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The heterogeneous nature of vesicles has been studies by evaluating exocytotic events released from the cell body of a dopamine-containing neuron of *Planorbis corneus*. Vesicular exocytosis has been elicited by stimulation with in situ application of elevated potassium and has been monitored electrochemically with a carbon fiber microelectrode placed on the cell body. These electrodes allow individual release events to be monitored and quantitated to reveal vesicular dopamine content. Statistical analysis of individual release events demonstrates that two classes of vesicles with specific bimodal distributions in dopamine content and vesicle size are observed after cell stimulation. The effect of psychostimulant on individual vesicular dopamine level has been studied by treating the cells with d-amphetamine. After a 20-min application of 10 μM amphetamine, changes in both vesicle content and size distributions are obtained with an overall decrease in vesicular dopamine level of 40%. The altered distribution show trimodal shapes indicating a third class of vesicles is created by amphetamine treatment. Our data appears to indicate that multiple classes of vesicles are released from the cell body of the dopamine-containing neuron and vesicular dopamine level can be manipulated by the application of the lipophilic weak base amphetamine.

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Multiple Classes of Catecholamine Vesicles Observed During Exocytosis from the *Planorbis* Cell Body

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Abstract

The heterogeneous nature of vesicles has been studied by evaluating exocytotic events released from the cell body of a dopamine-containing neuron of *Planorbis corneus.* Vesicular exocytosis has been elicitied by stimulation with in situ application of elevated potassium and has been monitored electrochemically with a carbon fiber microelectrode placed on the cell body. These electrodes allow individual release events to be monitored and quantitated to reveal vesicular dopamine content. Statistical analysis of individual release events demonstrates that two classes of vesicles with specific bimodal distributions in dopamine content and vesicle size are observed after cell stimulation. The effect of a psychostimulant on individual vesicular dopamine level has been studied by treating the cells with d-amphetamine. After a 20-min application of 10 μM amphetamine, changes in both vesicle content and size distributions are obtained with an overall decrease in vesicular dopamine level of 40%. The altered distribution show trimodal shapes indicating a third class of vesicles is created by amphetamine treatment. Our data appears to indicate that multiple classes of vesicles are released from the cell body of the dopamine-containing neuron and vesicular dopamine level can be manipulated by the application of the lipophilic weak base amphetamine.

Key words: dopamine, dopamine exocytosis, cell body, vesicles, vesicle content, vesicle size, multiple classes of vesicles, distributions, *Planorbis corneus*

1. Introduction

Ultrastructural studies of membrane specialization have revealed that multiple classes of clear and dense cored vesicles are observed in synaptic terminals and cell bodies [7, 9, 21, 22, 38, 39, 41, 44, 49]. Although these vesicles, heterogeneous in nature, have been suggested to correlate to the heterogeneous nature of synaptic functions [7, 38, 49], little is known concerning whether multiple classes of vesicles are functional during neurotransmitter release. In the classical quantal release hypothesis, an equal amount of material or quantum is thought to be released from each vesicle in the synaptic cleft during exocytosis. This hypothesis is based on the observations of equal amplitudes of postsynaptic miniature end-plate-potentials (MEPP) observed in electrophysiological recordings [8, 20, 30, 43]. However, the homogeneous MEPPs for neurotransmitter release do not seem reasonable considering the heterogeneous nature of synaptic vesicles; often found in a broad distribution or in multiple classes. A direct measurement of how much material being released from each individual vesicle during exocytosis is needed to test whether neurotransmitter release is quantal or if there exists a broad distribution or even multiple classes of heterogeneous vesicles. Electrochemical techniques developed in recent years have provided a unique approach to challenge the above questions due to their ability to quantitate the contents of individual vesicles during exocytosis.

Electrochemical techniques have previously been used to study the exocytosis from single adrenal cells [14, 33, 50, 52], mast cells [1], pheochromocytoma cells [11], pancreatic β cells [27] and the cell bodies of dopamine-containing neurons [12]. These techniques involve the use of small

carbon fiber electrodes to monitor and quantitate the electroactive neurotransmitters (catecholamines, serotonin etc.) released from individual exocytotic events at the cell membrane. Due to the small structure, high sensitivity, and high spatial and temporal resolution of the microelectrode, dynamic measurement of fast exocytotic events can be obtained. Assuming that each current transient detected by amperometry represents a single vesicular release event, one can calculate the chemical content of each vesicle by evaluating the corresponding current transient according to the electrochemical oxidation reaction. This assumption has been verified by comparing the average vesicle content of bovine adrenal cells obtained by electrochemical techniques to vesicle content obtained by other techniques [14, 42, 50, 51].

Previous experiments using electrochemical techniques have shown that a broad range of neurotransmitter amount is present in each individual vesicle during exocytosis [11, 14, 25, 50]. These variations in vesicle content can be as large as 600-fold [25] and the distribution of the number of exocytotic events vs the vesicle content is skewed. The skewed distribution obtained by electrochemical techniques cannot be evaluated by overlapping multiple unit gaussians into Poisson statistics as obtained in MEPP distributions [8, 16]. Thus, the broad range in vesicle content cannot be explained by a quantal release hypothesis. Furthermore, the preliminary evaluation of the distribution of vesicles from the cell body of the dopamine-containing neuron of the pond snail *Planorbis corneus* [12] has suggested that two classes of vesicles with distinct distributions are released after chemical stimulation.

In this paper, we use amperometry at microelectrodes to explore what appear to be different classes of vesicles (defined by different distributions of the amount of catecholamine released) in the *Planorbis* dopamine-containing neuron after it is stimulated. Furthermore, amphetamine is used to test the hypothesis that the size and distribution of different classes of vesicles can be individually manipulated. According to a recently proposed model [47, 48], amphetamine acts as a lipophilic weak base that disrupts the pH gradient across the vesicular membrane, thereby redistributing neurotransmitters from vesicles to the cytosol. In agreement with this weak base model, we have found that amphetamine substantially decreases the overall vesicle content. More interestingly, we have found that amphetamine differentially affects one class of vesicle over the other and may reveal the third class of vesicles after its action.

2. Materials and methods

Electrodes and Voltammetric Procedures

Working electrodes were prepared from 10-μm-diameter carbon fibers sealed in glass capillaries [26]. The sensing tip of the electrode was exposed by cleaving with a surgical blade. The reference electrode was a sodium-saturated calomel electrode (SSCE). Constant-potential amperometry was performed with a commercial potentiostat (EI-400, Ensman Instrumentation, Bloomington, IN). The applied potential was 700 mV. The output was connected to an A/D converter (Labmaster, Scientific Solutions, Solon, OH) interfaced with a GATEWAY 486 personal computer.

Cell Procedures

Planorbis corneus were obtained from NASCO (Fort Atkinson, WI) and were maintained in aquaria at room temperature until used. The snails were dissected under a snail Ringer solution (39.5 mM NaCl, 1.3 mM KCl, 4.5 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 6.9 mM $NaHCO_3$) adjusted to pH 7.4. The dissection procedure and the identification of the dopamine containing neuron were described previously [2, 13, 34-36, 40, 45]. Snail shells were broken and removed before snails were pinned with the ventral side uppermost in a wax-filled petri dish. The skin of the head part was cut open to reveal the brain. The ganglia ring was pinned at the connective tissue to the wax with 7-10 pins to extend the brain and expose individual cells. The outer connective tissue surrounding the brain was transparent and the orange-colored neurons could be seen beneath using a binocular microscope (70 x magnification). The outer connective tissue was removed from the surface of the pedal ganglion by a home-made fine pin with a 90-degree hook. The dopamine neuron is usually the largest cell (75-150 μm in diameter) in the left pedal ganglion and is easily identified by its specific location close to the statocyst [4, 36, 37, 40, 41]. Experiments were performed immediately after dissection.

The petri dish containing the dissected snail was placed on the stage of a stereomicroscope (Nikon Corporation, Tokyo, Japan). A working electrode was positioned over the top of the cell body of the dopamine containing neuron with a three-dimensional micromanipulator (Mertzhauser, Zeiss, Germany). The electrode was manipulated so that it lightly touched the center of the cell body. Release of dopamine from the cell body of the dopamine neuron was induced through short-duration (2 s to 6 s) local applications of 1 M KCl solution prepared in the snail Ringer solution (this corresponded to injections of 170 to 530 nL). For experiments using d-amphetamine, a 10 μM d-amphetamine solution prepared in the normal snail Ringer solution was used to incubate the whole snail for 20 min. The amphetamine solution was then washed away and replaced by the normal snail Ringer solution by a perfusion system built-in-house. A 5-min waiting time was used after changing the solutions and before the next measurement using 1 M KCl as the stimulant. All stimulation solutions were delivered by glass micropipettes controlled by a two-channel pressure application device (Picospritzer, General Valve Corporation, Fairfield, NJ). Typical tip dimensions of micropipettes were 10 μm. They were positioned with a micropositioner (Medical Systems Corporation, Greenvale, NY) to a desired distance (5 to 20 μm) from the cell body. All data were digitized and filtered with a 100 Hz two-pole low pass filter. Experiments were carried out at room temperature.

Data Treatment

Transient current responses obtained in the amperometric mode were evaluated by locally developed peak-detection-integration software. Only transients with peak widths less than 40 ms and larger than 4 ms and peak heights larger than twice the peak-to-peak noise (dependent on the experiment) were considered. These transients were integrated by a trapezoidal method with respect to time to determine the amount of charge in units of picocoulombs (pC). The vesicle content was calculated by $N = Q/nF$, where Q is the charge under each transient in coulombs, n is the number of electrons in the dopamine oxidation reaction, F is the Faraday constant (96,485 coulombs/mole) and N is the number of moles. Histograms of the frequency of release events vs

the amount of neurotransmitter released were obtained by sorting current transients in different size groups. All reported values are given with the standard deviation (s.d.).

Reagents and Drugs

Dopamine (DA) was obtained from Sigma (St. Louis, MO) and was used as received. Dopamine solutions were deoxygenated for 20 min prior to experiments and a blanket of nitrogen was then maintained over the solution. d-Amphetamine was also purchased from Sigma (St. Louis, MO) and was prepared in normal snail Ringer solution. All solutions were prepared with doubly distilled water.

3. Results

Dopamine Exocytosis from the Cell-Body Following Chemical Stimulation

It has been shown previously [12] that current transients corresponding to individual dopamine exocytotic events can be obtained by microelectrode amperometry from the cell body of the dopamine-containing neuron of *Planorbis.* Exocytosis is observed following stimulation of the cell body with either elevated external potassium ion or intracellular sodium ion. In the work reported here this dopamine containing neuron was stimulated with elevated extracellular potassium ion. Figure 1A shows the response obtained from a (120 μm) neuron after 3-s stimulation with elevated potassium. These current transients have rapid rise times (2 to 4 ms) as shown in the expanded plot in Figure 1B, which correspond to exocytotic events expected to occur on the millisecond time scale [20, 43]. These transients are not observed when calcium is not contained in the medium and in the stimulation solution indicating the calcium-dependent nature of exocytosis from the cell body of the *Planorbis* dopamine neuron. In addition, the released substance has been identified as dopamine previously by fast-scan voltammetry and capillary electrophoresis [12].

Frequency of Transient Events vs Vesicle Contents

Since dopamine released under the electrode is quantitatively oxidized, the amount of neurotransmitter released from each individual exocytotic event can be determined by integrating the current under each transient. This produces a solid estimate of the material in each vesicle. Figure 2 is a histogram of the frequency of the detected transients vs the amount of dopamine

released from individual vesicles (data from 21 cells). Clearly, a broad distribution of vesicle content is available from stimulated release with the largest content corresponding to 1400-fold that of the smallest one. The major part of this histogram is a broad and skewed distribution, which is similar to that observed from other systems [11, 14, 25, 50] and cannot be overlaid by multiple unit gaussians. To further explore the possibility that two distinct classes of vesicles coexist during exocytosis, we replotted the same transients into a dramatically different histogram as shown in Figure 3. The x-axis is the cubed root of vesicle content which is considered to be directly proportional to the radii of the vesicles. Assuming that the total amount of neurotransmitter contained in each vesicle is proportional to the volume and that vesicles have a spherical shape, then the cubed root of vesicle content should be proportional to the radius of the vesicle. The histogram (Figure 3) shows two symmetrical gaussian distributions. The small gaussian can be directly related to the left sharp distribution of Figure 2 because the number of transients in these two groups represent 11% of the total transients in each case. Likewise, the dominant gaussian in Figure 3 can be correlated to the major distribution in Figure 2.

Effect of Amphetamine on Vesicle Size

To further characterize the different classes of vesicles occurring from stimulated release of dopamine from *Planorbis*, we used d-amphetamine to investigate if average vesicle content and the distribution could be affected. Before amphetamine treatment, a potassium stimulated control was performed in normal snail Ringers solution. The cell was then incubated with amphetamine and stimulated again. Figure 4 shows a histogram of the frequency of current transients vs vesicle contents obtained for the control and amphetamine treated experiments (n = 5 cells). A bimodal

distribution similar to that shown in Figure 2 is clearly observed before amphetamine treatment. The distinctly different distribution after amphetamine treatment (Figure 4) appears to indicate that amphetamine decreases the average dopamine level in vesicles. Indeed, quantitation of the average vesicle content reveals a 40% decrease in dopamine content after the amphetamine treatment. Control experiments involving two consecutive stimulations provide histograms that are essentially identical for both stimulations and the average quantal size does not vary. To explore whether amphetamine equally affects vesicular dopamine level in both classes of vesicles, we plotted the histograms of the frequency of current transients vs the cubed root of vesicle contents (Figure 5). The two symmetrical gaussian distributions shown in Figure 5A demonstrate that control cells have similar distributions of vesicle content to those investigated previously (Figure 3). However, the distribution obtained after amphetamine treatment (Figures 5B and C) has a trimodal shape with no significant change in the small vesicle group. Furthermore, the effect of amphetamine on large vesicles is apparently not uniform since the majority of vesicles still have a similar distribution to that of the untreated vesicles.

4. Discussion

The data presented here provide direct observation of two distinct classes of vesicles (defined by different distributions in the amount of catecholamine released) coexisting in depolarization-induced exocytosis from the cell body of a dopamine-containing neuron of *Planorbis*. If we assume that vesicular release results in the complete emptying of all the transmitter in each vesicle into the extracellular space, our observation of two classes of vesicles should support previous observations of multiple classes of vesicles at synaptic terminals [7, 9, 21, 22, 38, 39, 41, 44, 49]. These results suggest that packaging of neurotransmitters into vesicles is not invariant. If these heterogeneous vesicles undergo exocytosis, they should release different amount of neurotransmitters, therefore eliciting different effects on the postsynaptic neurons. Studies on quantal release have suggested that neurotransmitter released from presynaptic terminals might saturate the postsynaptic receptors [17-19, 24, 30, 32, 43] and therefore, the MEPPs measured might not represent the actual amount of neurotransmitter released from presynaptic terminals. Recent evidence concerning neurotransmitter diffusion from catecholamine synapses [23] further supports that observed MEPPs do not quantitatively correspond to neurotransmitter released during individual vesicular events. The interesting part of the histogram of release from the *Planorbis* cell body is that it contains a sharp distribution to the left of the major distribution, which has also been shown in our preliminary observation [12]. This sharp distribution contains vesicles with smaller content and may represent a different class of vesicles from that of the major distribution. The broad distributions of vesicle content obtained by electrochemical techniques as shown here from the cell body of the dopamine-containing neuron of *Planorbis*, as well as from

other cells [11, 14, 25, 27, 50] suggests that vesicles with different sizes contain different amounts of neurotransmitters. These distributions cannot be simply evaluated by superimposing the unit gaussians into a poisson-like distribution [8, 16]. All these results collectively and convincingly demonstrate that neurotransmitter release from vesicles is not a simple quantal phenomenon.

Gaussian distributions of vesicles sizes have been previously reported from a number of systems [15, 21, 22, 39, 44]. The results presented here (Figures 3 and 5) show that distributions of the cubed root of vesicle contents provide typical symmetric gaussians similar to those observed from other systems [15, 22, 39, 44]. These distributions appear to indicate that vesicle content can be correlated with a random collection of vesicles about the linear vesicle dimension in contrast to vesicle volume. If we assume that vesicles have a spherical shape, the cubed root of vesicle content is directly proportional to the vesicle radius. The two symmetrical gaussian distributions shown in both Figures 3 and 5A strongly suggest that there are two distinct classes of vesicles which differ in size and content in the cell body of the dopamine-containing neuron of *Planorbis*. These two classes of vesicles may correlate with the clear and dense-cored vesicles observed in this cell by electron microscopy [3, 4, 41], although there is at this time no evidence for this. The cell body of this neuron contains a large number of granulated dense cored vesicles [3, 41]. This indicates that the majority of vesicles are of the large size which appears to match our observation that about 89 % of vesicles detected in our experiment are in the major distribution (Figures 3 and 5A). An alternative hypothesis is that catecholamine in these vesicles might vary in concentration with the size of vesicles remaining invariant. We feel this is less likely as electron microscopy has shown a variation of vesicle size in this cell [3, 41].

It is also interesting to consider these results in the context of multiple compartments of neurotransmitter that have different functions [5]. Two compartments of vesicles, releasable vs reserve stores of dopamine, have been previously postulated for this cell based on data from capillary electrophoresis separation of whole cell [31]. However, the data shown here are only representative of releasable stores of dopamine since the method is based on measuring exocytosis. Therefore, more than two classes of vesicles might be contained in this cell body. In support of this possibility, vesicle distributions from *Torpedo* electromotoneurons [39, 44] have been shown to contain three classes of vesicles. These include a class of empty vesicles, a class of fully filled vesicles and a class of filled but smaller vesicles. The possibility of the clear vesicles found in the *Planorbis* dopamine-containing neuron corresponding to empty vesicles cannot be excluded. The two classes of vesicles observed here may only represent the fully filled and partially filled vesicles which are releasable by depolarization-induced stimulations or different populations of filled vesicles with distinct classes of vesicle size.

The effect of amphetamine on vesicular dopamine content and size (Figures 4 and 5) appears to indicate that neurotransmitter levels in vesicles of this neuron can indeed be manipulated as observed in other systems [29, 46]. Whether amphetamine acts principally at the plasma membrane or at synaptic vesicles has been controversial. Our results confirm that amphetamine decreases the vesicular dopamine stores, which is in agreement with the weak base model [47, 48] and with experiments performed on pheochromcytoma cells [46]. The trimodal distribution of vesicles after amphetamine treatment (Figures 5B and 6) appears to suggest; however, that this

drug selectively affects a specific type of vesicle with minimal effect on the other vesicles since more than half of the vesicles in the major distribution still have a similar distribution to that prior to the amphetamine treatment. This distribution suggests that amphetamine mainly affects the large vesicles and may produce a third class of vesicles after its application. If amphetamine equally affected all the vesicles, a bimodal distribution similar to that of Figure 5A with a shift to a smaller vesicle amount would be expected. The center distribution created after amphetamine treatment appears to represent a third class of vesicles and only these vesicles are depleted by amphetamine. It is possible that if these cells are incubated for longer times or with higher concentrations of amphetamine, then all the vesicles could be affected. However, it is clear that one class of vesicles is preferentially depleted by amphetamine under the conditions used here.

The data presented here demonstrates that multiple classes of vesicles participate in exocytosis from the cell body of a dopamine-containing neuron of *Planorbis*. To our knowledge, this is the first demonstration that multiple classes of vesicles are simultaneously released and that the content of these vesicles can be independently manipulated by a psychostimulant. The rationale for different classes of vesicles is at present unclear. However, recent experiments have suggested different classes of vesicles may associate with different synaptic function as different classes of vesicles have been found in different functional synaptic terminals [7, 38, 49]. In addition, the distinct classes of vesicles observed here might be important in understanding the rationale for neurotransmitter exocytosis from the cell body of this neuron.

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Figure Legends

Fig. 1. Dopamine current transients detected by carbon fiber disk electrodes following cellular stimulation. (**A**) An example of current transients recorded with the amperometric method. A large *Planorbis* dopamine-containing neuron (diameter about 120 μm) was stimulated with a 3-s potassium chloride (1 M) pulse (260 nL) delivered from a glass pipette which was placed about 15 μm from the cell body. The stimulation is shown by an arrow below the trace. (**B**) Examples of the expanded secretory events which were randomly selected from (**A**).

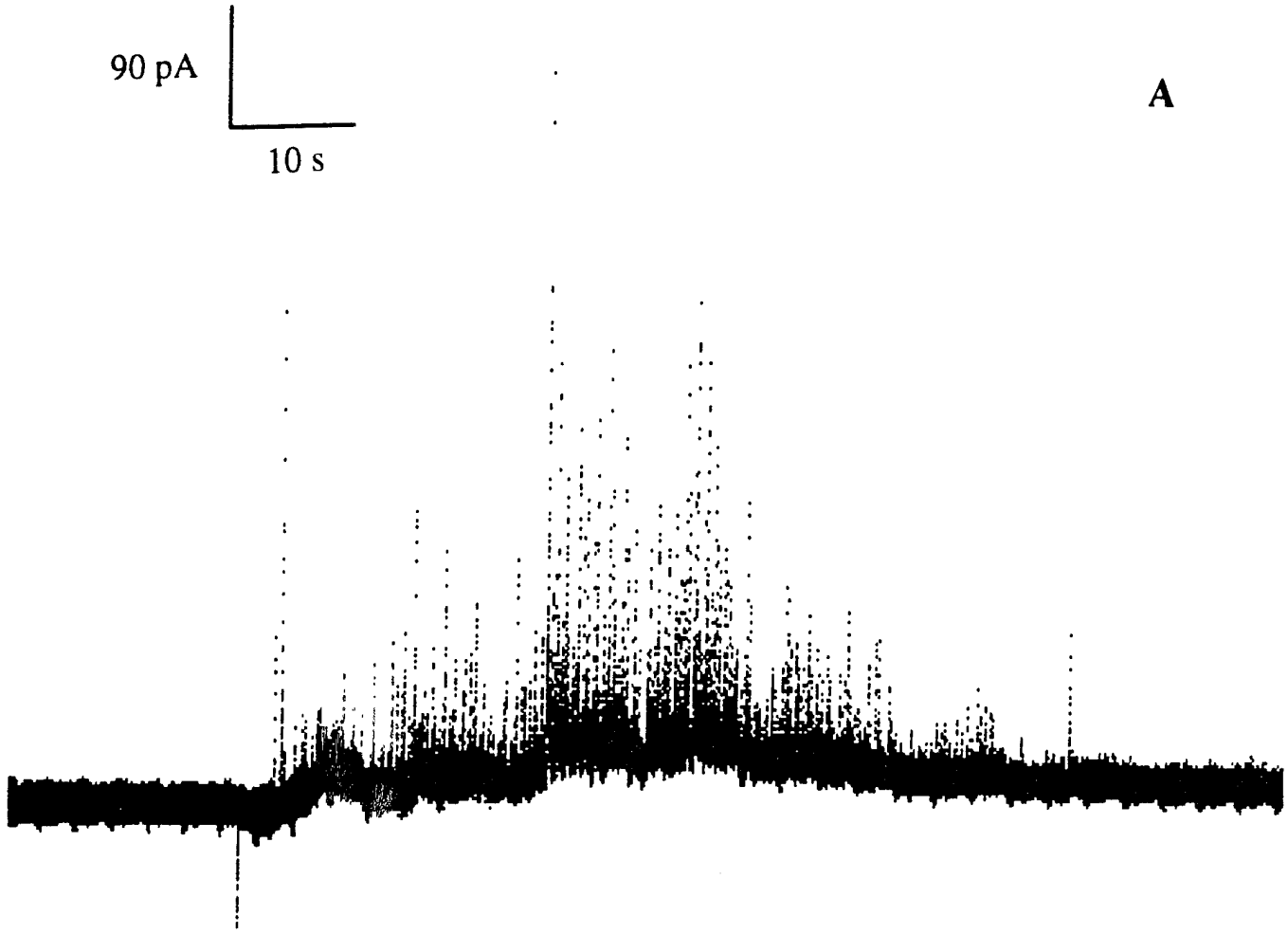
Fig. 2. Histogram of the frequency of release events vs attomoles of dopamine released per vesicle. Data were collected from 21 cells (23,524 transients). Experimental conditions were the same as in Figure 1.

Fig. 3. Histogram of the frequency of release events vs the cubed root of attomoles of dopamine released per vesicle. The same data set as in Figure 2 was used with all transients displayed.

Fig. 4. Histogram of percentages of total transients vs attomoles of dopamine released per vesicle. The empty bars are for response obtained in control experiments. The hatched bars are from experiments after amphetamine (10 μM) treatment. The difference in total number of current transients before and after amphetamine was normalized to give the percentage of total

transients. Data were collected from 5 cells (2669 total transients) before amphetamine and 5 cells (8987 total transients) after amphetamine.

Fig. 5. Histograms of percentages of release transients vs the cubed root of attomoles of dopamine released per vesicle. (**A**) Response obtained before amphetamine treatment. (**B**) Response obtained after amphetamine treatment. In both cases, same data sets as in Figure 4 were used. (**C**) Responses shown in A and B superimposed.



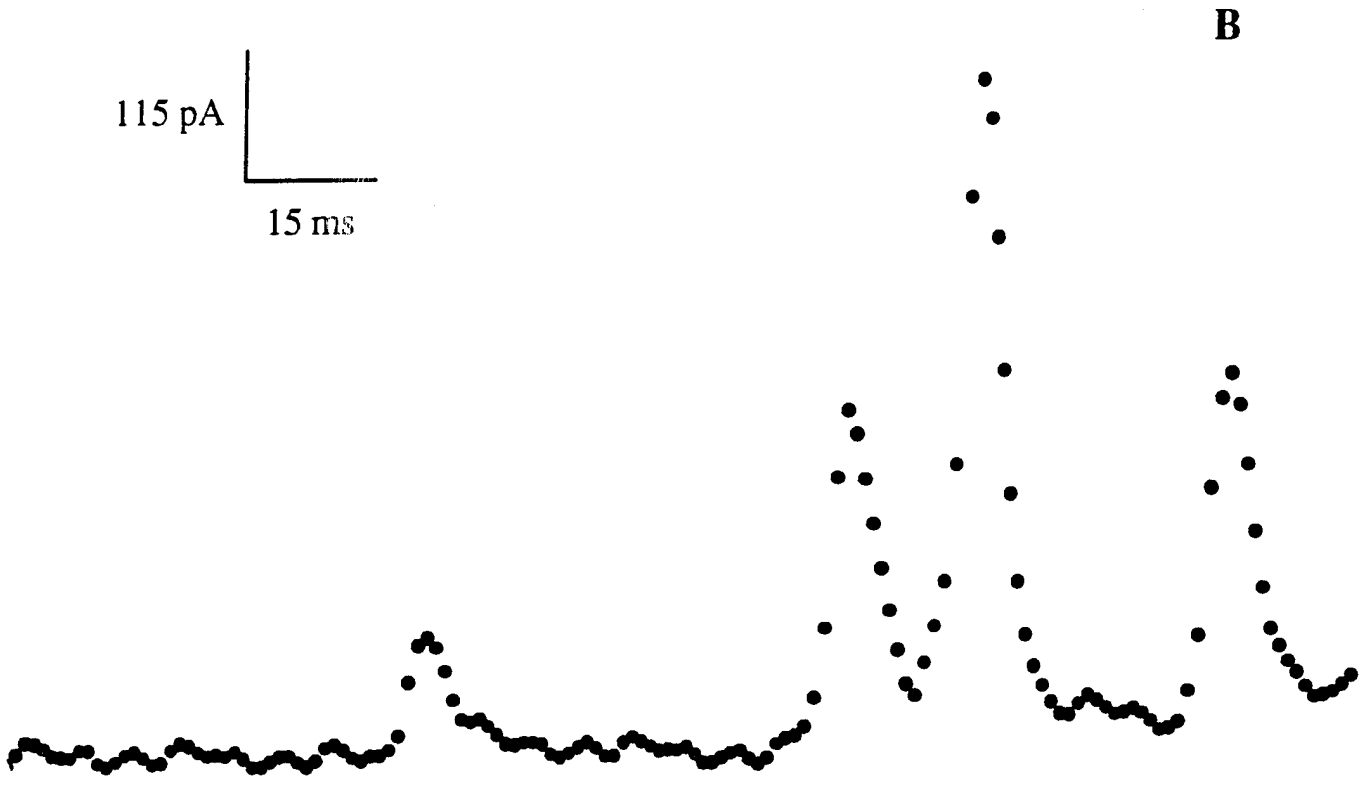


Figure 1

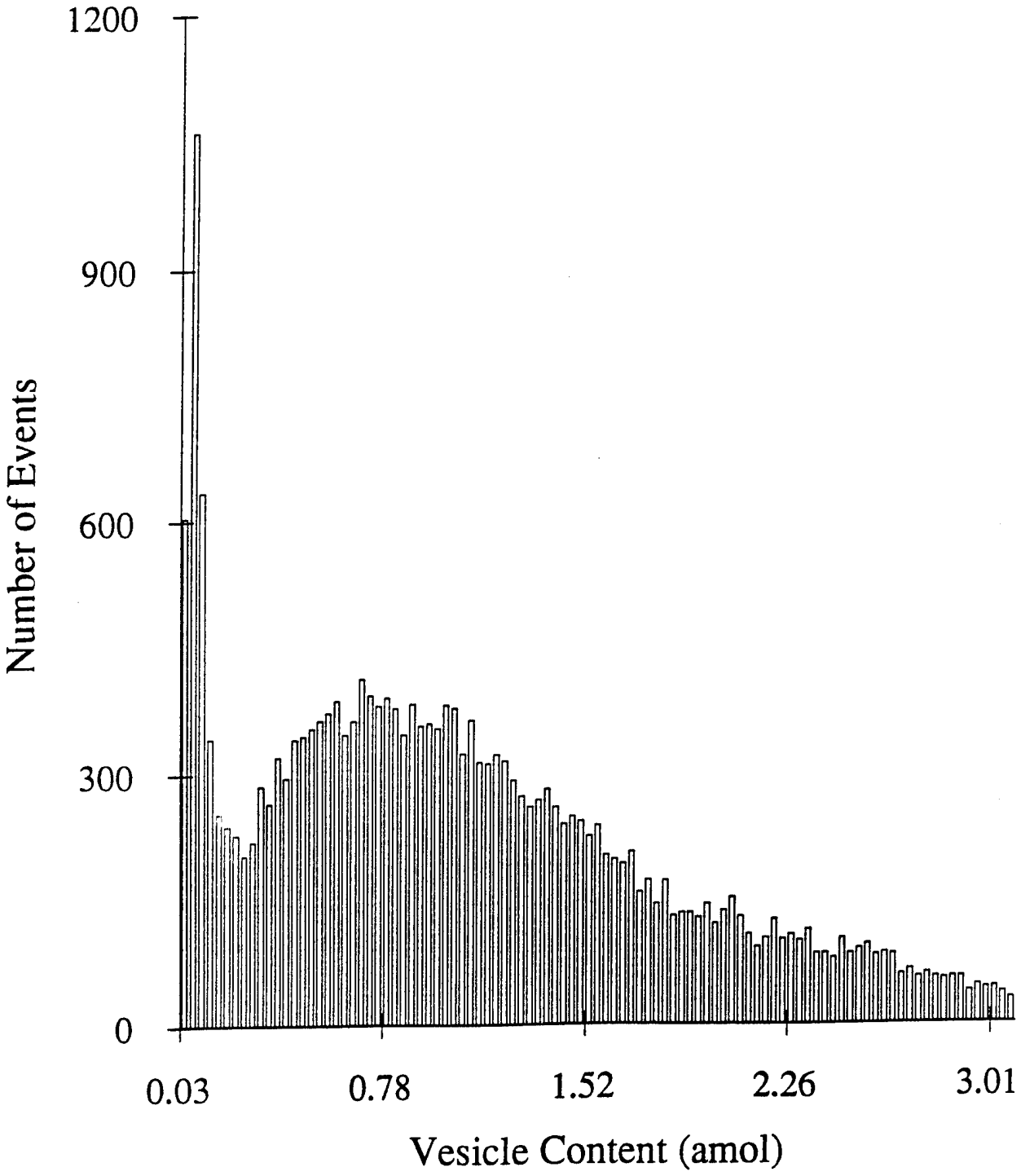

1200
900
600
300
0
Number of Events
0.03
0.78
1.52
2.26
3.01
Vesicle Content (amol)

FIGURE 2

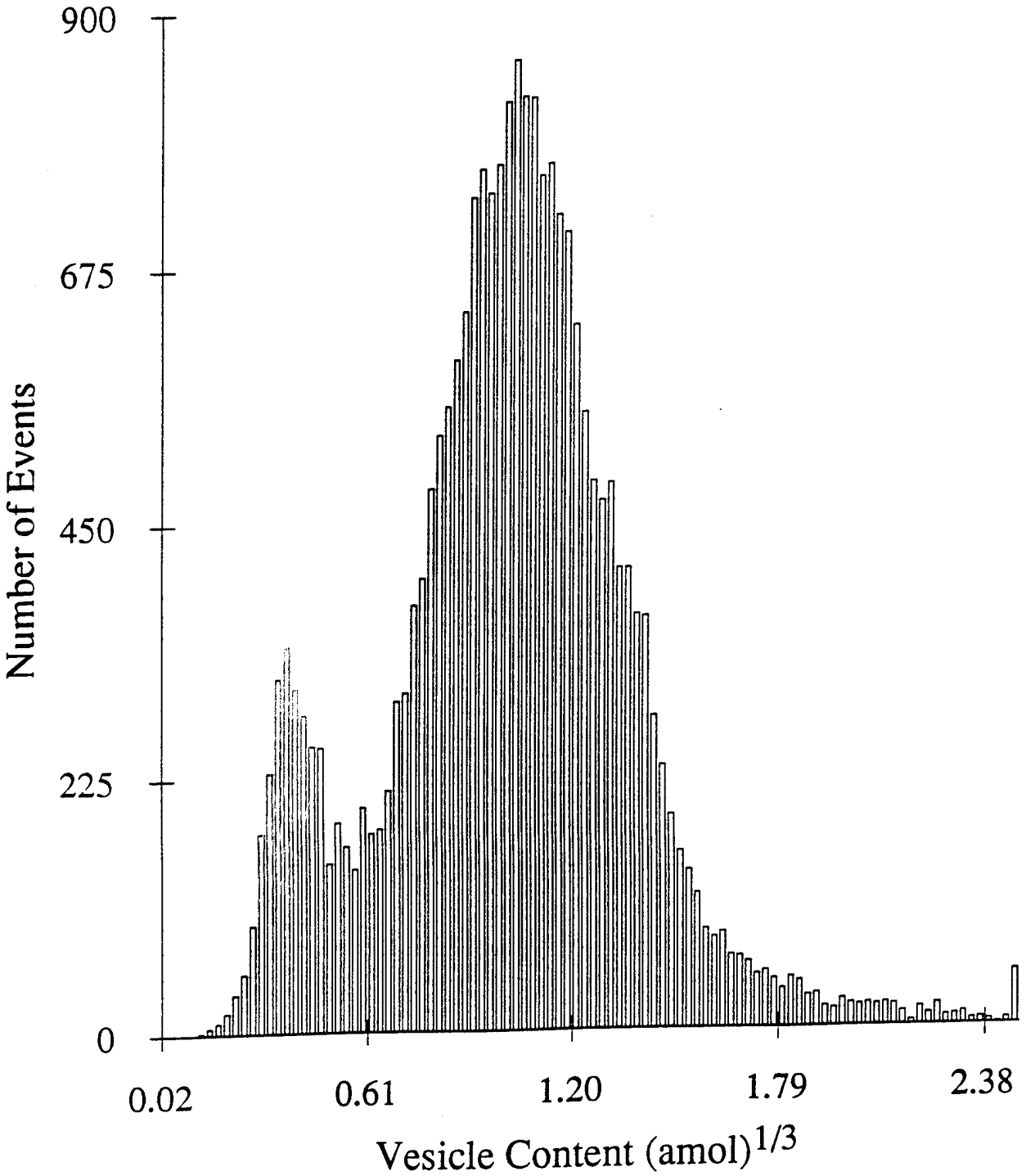

900
675
450
225
0
Number of Events
0.02
0.61
1.20
1.79
2.38
Vesicle Content (amol)1/3

FIGURE 2

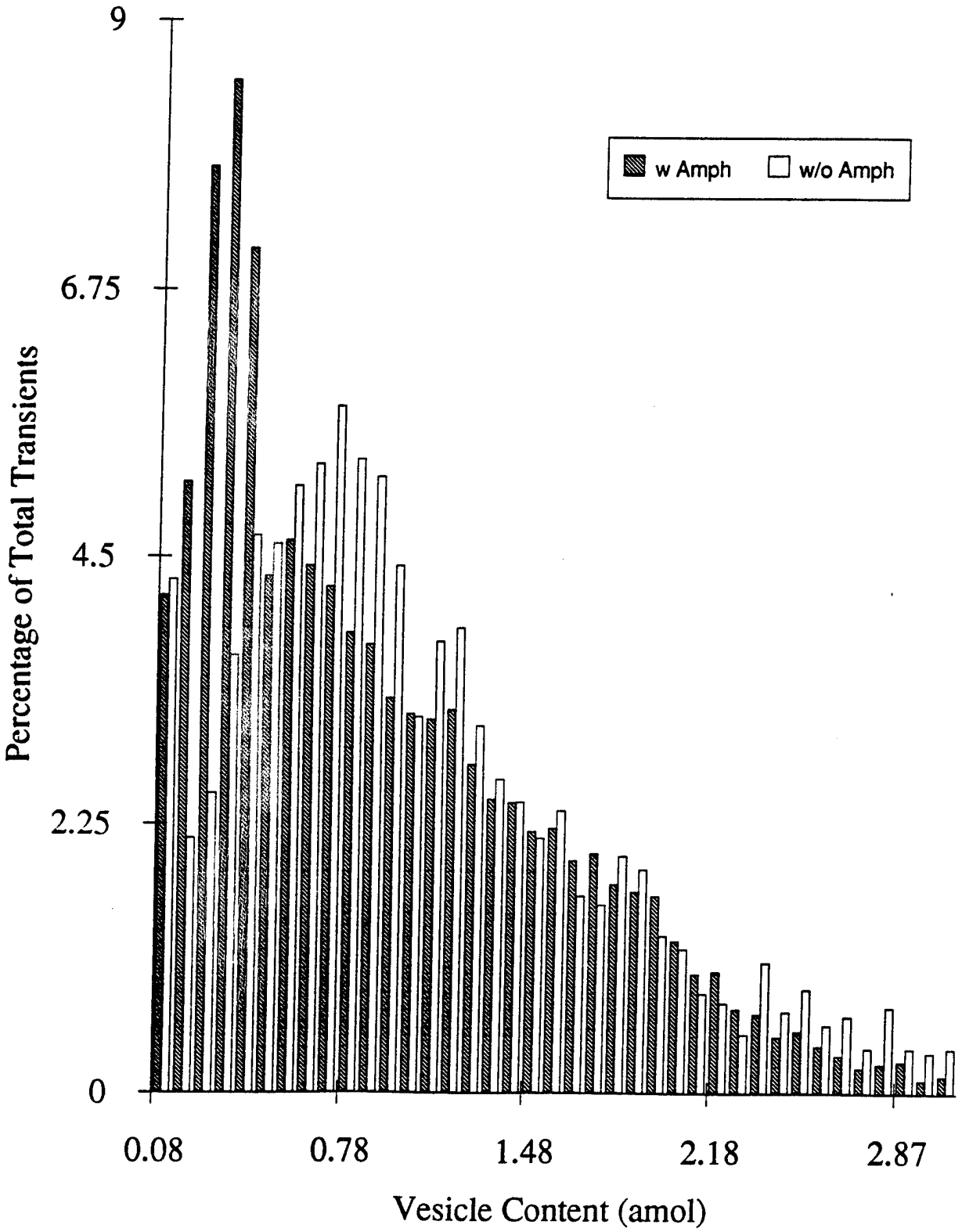
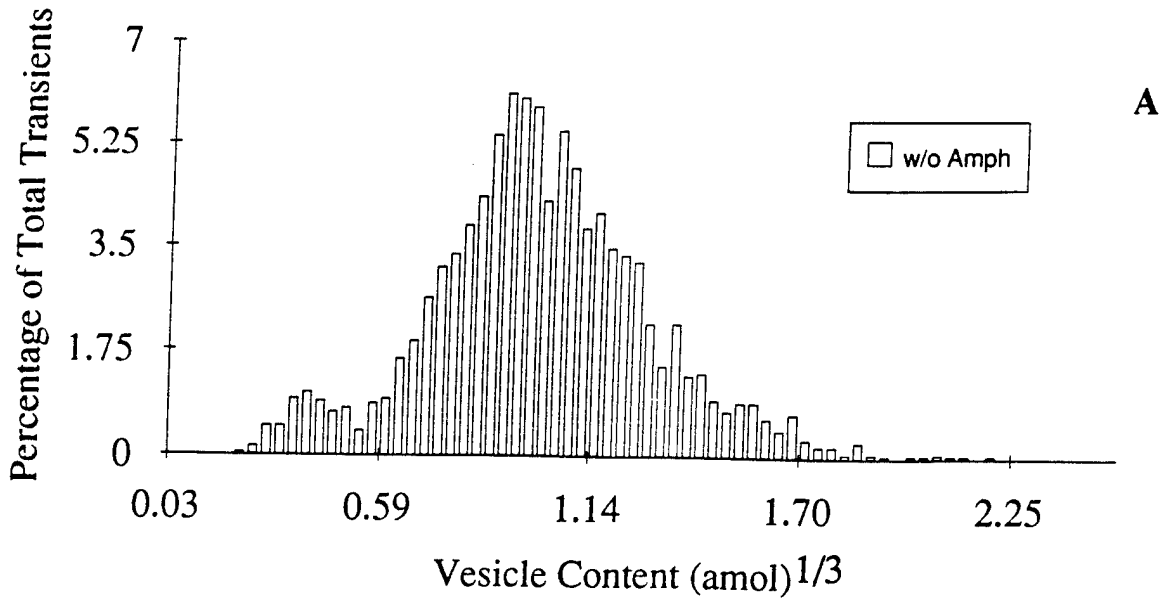
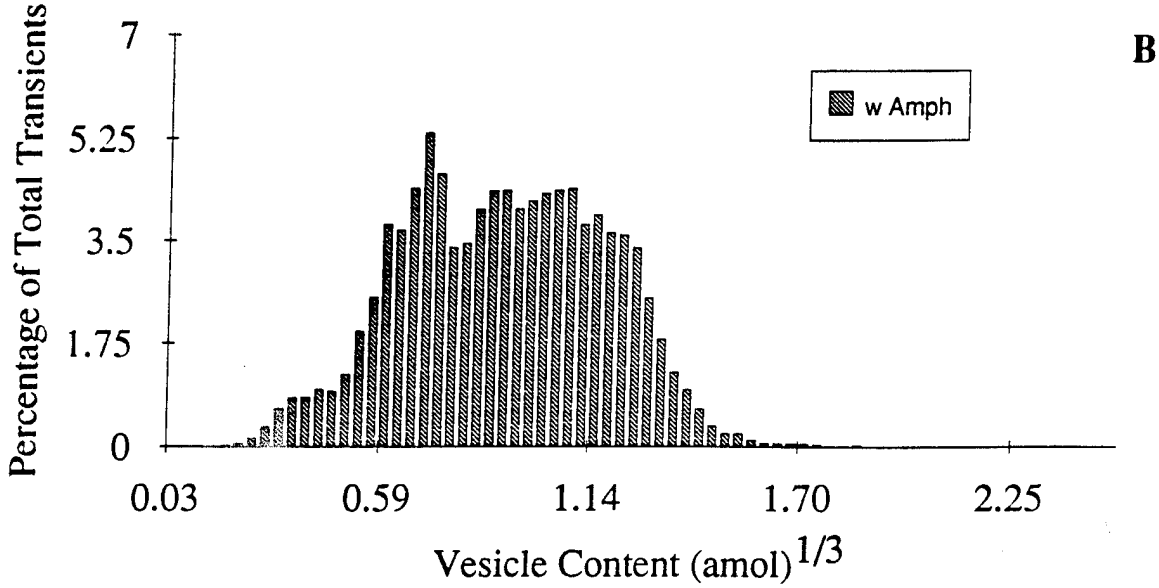

9
6.75
4.5
2.25
0
Percentage of Total Transients
w Amph
w/o Amph
0.08
0.78
1.48
2.18
2.87
Vesicle Content (amol)

FIGURE 4


A
Percentage of Total Transients
7
5.25
3.5
1.75
0
w/o Amph
0.03
0.59
1.14
1.70
2.25
Vesicle Content (amol)1/3


B
Percentage of Total Transients
7
5.25
3.5
1.75
0
w Amph
0.03
0.59
1.14
1.70
2.25
Vesicle Content (amol)1/3

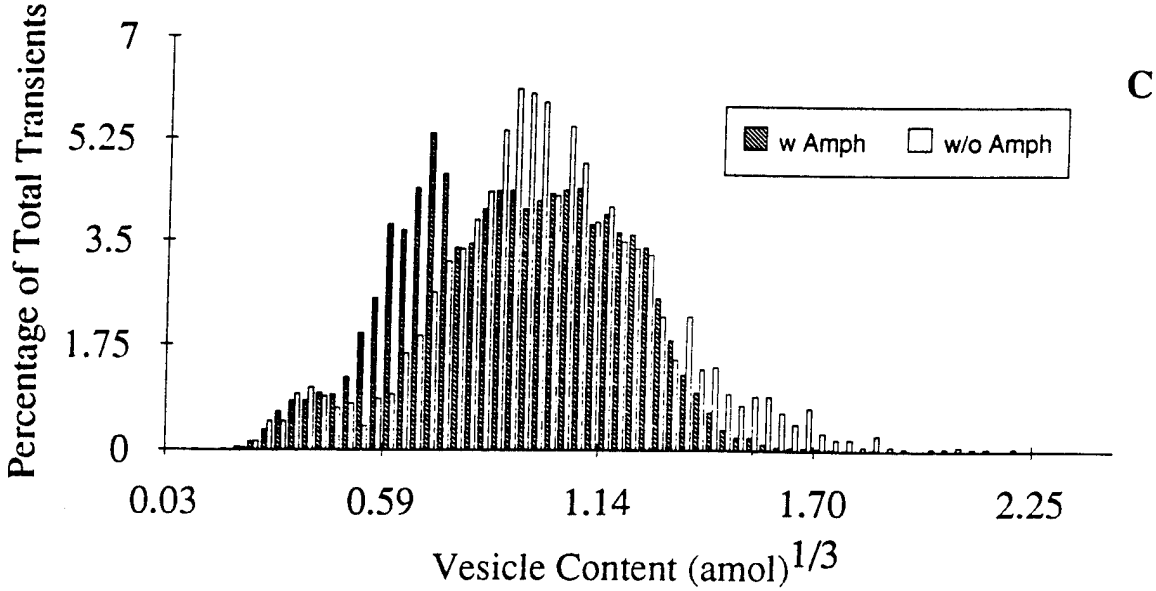

C
Percentage of Total Transients
7
5.25
3.5
1.75
0
w Amph
w/o Amph
0.03
0.59
1.14
1.70
2.25
Vesicle Content (amol)1/3

FIGURE 5