with L-DOPA (Fig. 3). These results show that, although quantal size appears to be related to catecholamine availability, the availability of catecholamines is not responsible for the cessation of vesicular release. Since the depletion of the releasable pool of vesicles by depolarization parallels the depletion of the ionomycin-releasable pool and since recovery of the releasable pool takes much longer than the time constant for charging of the vesicles in the presence of L-DOPA (Fig. 3A), it is concluded that appreciable cycling of large dense-core vesicles does not occur within the time span of the experiment.

Vesicle heterogeneity. In previous studies, distributions of the cube root of vesicle contents of PC12 cells, chromaffin cells, mast cells and pancreatic β cells have been fitted using single Gaussian functions (11, 15–17). All distributions of vesicle contents of PC12 cells were based on data from multiple cells in order to obtain sufficiently large numbers of events. However, the present results demonstrate large (6- to 7-fold) differences in mean vesicle contents between cells. Maximum likelihood analysis of single cell data (Fig. 4) shows that double Gaussian curves are generally required to describe the distribution of the cube root of vesicle contents of single PC12 cells and demonstrates the existence of two distinct classes of catecholamine-containing vesicles, which differ in their median vesicle contents by a factor of ~2. This difference is much smaller than the difference in mean vesicle contents between cells, and may be obscured in the distributions of pooled data that were considered previously (Fig. 1C).

Vesicle heterogeneity should be reflected in either differences in vesicle size or in differences in vesicular catecholamine contents. Morphometric measurements did not reveal distinctly sized classes of large dense-core vesicles in PC12 cells, as the distribution of vesicle diameter is approximately Gaussian (20). However, like previous results on the distribution of vesicle contents, the distribution of vesicle size is also based on data pooled from multiple cells. If it is assumed that the observed twofold difference in vesicle contents is exclusively caused by differences in vesicle size, the mean diameter of the large and small populations of vesicles would differ by a factor of 1.28. Such a difference would be difficult to resolve from the published distribution of vesicle diameter, since its standard deviation is approximately 25% of the mean (20).

The twofold difference in vesicle contents could be explained by release of aggregated vesicles, as observed at a relatively high frequency of 30% in mast cell degranulation (26), or by compound fusion of vesicles, which may occur at the rare incidence of ~0.4% in chromaffin cells (27). In order to obtain a number of large events equal to 50% of the total number of events (Fig. 4), 67% of the vesicles should be aggregated or be released simultaneously. Although this seems an unlikely high percentage, vesicle aggregation and compound fusion cannot be excluded at present.

It is known that PC12 cells release the catecholamines dopamine and norepinephrine (5), and that the large dense-core vesicles in these cells contain catecholamines as well as ATP (6). The question whether the different classes of vesicles contain different types of catecholamines or contain ATP and catecholamines at different ratios cannot be answered at present and would require a quite advanced analysis of single vesicle contents.

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