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Review

Quantitative Measurements of Released Amines from Individual Exocytosis Events

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Abstract

Chemical analysis of single cells is an area of great interest in the biological sciences. Single-cell systems are being utilized as a model to understand in vivo processes better. One method that is moving to the forefront in cellular analysis is electrochemistry. Owing to their rapid response time and small dimensions, voltammetric microelectrode techniques, such as amperometry and fast-scan voltammetry, have made it possible to monitor minute amounts of biological compounds and transiently occurring chemical events in cellular systems. The application of these methods to the quantitation of individual vesicular release events from single cells is overviewed here. The application of electrochemical monitoring to several types of cultured cells, including bovine adrenal chromaffin cells, rat pheochromocytoma (PC12) cells, beige mouse mast cells, superior cervical ganglion neurons, and human pancreatic β-cells, as well as to the invertebrate systems, the leech *Hirudo medicinalis*, and pond snail *Planorbis corneus* has provided a wealth of new information concerning exocytosis. Results obtained from the studies highlight the potential of electrochemical techniques in cellular analysis to contribute to our understanding of molecular and pharmacological effects on exocytosis. This article overviews work done on all the above cell types with an emphasis on PC12 cells.

Index Entries: Exocytosis; quantitation; catecholamines; PC12 cells; mast cells; adrenal cells; pancreatic β-cells.

Introduction

Exocytosis, a key component of neuronal communication, is a process that has been investigated extensively for several decades (Valtorta et al., 1990; Stamford et al., 1996). The process of exocytosis can be summarized as the docking of a vesicle (storage compartment) to the cell membrane, and subsequent release of the contents by fusion of the vesicle and cell membranes. This process allows the conversion of an electrical signal (action potential) to a chemical signal, which is necessary for communication between cells. The molecular mechanism of exocytosis has been the subject of a great deal of debate (Monck and Fernandez,

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1994; Schroeder et al., 1996), and methods to observe and quantitate individual events have traditionally revolved around electron microscopy and patch-clamp capacitance measurements (Neher and Marty, 1982). In the last few years, it has become possible to monitor directly individual exocytosis events involving easily oxidized messengers using amperometric measurements at microelectrodes (Wightman et al., 1991). These measurements allow precise quantitation of the messenger released during each exocytosis event.

The release of chemical messengers has been studied on the whole-cell level to quantitate an average release phenomenon and, at the level of a single vesicle, to quantitate individual exocytotic events. Investigations of the whole cell have been accomplished by capillary electrophoresis (Chen et al., 1995) and high-performance liquid chromatography (HPLC) (Cooper et al., 1994; Pothos et al., 1996) with electrochemical detection. These techniques provide a means for sampling small volumes and yield important information about the vesicular chemical content. However, these methods lack the time and spatial resolution needed for detection of individual release events.

Detection of neurotransmitters from single exocytotic events has recently been made possible by significant advances in microelectrode technology. The sensitivity of microelectrodes provides a means to detect low levels of easily oxidized neurotransmitters secreted from single vesicles, while also providing selective detection in a complex biological matrix. The rapid response time of these sensors makes them useful for real-time studies of release. Another advantage is their small structures, which makes them ideal for monitoring dynamic chemical processes, like exocytosis, at a single-cell surface. Stimulation of a cell when a microelectrode is placed on the surface results in a series of current spikes resulting from the oxidation of catecholamines (Fig. 1). Wightman et al. (1991) have shown that each current spike represents the oxidation of neurotransmitter from a single exocytotic event. Microelectrode techniques can also provide chemical information about the released neurotransmitter using fast-scan cyclic voltammetry (Fig. 1, inset). Thus, amperometric detection appears to be a highly valuable tool for quantitation of quantal communication events.

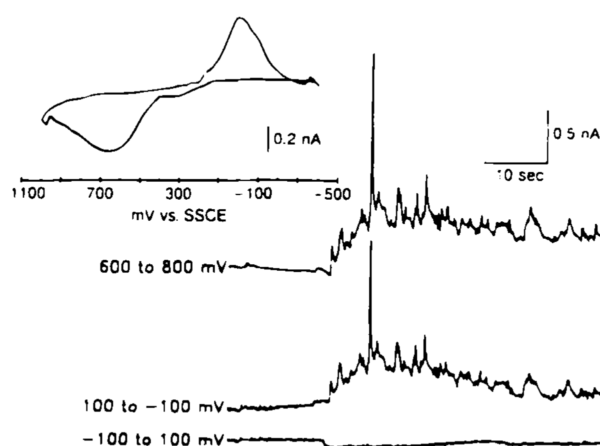


Fig. 1. Voltammetric data (scan rate, 200 V · s^{-1}; repetition rate, 10 Hz) obtained with a carbon-fiber electrode during exposure of a single cell to 100 μ*M* nicotine (3-s pressure ejection). The subtracted voltammogram obtained following nicotine exposure is shown in the upper left portion of the figure. The voltammogram is 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. Voltammograms obtained prior to stimulus represent only background. Thus, the subtracted voltammograms represent the change in the concentration of catecholamine. The traces show average currents obtained from successive voltammograms. The potential regions examined are 600–800 mV on the positive scan (top trace), 100 to –100mV 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. Reprinted with permission from Wightman et al. (1991).

Understanding communication between mammalian neurons is the ultimate goal in all these studies; however, at current levels of detection, the extremely minute amounts of chemical transmitter released at a single synaptic vesicle are difficult to detect. The conven-

tional size of a vesicle in a nerve terminal is approx 50 nm, and the estimated neurotransmitter is 1–2000 molecules (Siegel et al., 1994). In addition, an estimate of 1000 molecules has been used for modeling the efflux of dopamine measured electrochemically in the rat striatum (Garris et al., 1994). Mammalian neurons also have an added complexity of the cellular environment in vivo and the inconvenience that mature neurons stop proliferation. Add to this the need for nanometer-size electrochemical probes to access the small synaptic gap and it is evident that technology is not yet available to achieve this goal.

For these reasons, model systems with larger vesicles and higher concentrations of neurotransmitter have been used to investigate individual exocytotic events. In this article, we summarize the different cell types that have been used as model systems for amperometric exocytosis measurements and describe some of the questions being addressed. The nonsynaptic model systems that will be covered include bovine adrenal chromaffin cells, beige mouse mast cells, human pancreatic β-cells, and rat pheochromocytoma (PC12) cells. Two invertebrate neuronal systems that form functional synapses and one mammalian neuronal system will also be discussed. Quantitation of vesicular release for these cellular systems using amperometry will be presented along with a more detailed discussion of exocytosis measurements using the pheochromocytoma cell line.

Adrenal Cells

The majority of work concerning exocytosis at the single-cell level has been carried out on bovine adrenal chromaffin cells. They are ideal models for the study of stimulation–secretion processes because of their similarities to postganglionic sympathetic neurons (Adams, 1990). Like neurons, they possess vesicles containing catecholamines that are secreted in a quantal fashion upon chemical or electrical stimulation. The first direct chemical evidence for time-resolved detection of individual exocytosis events has been observed at single adrenal cells by Wightman and coworkers using electrochemical detection (Fig. 1) (Wightman et al., 1991; Leszczyszyn et al., 1991). In this work, the current spikes in the amperometric response are described as individual quanta of neurotransmitters representing the release of a single vesicle. This is justified based on statistical evidence of spike overlap and analysis of histograms showing single distributions of spike frequency vs amount released. An average quantity of neurotransmitter per adrenal medullary chromaffin vesicle has been reported as 1–10 attomol. Finnegan et al. (1996) report a more precise value, 3.3 attomol (2.0×10^6 molecules), obtained by fitting the exocytotic response with a Gaussian distribution based on vesicular volumes (*vide infra*). Fast-scan voltammetry (Fig. 1, inset) has been used to identify the secreted substances as norepinephrine and epinephrine (Ciolkowski et al., 1992). In this case, the faster rate of cyclization of epinephrine to norepinephrine generates a second peak in its cyclic voltammogram and can be used to distinguish the two compounds. Conclusions reached by measurement of individual exocytosis events at adrenal cells are summarized in the paragraphs below.

Manipulations of microelectrodes and application of different chemical stimulants have led to further insight behind the exocytotic process. One perturbation of the microelectrode has been a decrease in size. Etched carbon fibers, which produce electrodes having ~1 μm diameter, have been investigated for use in cellular analysis (Kawagoe et al., 1991; Strein and Ewing, 1992). Spatially localized zones of exocytotic release have been unveiled by mapping the surface of adrenal cells with these small sensors (Schroeder et al., 1994).

The use of amperometric monitoring of catecholamine release combined with application of receptor agonists and antagonists has been used to identify autoreceptors on the cell surface. Zhou et al. (1994) have applied this method to adrenal cells, and confirmed that autoreceptors exist and operate by a negative feedback mechanism. Similar to the α_2-autoreceptor antagonist piperoxan, amphetamine is

shown to increase the number of catecholamine exocytosis events, suggesting that the pharmacology of amphetamine includes blockade of catecholamine autoreceptors.

A chelating fluorescent probe that allows detection of intracellular divalent cations, Fura-2 AM, has been used in conjunction with electrochemical detection of catecholamine release to correlate calcium responses to various chemical agents (Jankowski et al., 1994; Finnegan and Wightman, 1995; Robinson et al., 1995). It has been observed that secretion by exocytosis is strongly coupled to elevation in cytosolic Ca^{2+} (Finnegan and Wightman, 1995). Secretory vesicle catecholamine content has been manipulated by changes in the extracellular ionic composition and by addition of secretogogs. It has been shown that concentrations in the vesicle are affected as well as the kinetics of the release mechanism (Jankowski et al., 1994).

Chow and coworkers (1994) combined amperometry with electrical capacitance measurements to correlate the change in calcium concentrations with exocytosis measurements on depolarization of the adrenal cell membrane. Based on the lower concentrations of calcium and slower increases in calcium concentration, it has been suggested that calcium channels and vesicles are not closely apposed in the chromaffin cell, unlike synapses. Figure 2 shows the two possibilities, molecular coupling and no molecular coupling, for localization of the exocytosis machinery. This conclusion contrasts that obtained with calcium imaging combined with small electrochemical probes (Finnegan and Wightman, 1995; Robinson et al., 1995).

Temporal characteristics of vesicular release with respect to diffusional broadening have been studied by Wightman et al. using bovine adrenal cells. Exocytosis has initially been described as a diffusion-controlled process (Schroeder et al., 1992), and the amplitude and time-course of the signal described as correlated by the distance between the electrode and the cell (Kawagoe et al., 1991). Recently, additional studies with the electrode in direct contact with the cell surface have suggested that diffusion is not the only process occurring. Delay in vesicle fusion compared to neuronal systems has been reported by Chow et al. (1995), as well as a small peak or "foot" at the onset of the current transient for vesicular release. A more detailed study of the foot region of the amperometric response at chromaffin cells shows rapid fluctuations in transmitter release, which are attributed to opening and closing of the fusion pore prior to complete exocytosis (Zhou et al., 1996). In addition, Wightman et al. (1995) have studied in detail the temporal broadening of the exocytosis event and described a rate-limiting kinetic step that involves dissociation of the neurotransmitter once released. Three distinct stages of exocytosis have been described (Schroeder et al., 1996), which include formation of the fusion pore, pore expansion to release catecholamine, and finally dissociation of the intravesicular matrix. A typical current transient with each stage in the exocytosis

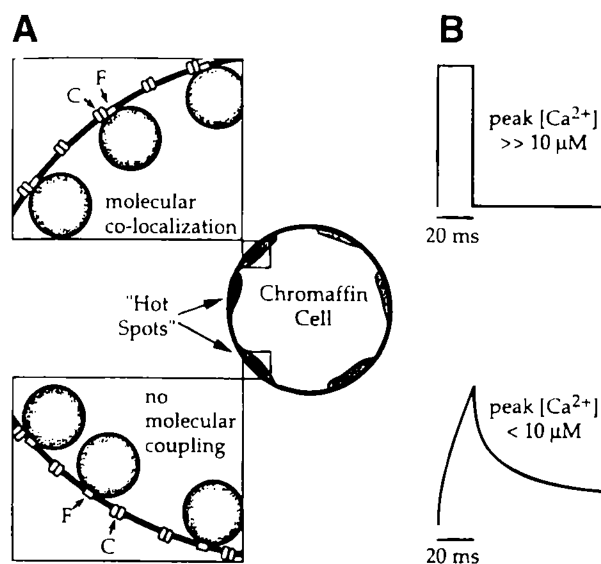
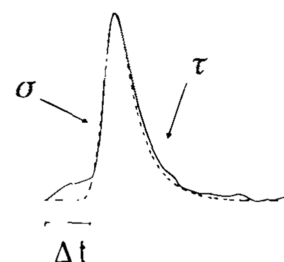


Fig. 2. **(A)** Schematic comparison of molecular coupling of vesicles and channels (upper box) and noncoupled vesicles and channels (lower box). F, fusion machinery; C, calcium channel. Methods having micrometer-level resolution (such as optical imaging) would not allow one to distinguish between these two cases. **(B)** Diagrammatic comparison of $[Ca^{2+}]_i$ time-courses at the vesicles for the cases shown to the left in A, for 20-ms depolarizations. Reprinted with permission from Chow et al. (1994).

process identified is shown in Fig. 3. Adrenal cells appear to store catecholamines in a matrix of chromogranin A, which is solubilized at extracellular pHs, but is polymeric at the more acidic pH inside the vesicle. When the vesicular matrix is expelled, the change in pH causes the chromogranin A to dissolve, thus releasing catecholamine.

Mast Cells

Beige mouse mast cells have also been utilized for amperometric investigations of the secretory agents 5-hydroxytryptamine (serotonin) and histamine. Histamine and serotonin are easily oxidizable molecules that are important in the nervous and immune systems of mammals. One advantage of using this cell system is the large size of the vesicles present. The larger vesicles allow release to be correlated with simultaneous changes in membrane capacitance (de Toledo et al., 1993). This makes mast cells especially useful for studies involving the fusion process of exocytosis. Quantitation of both agents has been described for whole mast cells using capillary chromatography with electrochemical detection yielding 150 ± 18 and 3.8 ± 1.3 fmol of histamine and serotonin/cell, respectively (Pihel et al., 1995). Serotonin and histamine have been shown to corelease from a single vesicle. An average serotonin vesicle content of 3.8 m*M* has been reported by Finnegan et al. (1996). Taking into consideration an average vesicle radius of 770 nm, an average of 7.3 amol (4.4×10^6 molecules) of serotonin is present in a single vesicle of the mast cell. The mast cell appears to be a promising model system for which amperometric detection of release events can be used to understand exocytosis further.



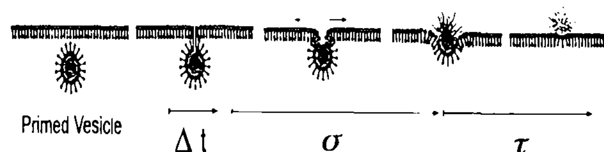


Fig. 3. A single secretory event with an exponentially modified Gaussian function superimposed. Cartoon: Proposed stages in exocytosis. In response to stimulation, a vesicle fuses with the plasma membrane, resulting in a pore connecting the vesicular contents to the external medium. Flux of free intravesicular catecholamine is almost steady-state at this time because of restriction by the pore diameter. The duration of this stage is given by Δt. Next, the fusion pore expands, which leads to a Gaussian flux from the vesicle characterized by a time constant σ. The dissociation rate of catecholamine from the protein matrix retards the release and is reflected in the parameter τ. Reprinted with permission from Schroeder et al. (1996).

Pancreatic β-Cells and Rat Melanotrophs

The electrochemical techniques discussed above have also been extended to other secretory cells, including human pancreatic β-cells and rat melanotrophs. Pancreatic β-cells secrete insulin based on the level of glucose in the body. Membrane potential experiments with intracellular microelectrodes (Meissner, 1990) and amperometric monitoring (Kennedy et al., 1993) of insulin secretion from individual cells have been carried out in efforts to gain insight into the function of these important cells in the regulation of blood sugar levels in the body. The latter work has been extended to show that material secreted during exocytosis is indeed insulin using chromatographic methods (Huang et al., 1995). The fact that insulin is stored within vesicles provides a common link between exocytosis from β-cells and cells of the nervous system. Exocytosis from these cells has also shown a

calcium ion dependence similar to the other secretory cells described. The insulin found within a vesicle is stored in a solid form (crystalline zinc–insulin complex) that must dissociate after extrusion from the vesicle (Orci, 1982). Kennedy et al. (1996) have used amperometry to detect released insulin, while varying the extracellular pH to determine if extracellular composition plays a role in the matrix dissociation. Their results show that changing the extracellular pH does not affect vesicle docking or release, but does affect the amount of soluble insulin available for detection. The average quantity of a single vesicle has been determined to be 1.7 amol (1.0×10^6 molecules) for human β-cells, which is similar to the values reported for catecholamine in adrenal cells (Finnegan et al., 1996).

Peptide release has also been quantitated for single exocytosis events at rat melanotrophs. Peptide hormones are necessary for many biological functions, so understanding their secretion and storage characteristics is very important. The secretion of peptide hormones has been detected electrochemically by oxidation of tryptophan and tyrosine residues found in the pro-opiocortin cleavage products. Calcium-mediated release can be measured at a carbon-fiber electrode placed on a single cell in culture, and quantitation of release from single vesicles yields an average of 0.32 amol (190,000 molecules) of α-melanocyte-stimulating hormone (Paras and Kennedy, 1995).

Invertebrate Neuronal Systems

Exocytosis has also been quantitated from invertebrate neuronal systems, such as the leech *Hirudo medicinalis* and the pond snail *Planorbis corneus*. These model systems have relatively large cells in comparison to mammalian neurons, which facilitates electrode placement and cell identification in these systems. Bruns and Jahn (1995) have described the electrochemical detection of serotonin from synaptic vesicles in the leech *H. medicinalis*. The leech neuron has been cocultured with a postsynaptic neuron and the carbon-fiber electrode placed at the axonal stump. Two types of exocytotic responses have been proposed, one for small clear and the second for large dense core vesicles. Release of the small clear vesicles appears to occur more rapidly (faster time constant) than the large dense core vesicles, which are more randomly distributed throughout the cell. Determination of the quantities of neurotransmitter stored in the small clear and large dense vesicles resulted in 8 zmol (8×10^{-21} mol, 4800 molecules) and 13 amol (7.8×10^6 molecules), respectively (Bruns and Jahn, 1995). For the large dense core vesicles, a "foot" is observed, suggesting the formation of a fusion pore. It is suggested that the faster rates observed for the small clear vesicles are owing to the discharge of their contents on a submillisecond time scale through the undilated fusion pore.

In examining another invertebrate system, the pond snail *P. corneus* has been shown to release a significant amount of catecholamine directly from the cell body of the large dopamine neuron (Chen and Ewing, 1995). Capillary electrophoresis and fast-scan voltammetry have been used to identify the neurotransmitter as dopamine (Chen et al., 1995). Individual exocytosis events are observed with amperometry and are only observed if calcium is present during stimulation. The average amount of dopamine released from a single vesicle has been reported at 1.36 amol (818,000 molecules). Excitingly, as many as 89,000 exocytosis events have been observed from a single stimulation of the neuron (Chen and Ewing, 1995). In addition to the large number of vesicles released from the cell body, the amount of catecholamine released from these vesicles has a bimodal distribution (Chen and Ewing, 1995), suggesting that at least two distinct classes of releasable vesicles are present in this cell (Fig. 4). The smaller Gaussian makes up only 9% of the total release events and has a considerably smaller average vesicle catecholamine content (4%) than that of the dominant Gaussian. The *Planorbis* system appears to be highly interesting pharmacologically, since amphetamine has been shown to alter catecholamine content dif-

neurons. Like sympathetic neurons, PC12 cells synthesize acetylcholine, as well as the catecholamines, dopamine, and norepinephrine (Schubert and Klier, 1977). They also store, release and reuptake these neurotransmitters, as well as exhibit Ca^{2+}-dependent exocytosis (Greene and Rein, 1977; Baizer and Weiner, 1985; Chen et al., 1994). A widely applied characteristic of these cells is that they also have the ability to differentiate into neuronal-like cells on treatment with nerve growth factor, thus providing a model of the developing nerve cell.

Studies of these cells have involved the detection of zeptomole quantities of dopamine (Chen et al., 1994) from undifferentiated cells as well as from the varicosities of differentiated cells (Zerby and Ewing, 1996a). The initial electrochemical studies on PC12 cells have focused on the quantitation of dopamine release. Current transients for the oxidation of dopamine are on the time scale expected for exocytotic release of catecholamines (9.3 ms half-width) and are thus similar to events observed at adrenal cells. However, catecholamines are present in much lower levels in vesicles of PC12 cells. This is partially owing to the smaller size of the PC12 vesicle with a mean vesicular radius of 79 nm (Schubert et al., 1980) compared to the adrenal cell vesicle at 300 nm (Coupland, 1968). The quantity of dopamine oxidized from a single vesicle after stimulated release has been reported to be 190 zmol (114,300 molecules) for a specific passage of PC12 cells (Chen et al., 1994). Since PC12 cell lines vary from laboratory to laboratory, it is important to note that this value might vary. For this cell line (American Type Culture Collection, Rockville, MD), it is approx 18 times smaller than the amount of release observed from a single adrenal cell vesicle (*vide supra*).

Quantitation and Generating Histograms

The experimental protocol is similar for quantitation of exocytosis in all the cell types discussed. A microelectrode is gently placed on a single cell where exocytosis is monitored on pressure pulse ejection of stimulant through a micropipet. Detection of the released components is accomplished with the microelectrode poised at a potential where oxidization of released messengers occurs. Figure 5A depicts typical current transients observed for PC12 cells after stimulation. Quantitation is accomplished by integrating the charge (Q) under individual current transients obtained using amperometry. Using Faraday's law: $Q = nFN$, where n is the number of electrons in the electro-oxidation ($n = 2$ for catecholamines), and F is Faraday's constant (96,485 C/Eq), the number of moles (N) of electroactive molecules released, such as the catecholamines, serotonin, histamine, insulin, and certain peptides, can be determined. The reported quantity of neuromessenger from individual vesicles in a cell type is an average value. As shown above, distribution of values exists and can be easily visualized by plotting a histogram of the total cellular exocytosis data. Histograms are generated by plotting the percentage of total release events vs charge or number of moles in each bin. Figure 5B shows a typical histogram generated from exocytotic events measured from PC12 cells. The average vesicle content for this set of PC12 cells is 199 ± 14 zmol (Zerby and Ewing, 1996a); however, the distribution ranges from 20 to 800 zmol (Fig. 5B). Histograms plotted in this manner give a skewed (non-Gaussian) distribution and have been modeled mathematically by Wightman et al. (1991) for adrenal cells. Several different mechanisms could lead to the skewed distributions observed, with some events being much larger than average. The most feasible explanation for this distribution is that vesicles have a range of radii with relatively uniform concentrations of neuromessenger (Wightman et al., 1991). Distribution of vesicle radii in adrenal chromaffin cells has been measured and is approximately Gaussian (Coupland, 1968). Using the assumption that vesicles are spherical and have a constant concentration of catecholamine, the observed areas for transients in units of charge can be directly related to vesicle radii using Faraday's law as shown below:

$$Q = \tfrac{4}{3}\pi n F C r^3 \qquad (1)$$

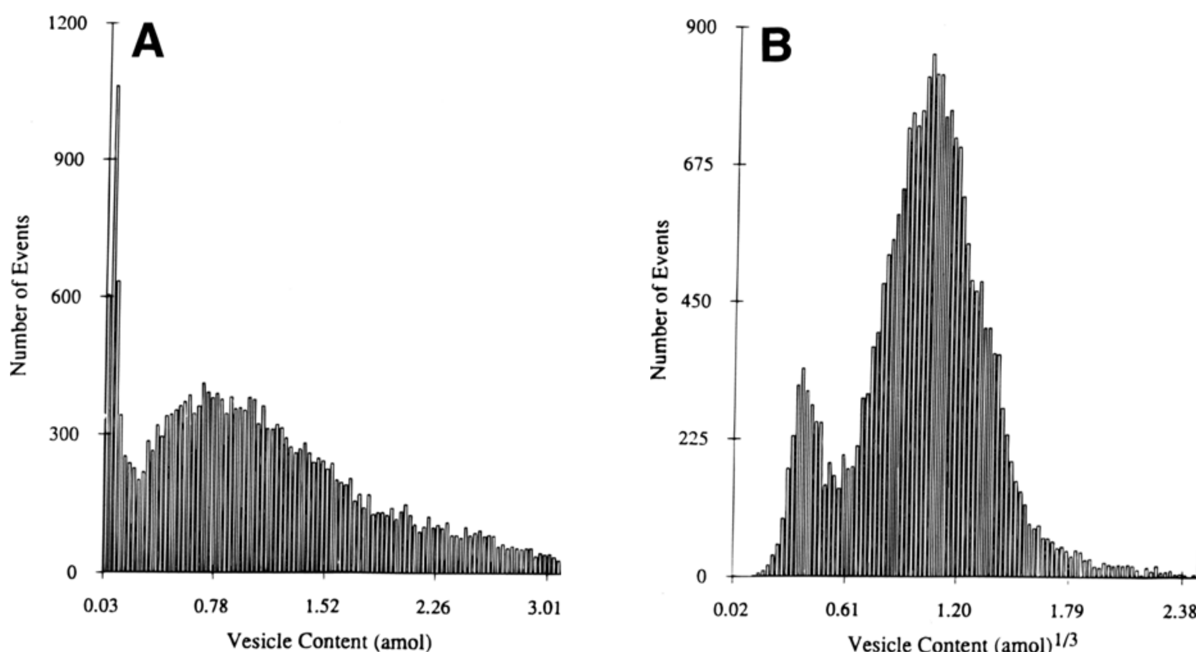


Fig. 4. **(A)** Histogram of the frequency of release events vs attomoles of dopamine released per vesicle for stimulated release from the cell body of the dopamine neuron in *P. corneus*. Data have been collected from 21 cells (23,524 transients). **(B)** Histogram of the frequency of release events vs the cubed-root attomoles of dopamine released per vesicle. Reproduced with permission from Chen and Ewing (1995).

ferentially in the two classes of vesicles (Chen et al., 1995) and physiologically where it appears that stimulus-release coupling does not result in a random burst of exocytosis, but rather in controlled events with regular time intervals between individual events (Chen et al., 1996).

Mammalian Neuronal Systems

Developing superior cervical ganglion neurons from neonatal rats have been investigated with amperometric methods (Zhou and Misler, 1995). Real-time electrochemical detection has been accomplished using carbon fiber electrodes placed at varicosities on the axon of the extending neuron. Local application of potassium or black widow spider venom has been shown to initiate quantal release of catecholamines. The electrochemical system and electrodes used in this work have been optimized to detect the minute amounts released. Zhou and Misler (1995) report a median spike charge of 11.3 femtocoulombs, which corresponds to 35,000 catecholamine molecules per release event (58 zmol). These values are on the order of those obtained for small clear vesicles in the in the leech *H. medicinalis* (Bruns and John, 1995). Electrochemical systems are continually being optimized to decrease the amount of catecholamine that can routinely be detected; however, major advances are still required to approach the ~1000 molecule detection needed for measuring exocytosis at nerve terminals.

Rat Pheochromocytoma (PC12) Cells

The PC12 clonal cell line, derived from a cancerous rat adrenal gland (Greene and Tischler, 1976), has been widely used as a model for adrenal chromaffin cells and sympathetic

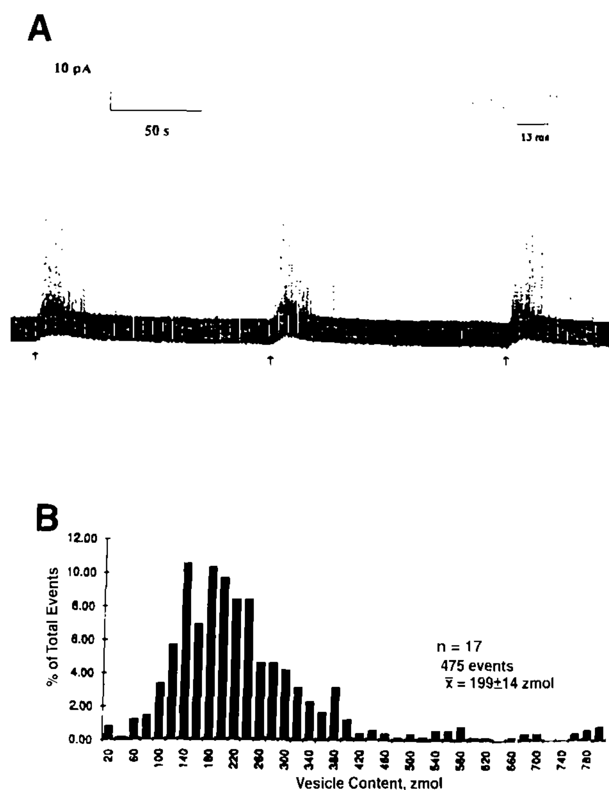


Fig. 5. **(A)** Current time trace for exocytosis at a single undifferentiated PC12 cell. A 6-s ejection of 105 m*M* potassium chloride from the microinjector is administered at each arrow. The resulting current transients correspond to the oxidation of dopamine at the electrode tip as it is released from the cell. The area under each current transient is equivalent to the total charge produced by the oxidation of the dopamine content of one vesicle. Detection is performed in the amperometric mode at 650 mV vs SSCE. Also shown in the inset is an example of a typical current transient on an expanded time scale. **(B)** Distribution of the amount of catecholamine released following potassium stimulation of undifferentiated PC12 cells. The area under each current transient observed in the first 40 s of potassium-stimulated release is converted into moles of dopamine detected per vesicle, using Faraday's law ($Q = nNF$). The total number of moles of dopamine detected for each exocytosis event are collected into bins having increments of 20 zmol and plotted as the percent of the total number of vesicles undergoing exocytosis. Reproduced with permission from Zerby and Ewing (1996a).

where C is the vesicular concentration of catecholamine. If the cube root of the vesicular content ($Q^{1/3}$ or mole$^{1/3}$) is utilized for generation of the histograms, then a normal (Gaussian) distribution should be obtained. The $Q^{1/3}$ histogram and a Gaussian fit to the distributions are shown for three different cell types that have been examined by this procedure (Fig. 6) (Finnegan et al., 1996). Small deviations in the Gaussian distribution are possibly owing to variation in the vesicular concentration, which is assumed to be constant. Vesicular volumes have been shown to change over time in culture (Finnegan et al., 1996) and also result from chemical perturbations (*vide infra*) (Sulzer et al., 1995; Pothos et al., 1996). However, adrenal cells, mast cells, PC12 cells, and the invertebrate cells discussed above all show a Gaussian distribution for the $Q^{1/3}$ plots, strongly suggesting that a Gaussian distribution of vesicular radius is ubiquitous in nerve-like cells.

Single Events at PC12 Cell Varicosities

Quantitation of exocytosis has been used to investigate changes in the location of release following cell differentiation. PC12 cells are ideal for studying neuronal differentiation and the changes that occur during development and maturation, since they can be induced to become more neuronal-like both morphologically and physiologically by exposure to nerve growth factor (NGF) (Levi-Montalcini and Angeletti, 1968). Thus, NGF-treated PC12 cells have been used to probe the site of exocytosis during the differentiation process (Zerby and Ewing, 1996a). On treatment with NGF, PC12 cells extend processes, and along these processes or neurites, varicosities form (bulbous regions, 1–2 µm in diameter). Varicosities have been previously shown to contain aggregates of small vesicles (20–70 nm in diameter) (Greene and Tischler, 1976). Experiments carried out on d 10–14 of culture show no release from the cell body ($n = 3$), only very infrequent responses from the smooth regions of the neurites ($n = 5$), and frequent release when the electrode is located at a varicosity ($n = 16$). This response is most frequently observed at varicosities located at

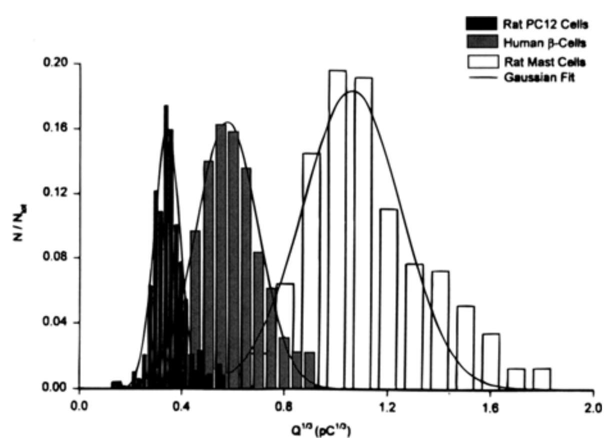


Fig. 6. Cubed-root histograms for amperometric spikes from rat mast, human pancreatic β-, and rat PC12 cells. Histograms are from 234 A23187-induced spikes from 7 rat peritoneal mast cells (open columns), 228 tolbutamide-induced spikes from 6 human pancreatic β-cells (gray columns), and 475 K^+-induced spikes from 17 rat PC12 cells (solid columns). The fitted Gaussian curves had mean ± SD values of 1.06 ± 0.19, 0.58 ± 0.12, and 0.34 ± 0.050 $pC^{1/3}$, respectively. Reproduced with permission from Finnegan et al. (1996).

the intersections of several neurites. Exocytosis from differentiated cells occurs preferentially at the varicosities as might be expected. The average vesicular catecholamine content observed for exocytosis at varicosities is 178 ± 9 zmol (107,000 molecules) and is not significantly different from that observed at undifferentiated cells; however, a more narrow distribution is observed at the former (Fig. 7). This study demonstrates that functional changes occur during differentiation, specifically the relocation of the site of exocytosis without significant alteration in the overall mean vesicle catecholamine content, although the narrow distribution in the released amount might indicate that a tighter distribution of vesicle radii is necessary to pack them into the relatively small varicosities.

Latency of Release Following Chemical Stimulation

Quantitation of exocytosis at PC12 cells has been used to investigate the effect of different mechanisms of stimulation on the latency of exocytosis following stimulation (Zerby and Ewing, 1996b). PC12 cells possess both nicotinic and muscarinic receptors that trigger exocytosis through two different mechanisms on activation. Application of nicotine causes conformational changes in the nicotinic receptor, which opens sodium channels. Sodium influx causes sufficient depolarization of the cell membrane to open voltage-sensitive calcium channels, allowing rapid influx of calcium to cause exocytosis (Stallcup, 1979). The application of muscarine activates muscarinic receptors that act through intracellular second messengers to release calcium from intracellular stores triggering exocytosis (Berridge and Irvine, 1984). To obtain rapid membrane depolarization of the cell, KCl can be applied to promote exocytosis by direct membrane depolarization. The amperometric response for stimulation with each of these agents is shown in Fig. 8A. The average vesicle catecholamine content is unaltered by the mechanism of release, but the latencies (time between application of the stimulant and secretion events) vary significantly. The mean latencies for each type of stimulation have been observed to be: 6 ± 1 s (105 m*M* K^+); 37 ± 5 s (1 m*M* nicotine); and 103 ± 11 s (1 m*M* muscarine). Figure 8B is representative of the histograms obtained for the three mechanisms of stimulated release. The 6-s delay until release following potassium stimulation apparently represents the diffusion time from the stimulation pipet. However, the relatively long latencies until the onset of exocytosis after nicotine and muscarine are surprising, since one usually expects these events to occur on the millisecond time scale. The longer times might reflect a reduced number of sodium channels on these cells or a slow step in the G-protein coupling by these receptors in PC12 cells, as well as other potential rate-limiting mechanisms. These measurements hold great promise as a tool to study the different receptor mechanisms leading to exocytosis.

Chemical and Pharmacological Modulation of Messenger Vesicles

The mechanism of action for amphetamine has been a long-standing question. Two pro-

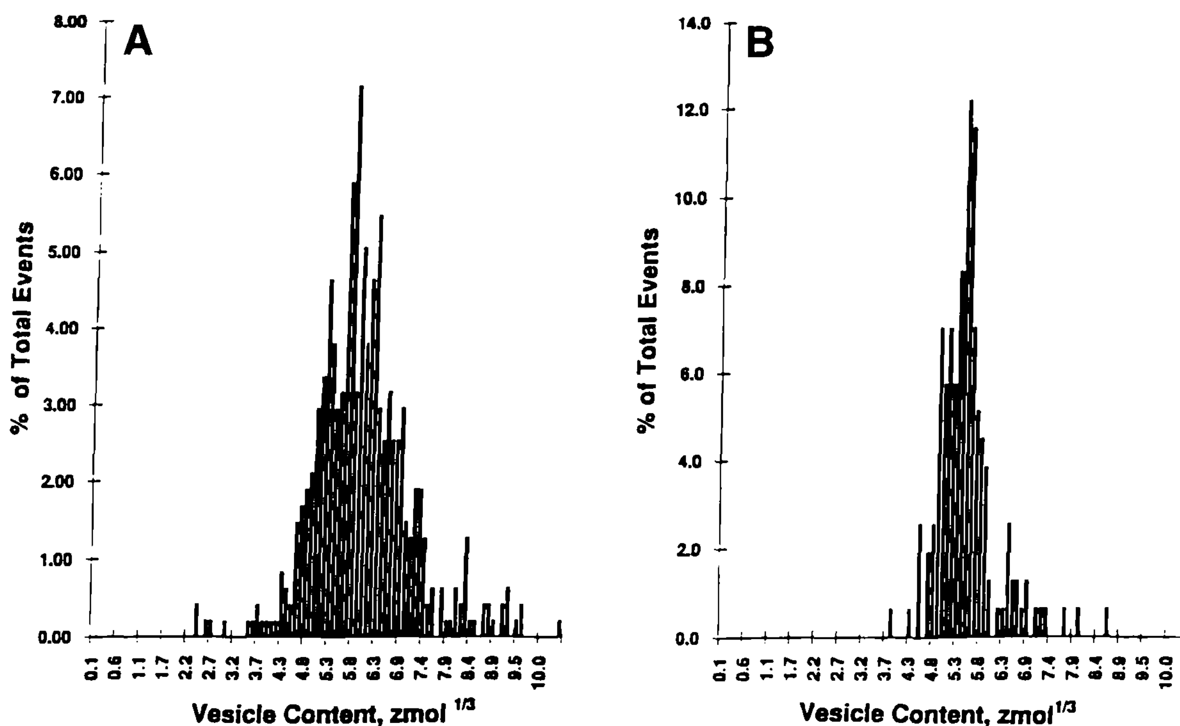


Fig. 7. Distribution of vesicle content for potassium-stimulated release at varicosities plotted as the cube root of catecholamine released. Plots of the percent of total events observed in the first 40 s following initiation of release vs the cube root of the amount of catecholamine released on elevated potassium stimulation for **(A)** 17 undifferentiated PC12 cells (475 total release events) and **(B)** 16 differentiated PC12 cells (156 total release events). Reproduced with permission from Zerby and Ewing (1996a).

posed mechanisms exist to explain amphetamine action: (1) exchange diffusion (Seiden et al., 1993) and (2) vesicle depletion (Sulzer et al., 1993). The exchange-diffusion model involves attachment of amphetamine to the dopamine transporter, transport of the amphetamine into the cell, and transport of dopamine out of the cell. This model appears to predominate at low concentrations of the drug. The second mechanism of action of amphetamine observed, apparently operational at high dosages, involves depletion of catecholamine from vesicles followed by reverse transport out of the cell (Sulzer et al., 1993). Measurement of individual events at PC12 cells provides a unique method to probe this second mechanism (Sulzer et al., 1995). PC12 cells have been incubated with 10 μM amphetamine followed by amperometric monitoring of stimulated release to evaluate changes in the amount of dopamine released per exocytotic event (Fig. 9). The amount of material stored in the vesicles decreases more than 50% on average after this treatment (compare Fig. 9A,B). The shift to smaller vesicular amount is clearly observed in the histograms for PC12 cells with and without amphetamine treatment in Fig. 9C. From these results, it is apparent that amphetamine indeed decreases the level of dopamine in the vesicles of dopamine-containing cells, thereby supporting this hypothesis.

Additional experiments to support the action of amphetamine have been conducted with *Planorbis* neurons (Sulzer et al., 1995). Using the *Planorbis* neuron, the increase of dopamine in the cytosol has been observed, and subsequent release owing to reverse transport has been reported. The large size of the *Planorbis* neuron (200 compared to 10 μm for the PC12 cell)

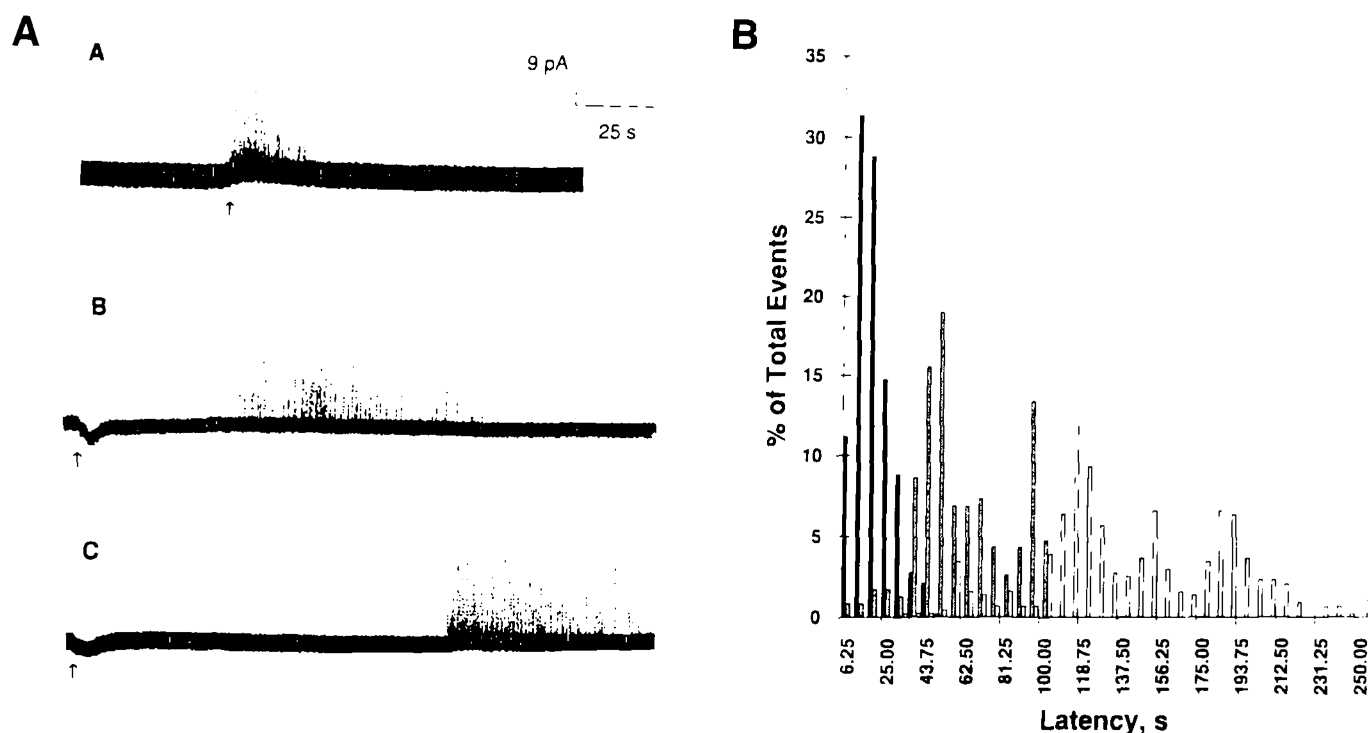


Fig. 8. **(A)** Current time traces for exocytosis at single PC12 cells. A 6-s ejection of stimulant (a) 105 m*M* K^+, (b) 1 m*M* nicotine, or (c) 1 m*M* muscarine from a microinjector is administered at each arrow. The resulting current transients correspond to the oxidation of dopamine at the electrode tip as it is released from the cell. Detection is performed in the amperometric mode at 650 mV vs SSCE. **(B)** Distributions of latencies observed between stimulation and secretion. The time between the application of the stimulus and the detection of released dopamine for each exocytosis event in the first 40 s of release is plotted vs the percentage of the total number of events observed for each type of stimulus. The differences in the time-courses of release stimulated by 105 m*M* K^+ (dark bars; *n* = 17 cells; 475 events), 1 m*M* nicotine (shaded bars; *n* = 16 cells; 232 events), and 1 m*M* muscarine (open bars; *n* = 19 cells; 439 events) are apparent. Reproduced with permission from Zerby and Ewing (1996b).

makes it possible to insert dopamine directly into the cell to bypass the uptake transporters. On injection of amphetamine, nonexocytotic dopamine release has been observed from the *Planorbis* cell body, demonstrating that amphetamine can act intracellularly as part of its overall mechanism.

Quantal size has been investigated using other chemical agents, such as L-3,4-dihydroxyphenylalanine (L-DOPA). Addition of L-DOPA to cultured cells can increase the levels of dopamine within the vesicles markedly. This loading effect has been demonstrated using PC12 cells (obtained from Lloyd Greene, Columbia University) and ventral midbrain neurons in culture (Pothos et al., 1996). Overall levels of dopamine in an entire culture have been determined using high-performance chromatography showing a >10-fold release of dopamine in ventral midbrain neurons following a 1-h incubation with 100 μ*M* L-DOPA. A similar effect has been observed for PC12 cells where a 370% increase in dopamine release is observed after a 1-h L-DOPA incubation. Experiments conducted with nomifensine, a dopamine transport inhibitor, suggest that reverse transport does not make a significant contribution to release in these studies. To

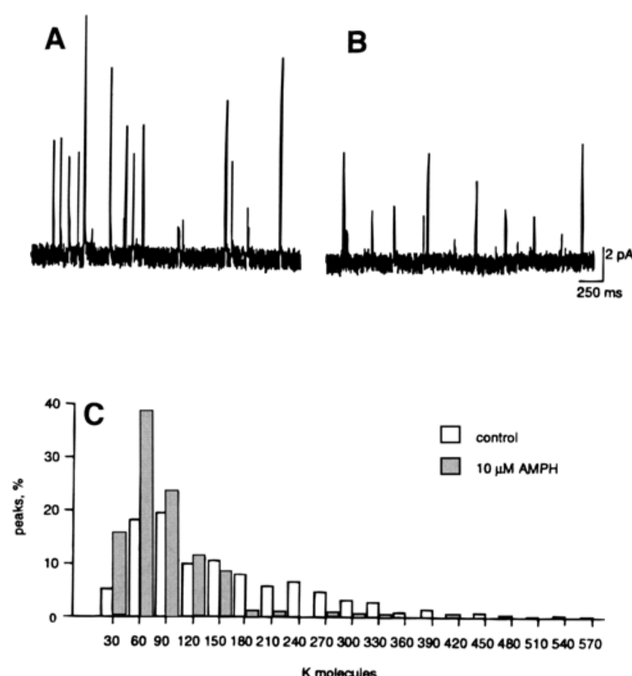


Fig. 9. Amphetamine (AMPH) exposure decreases the quantal amplitude of individual release from PC12 cells. Randomly chosen electrochemical records of quantal release for **(A)** a control cell and **(B)** a cell that has been incubated in 10 µ*M* *d*-AMPH for 10 min. Cells are stimulated by local perfusion (30 nL of 1 m*M* nicotine in 105 m*M* KCl saline) to induce vesicular exocytosis. **(C)** A histogram shows the percentage of peak sizes in paired control and AMPH-treated cells. Peaks are combined in intervals of 30,000 molecules. The lower limit of each bin size is shown on the abscissa. Reproduced with permission from Sulzer et al. (1995).

quantitate the amount of dopamine at the vesicular level, amperometry with carbon microelectrodes has been employed. An amperometric trace for exocytosis after incubation with L-DOPA is shown in Fig. 10A. Figure 10B shows the cube root histogram for release from a control and L-DOPA-incubated cells. Incubation with L-DOPA does not appear to increase the number of exocytotic events, but does increase the vesicular content to a value 250% larger than the control. The average values for exocytosis from a single vesicle obtained for a PC12 untreated and treated with L-DOPA are 125 zmol (75,000 ± 11,000 molecules) and 310 zmol (187,000 ± 31,000 molecules) of dopamine, respectively.

The quantal size of a vesicle has been shown to depend on the extracellular conditions. Pharmacological manipulations of PC12 cells with L-DOPA and amphetamine have been shown to increase dopamine release; however, one acts by increasing dopamine in the vesicles and the other by increasing dopamine in the cytosol. These results are highly important, since they define a mechanism for neuroplasticity whereby the amount of messenger released might be modulated under different cellular conditions.

Future of Electrochemical Exocytosis Measurements

Currently many questions in neuroscience are being addressed using electrochemical techniques for quantitation of exocytotic release. Several model systems and experimental protocols have been developed utilizing amperometric measurements of exocytosis to study various cellular functions related to neurocommunication. With zeptomole quantitation, PC12 cells (Pothos et al., 1996; Zerby and Ewing, 1996a) or multiple mammalian neurons (Garris et al., 1994) can be sampled; however, it is at present difficult to look at exocytotic events at single mammalian neurons.

The future of exocytosis studies depends on the development of smaller electrodes and minimizing the level of detection with more sensitive instrumentation. With the development of smaller electrodes, it should be possible to insert an electrode directly into a functioning synapse to detect individual exocytosis events. It should also be possible to determine the amount of neurotransmitter that escapes the synapse and to investigate other regions of neurons that may be involved in neurotransmitter release. Another possibility for studying single synapses might involve inducing a neuron to grow (synapse) onto an electrode surface. In this kind of experiment, the electrode would

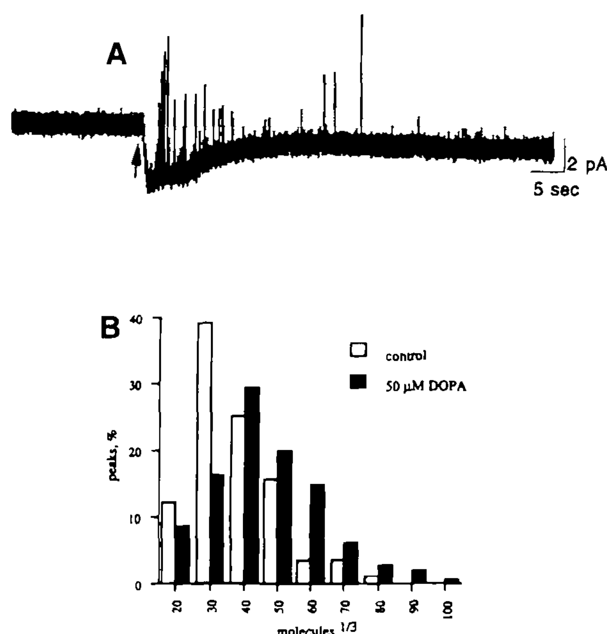


Fig. 10. **(A)** Amperometric trace from a PC12 cell incubated for 40 min in 50 µ*M* L-DOPA. Individual quanta are represented by the peaks after stimulation and the quantal size is determined by the charge of each peak, which is owing to molecular oxidation at a carbon-fiber electrode touching the cell. The sharp inflection in the baseline is the result of a 6-s perfusion application of the stimulation saline (arrow). The width at half-height ($t_{1/2}$) is determined by measuring the width of individual peaks at a point halfway between the baseline and maximal peak height. **(B)** Histogram of the cubed root of quantal sizes by percentage. L-DOPA exposure shifts the distribution of quantal sizes to the right. The lower limit of each bin size is displayed on the abscissa. Reproduced with permission from Pothos et al. (1996).

function as the postsynaptic side of the synapse and receive the signal after stimulation of the presynaptic neuron. In addition to smaller probes and more sensitive measurements, experiments in the future will require fast probes to measure nonelectroactive neurotransmitters. Perhaps, enzyme electrodes under development for glutamate (Kuhr and Pantano, 1993) and acetylcholine (Michael et al., 1993) will eventually be made fast enough and small enough for use in exocytotic measurements.

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