Recycling and refilling of transmitter quanta at the frog neuromuscular junction

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1. Fluorescent dyes have been used at the frog neuromuscular junction to label synaptic vesicular membrane. Retrieved membrane is reformed into vesicles, which are released along with pre-existing vesicles. Consequently, if vesicular refilling with acetylcholine (ACh) is depressed by inhibitors, two sizes of quanta should be released: normal and smaller. As recycling continues the fraction of smaller size quanta should increase exponentially.

2. We enhanced the rate of quantal release by elevating the K^+ concentration. The principal inhibitors were (−)-vesamicol (VES), hemicholinium-3 (HC3), and NH_4^+. Quantal size measurements were fitted to one and to two cumulative lognormal probability distribution functions. When two fitted better, the statistical significance assessment took into account the three additional parameters used in calculating the fit.

3. After recycling in the presence of inhibitor, many sets were fitted better by two lognormal functions. As recycling continued, the fraction of the miniature endplate potential voltage–time integrals (∫MEPPs) in the larger sub-population decreased exponentially.

4. The size of the releasable pool was estimated by counting the quanta released by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). This was compared to pool sizes calculated from the inhibitor experiments. The two estimates of pool size were indistinguishable, with mean values ranging from about 170 000 to 270 000.

5. With all of the treatments tested, the means of the sizes in the smaller sub-population of ∫MEPPs were about 1/3 those of the larger sub-populations.

6. Recycling synaptic vesicles appear to be incorporated into the releasable pool from which they have roughly the same probability of release as the pre-existing vesicles.

Most synapses transmit by the release of packets, or quanta, of neurotransmitter. The quanta are packaged within synaptic vesicles. After the quanta are released the membrane of the synaptic vesicles is retrieved and shaped to form new vesicles, which are then reloaded with transmitter. The recycled vesicles then become available for release. Within this broad picture, many details of the recycling and reloading processes remain fuzzy (Betz & Angleson, 1998).

Much has been learned by following the movements of vesicular membrane at the neuromuscular junction (NMJ) with styryl dyes, like FM1-43 (Betz & Bewick, 1992, 1993). The dyes are taken up by the outer leaflet of the presynaptic membrane. Once in the lipid they become intensely fluorescent. If the motor nerve is stimulated while dye is in the extracellular fluid, within minutes fluorescent spots are seen in the terminal. The spots are clusters of vesicles made from membrane recovered from the terminal (Henkel *et al.* 1996). Sufficient stimulation labels almost all of the vesicles. If the dye is then removed from the extracellular solution, and the nerve stimulated again, the fluorescence is lost, as the vesicles fuse with the terminal and the dye diffuses away. Recycled vesicles appear to mix indiscriminately into the total population of vesicles in the motor nerve terminal.

If the recycled vesicles are mixed randomly into the pool from which they have an equal probability of release, and if filling with transmitter occurs before the vesicles enter the pool, we can devise a hypothesis to be tested electrophysiologically. Suppose that vesicles are recycled in the presence of a drug that inhibits refilling. If refilling were completely blocked, then after stimulation we would observe only quanta of normal size. If the drug depresses but does not eliminate refilling, then after a significant proportion of the initial pool has been released and recycling has formed new vesicles, there would be two distinct sizes of quanta: normal and a smaller size.

Much of the available evidence about the effects of inhibitors of vesicle loading appears to support neither of these possibilities. In most studies, the quanta are reported to become uniformly smaller (reviewed by Van der Kloot & Molgó, 1994). To choose a classic example, Elmqvist & Quastel (1965) studied the effects on the mammalian neuromuscular junction of hemicholinium-3 (HC3), an inhibitor of the high affinity choline transporter of motor nerve terminals. They measured the amplitudes of miniature endplate potentials (MEPPs) before and after bouts of nerve stimulation in the presence of HC3. At first, the amplitude did not change. Later, there was a gradual, progressive decrease in amplitude. Similar uniform reductions in MEPP size were reported when frog motor nerves were stimulated in the presence of vesamicol, an inhibitor of active ACh uptake into cholinergic vesicles (Van der Kloot, 1986; Whitton *et al.* 1986; Lupa, 1988; reviewed by Van der Kloot & Molgó, 1994). In contrast, Searl *et al.* (1991) found two distinct sub-populations of MEPP sizes at the rat NMJ after stimulation in a low concentration, 0·1 μM, of (−)-vesamicol (VES). Similar treatment in the presence of an inhibitor of the high affinity choline transporter troxypyrrolium did not subdivide quantal sizes. Obviously, the steps in the recycling mechanism remain unresolved.

Two possibilities have been suggested to account for the reported uniform decreases in quantal size. (i) The vesicular ACh contents are in equilibrium with the cytoplasmic ACh (Large & Rang, 1978; Williams, 1997). Consequently, when the cytoplasmic ACh concentration decreases, the quanta grow smaller uniformly. There is considerable evidence against ACh equilibrium between vesicle and cytoplasm (Naves *et al.* 1996). (ii) The recycled quanta are ordered into a queue for release, from which those filled first are released first (Elmqvist & Quastel, 1965).

Before speculating further about how a uniform decrease in quantal size is produced, it seemed prudent to look more closely at the data. Perhaps past experiments in the frog were not done with sufficient resolution to detect two size categories. Moreover, the number of quanta released in the presence of the drug before the measurements are made might be crucial, so varying periods of enhanced release should be tested. Visual examination of histograms of quantal sizes might not give clear and convincing evidence for two categories.

Therefore, we decided to re-examine the distribution of quantal sizes following a period of enhanced release in the presence of inhibitors of ACh loading into synaptic vesicles. We used several artifices to improve the chances of resolving the quantal sizes into two sub-populations. We tested a number of inhibitors, starting with (−)-vesamicol (VES), HC3 and NH_4^+ (Van der Kloot, 1987). NH_4^+ inhibits ACh uptake into vesicles by diminishing the proton gradient across the vesicular membrane, which is required for transmitter accumulation (reviewed by Parsons *et al.* 1993; Van der Kloot & Molgó, 1994). We recorded MEPPs in solutions containing primarily impermeant anions. This has two advantages, because the muscle membrane has a high Cl^- permeability (Hodgkin & Horowicz, 1959). (i) When Cl^- is replaced, the input resistance of the fibre is increased, so that the MEPPs are larger. (ii) Elevated concentrations of K^+ were used to enhance the rate of spontaneous quantal release. When Cl^- is present, it loads into depolarized fibres. When the K^+ is removed, the Cl^- comes out over a period of time, which prevents the fibre from repolarizing promptly. Depolarization requires large corrections for the decreased electrochemical gradient for Na^+ entry through the ACh receptor (AChR). With an impermeant anion, repolarization is prompt.

After recording MEPP sizes, the data sets were fitted to one and two probability distribution functions by a mathematical method. Quantal sizes are fitted better by lognormal than by normal distribution functions (Van der Kloot, 1987, 1991; Bekkers *et al.* 1990; Borst *et al.* 1994). Therefore, the data sets were fitted with one and two cumulative lognormal distributions. Suppose that the fit to two lognormal distributions is better than the fit to one. This still leaves an open question: whether this is because the hypothesis that there are two sub-populations is better, or simply because more parameters were used to fit the distribution to the data? This question can be resolved by using a statistical method for testing models that takes into account the number of fitting parameters (Horn, 1987). Without such a test, it would be impossible to rigorously test the hypothesis.

The results show that in the presence of the inhibitors of vesicular filling, a period of enhanced quantal release often produced MEPP populations composed of two sub-populations. The more quanta released, the larger the fraction in the smaller sub-population. The hypothesis that the partially filled, recycled quanta are mixed with quanta of normal size into a pool from which all quanta have an equal probability of release leads to a clear prediction. As recycling proceeds, the fraction of quanta of normal size that are released will decrease exponentially. When 37% (1/e) of the quanta being released are of normal size, then the total number of quanta released during the experiment is equal to the number of quanta initially in the pool.

As will be seen, the data satisfied the hypothesis that recycling, partially filled vesicles mix into the vesicular pool. Further experiments suggest that the partially filled vesicles and those loaded before the inhibitor was present have a similar probability of release. A brief account of some of these experiments has appeared (Van der Kloot & Molgó, 1999).

METHODS

Electrophysiology

Sartorius or cutaneous pectoris nerve–muscle preparations were dissected from *Rana pipiens*, killed by double-pithing in accordance

with University guidelines (Van der Kloot, 1996). Dissection was performed in a solution containing (mM): NaCl, 120; KCl, 2·0; $CaCl_2$, 2·5; Tes-NaOH at pH 7·4, 4·0; The impermeant anion used was gluconate, selected simply because it was readily available. The recordings were made in solution which is designated gluconate Ringer solution, containing (mM): sodium gluconate, 120; KCl, 2·0; $CaCl_2$, 2·5; Tes, 4·0; neostigmine bromide, 0·003; tetrodotoxin, 0·00003. Tetrodotoxin is essential to block the spontaneous contractions observed in Cl^--free solutions. The spontaneous MEPP frequency was elevated by placing preparations in an elevated potassium gluconate solution, containing (mM): sodium gluconate, 92; potassium gluconate, 30; $CaCl_2$, 2·5; Tes, 4·0; neostigmine bromide, 0·003; tetrodotoxin, 0·00003. Ca^{2+}-free gluconate Ringer solution contained the same constituents as the gluconate Ringer solution, except that $CaCl_2$ was replaced by $MgCl_2$.

The preparations were pinned on a layer of silicone rubber in an acrylic chamber with a volume of about 5 ml. MEPPs were recorded with bevelled glass microelectrodes, with DC resistances between 2 and 4·5 MΩ. The signals were detected with an Axon 2A voltage clamp amplifier, and passed on to an Axon CyberAmp 300 signal conditioner, which amplified them further over a bandwidth from 0·1 to 1000 Hz. The signal emerging from the CyberAmp was split, one branch going to an Axon AI 2020 event detector, the other to a ComputerBoards DAS-16 330 A/D converter (Mansfield, MA, USA). When a MEPP passed a threshold set just above the noise level, the event detector delivered a +5 V pulse to the A/D board, which digitized at 10 kHz. The 200 points before the +5 V pulse and the 800 after the pulse were output to a computer. The signal was observed online by an operator. If the MEPP was not contaminated by an overlapping MEPP or by electrical noise, its voltage–time integral, ∫MEPP, was calculated as the measure of the quantal size (Van der Kloot, 1997). In each data set at least 300 MEPPs were recorded, since trial and error showed that this was an appropriate sample size for the further calculations. Examples of the data are in Fig. 1*A*.

Curve fitting and statistics

The ∫MEPPs from each data set were sorted in ascending order and plotted as a cumulative curve. Each of these curves was fitted with one and two cumulative lognormal distributions, using the Levenberg-Marquardt method (Press *et al.* 1989). If the fit to two distributions appeared to be better, the next step was to determine whether improvement was because of the hypothesis, or simply because there were more fitting parameters (Horn, 1987). The ∫MEPPs, arranged in ascending order, were sorted into bins, each of which contained a minimum of five observations. The predicted values from the one and two lognormal curves were sorted into the same bins. The likelihood functions, $ll = f_{obs}\ln(f_{obs}/f_{pred})$ (where f_{obs} is observed number in each bin and f_{pred} is predicted number in each bin), were calculated for each bin. These likelihood functions were then summed, Σll_{one} and Σll_{two}. Then $G = 2(\Sigma ll_{one} - \Sigma ll_{two})$ (Horn, 1987). G is distributed as χ^2 with 3 degrees of freedom, because the two lognormal curve is calculated with three more parameters than the single one: the fraction in the second distribution, and the mean and S.D. of the second distribution. The probability that the data are best described by one lognormal curve rather than two is obtained from the χ^2 value and the degrees of freedom. Examples of data sets fitted by one and two lognormal curves are shown in Fig. 1*B*. The figure makes it clear how difficult it can be to detect the presence of two distributions by eye.

All of the means of data sets reported in the text and in the figures are given with ±95% confidence intervals.

Measuring the number of quanta released

When neuromuscular preparations are exposed to elevated K^+, the MEPP frequency, F_{MEPP}, continues to rise over a period of minutes, reaches an asymptote, and then progressively declines (Cooke & Quastel, 1973). To obtain reliable estimates of the total number of quanta released over time it is necessary to make repeated measurements. Furthermore, there is appreciable variation from one junction to the next in the extent to which F_{MEPP} is elevated

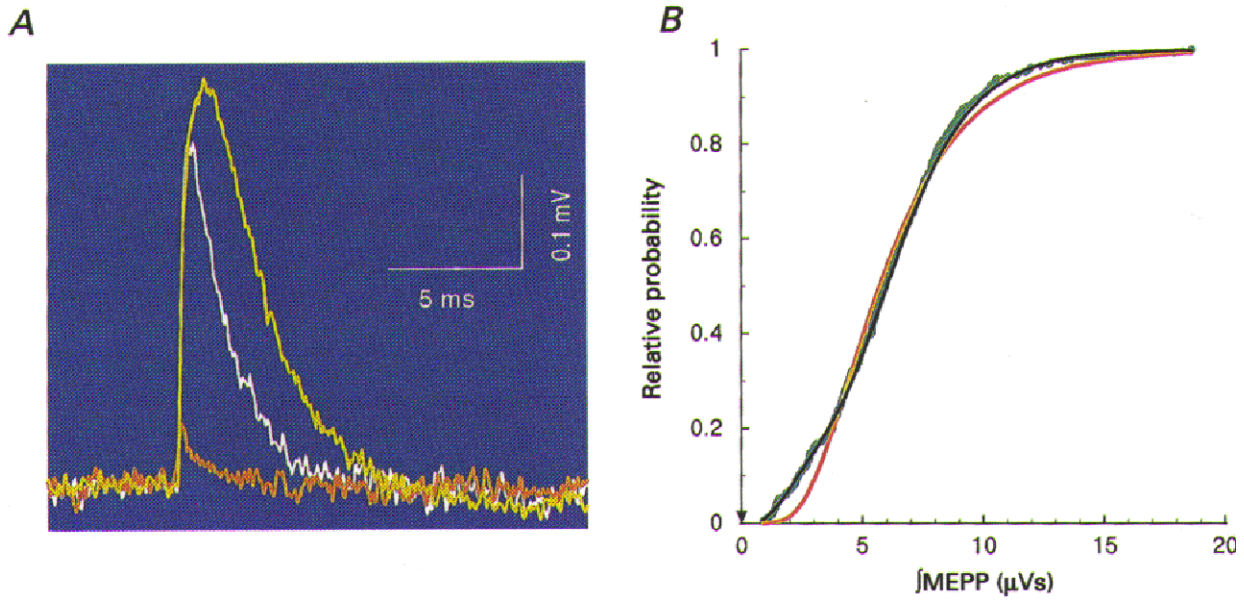

Figure 1. Examples of MEPP recordings and the fitting of distribution functions to the data

A, MEPP recordings in gluconate Ringer solution from a preparation that had been previously soaked for 20 min in 30 mM K^+ solution + 1 μM HC3. The examples were selected to illustrate the wide range of MEPP sizes. *B*, a cumulative plot of the data from a similar experiment (green circles), the fit to a single lognormal distribution (orange line), and the fit to two lognormal distributions (black line).

(Molenaar & Oen, 1988). Our method was to place preparations in elevated K^+ solution, digitize the MEPPs at an endplate at 2 kHz and display the trace on the computer screen. The operator counted the number of MEPPs seen on the trace. At the highest frequencies encountered undoubtedly some of the MEPPs were obscured and not counted. The display and counting were repeated until the total number of MEPPs tallied was greater than 100. In other words, 100 or more MEPPs were counted, rather than counting all MEPPs over a set time period. The F_{MEPP} and the time at which the measurement ended were recorded. Then another endplate was penetrated and the process repeated. The measurements were continued as long as the preparations were in elevated K^+ solution, which ranged from 2 to 120 min. Because of the problems of variation in time and from one junction to the next, the variance of the estimates of the number of quanta released in elevated K^+ solution as a function of time is high (see Results).

The number of quanta released following exposure to CCCP was measured as follows. An endplate was penetrated. CCCP was added to the bath to give a final concentration of 10 μM. Every 10 s the computer recorded a series of A/D points at 2 kHz extending for 0·64 s. If the membrane potential of the fibre fell to a level where seeing the MEPPs became problematical, the microelectrode was inserted into a new endplate. In some data sets all recording was from a single fibre; in others as many as four junctions were penetrated. Recording was terminated when the F_{MEPP} reached a low value, usually < 1 s^{-1}. After the recording was completed, the operator reviewed the records in the form of 64 ms segments, counting the number of MEPPs in each segment. The total number of MEPPs released was estimated assuming the measured rate persisted until the next set of measurements was taken in.

Ultrastructure

Cutaneous pectoris muscles were fixed for 1 h at room temperature with 2·5% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·2) and post-fixed with 1% OsO_4 in the same buffer. Blocks containing endplate-rich regions were dehydrated and flat-embedded in epoxy resin. Cross and longitudinal ultra-thin silver-grey sections were counter-stained with uranyl acetate and lead citrate and observed in a Hitachi H-500 electron microscope.

Chemicals

The drugs were obtained from Sigma, except for HC3 and (−)-vesamicol (RBI, Natick, MA, USA) and BAPTA AM (Molecular Probes, Eugene, OR, USA). Preparations were treated with BAPTA AM as described by Van der Kloot & Molgó (1993).

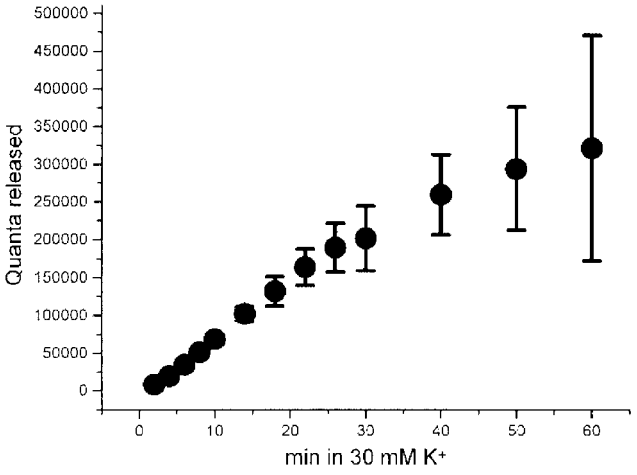

Figure 2. Estimates of the number of quanta released while stimulating quantal release in 30 mM K^+ solution
The error bars show the ±95% confidence limits.

RESULTS

Number of quanta released by 30 mM K^+ solution

MEPP frequencies were measured at different times during soaking in 30 mM K^+ solution containing 1 μM HC3, 2 μM VES, or 30 mM NH_4^+. The results of the measurements showed that the identity of the inhibitor did not affect the results (determined by ANOVA). Therefore, all of the results from 352 penetrations were merged to show the total number of quanta released as a function of time in K^+ solution (Fig. 2). The points on the curve were obtained by adding the estimated mean number released in all preceding time periods to the estimated mean number released in the time period under consideration. The 95% confidence limits were obtained in exactly the same way, but using either the +95% or −95% limits for each time period rather than the means. Because of the variability from junction to junction, the confidence limits become quite broad.

∫MEPP size

The sizes of ∫MEPPs recorded from different junctions from the same frog, from different frogs, and from frogs at different seasons of the year varied substantially. Pooling all of our measurements in gluconate Ringer solution in which the preparations had not yet been exposed to elevated K^+, the mean ∫MEPP was $8{\cdot}9 \pm 1{\cdot}15$ μV s (95% confidence limits). The coefficient of variation (CV) was 0·69 ($n = 111$). (The CV is the ratio of the standard deviation to the mean.) The largest mean was 32·9 μV s, the smallest was 2·1 μV s. This broad range of sizes shows why it is necessary to employ experimental designs in which muscles from the same frog serve as experimental and control preparations in studies on treatments that alter quantal size (Van der Kloot, 1991). It also is why, as will be shown next, mean values for the sizes of quanta after the treatments with inhibitors are not very revealing.

Figure 3*A* shows the mean values for ∫MEPP sizes after varying numbers of quanta had been released in the presence of VES, HC3 or NH_4^+, along with the 95% confidence limits. Size decreased significantly following the release of about 100 000 quanta, but there is little clear decrease in size with further release.

Coefficients of variation after exposure to 30 mM K^+ solution

After exposure for various times to elevated K^+ solution, there was often an increase in the CVs. Even with no inhibitor present the CV increased significantly as a function of the number of quanta released (Fig. 3*B*). The increases were quite variable, as shown by the broad 95% confidence limits on the measurements. When HC3 or NH_4^+ was included in the solutions, the CVs increased substantially more than in the controls (Fig. 3*B*). Similarly, the inclusion of VES produced a larger increase in the CV following

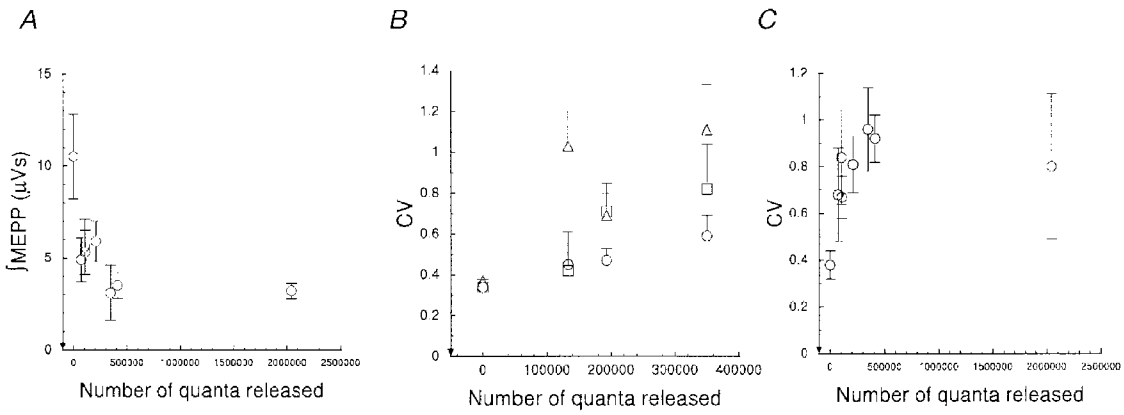

Figure 3. The properties of the quanta change when release is promoted in the presence of the inhibitors

A, the mean ∫MEPP becomes smaller in the presence of VES, NH_4^+ or HC3. The results with all three inhibitors have been pooled. The error bars show the ±95% confidence limits. *B*, the CVs of the ∫MEPPs increase. Without inhibitor (○), with HC3 (□), and with NH_4^+ (△). The error bars show the +95% confidence limits. *C*, changes in the CVs in the presence of VES; the error bars show the ±95% confidence limits.

periods of enhanced quantal release than that observed in the controls (Fig. 3*C*). The similar increases in the CVs observed with the three inhibitors was a major factor in the decision to merge the three data sets in the further analysis of the data.

One or two sub-populations

We first tested the reproducibility of the mathematical curve fitting method and the hypothesis-testing statistic (see Methods). This was done by working with preparations that had been exposed to 30 mM K^+ solution in the presence of one or another of the inhibitors. An endplate was penetrated and two or three sets, each containing at least 300 MEPPs, were recorded. The sets were then analysed and the data from the same junctions compared. In every example in which the analysis concluded that two lognormal distribution functions fitted better than one, the conclusion applied to all of the data sets recorded from the same junction. The estimates for each junction of the fraction of the ∫MEPPs that fell into the larger of the two distributions are shown in Fig. 4. In some examples the estimates were very close, in others there was an appreciable spread. The method appears to be effective, but not highly precise. Therefore, a considerable number of data sets were needed to reach reliable conclusions.

All of the sets of ∫MEPP measurements were tested to see whether the size distributions were fitted better by one or two lognormal probability distribution functions. Two criteria had to be met before it was concluded that the fit with two was better ($P > 0{\cdot}95$); (i) the result of the hypothesis test (see Methods) had to be positive, and (ii) the ratio of the mean of the larger sub-population to the mean of the smaller had to be greater than 1·5. By these criteria, the percentage of the sets fitted better with two sub-populations is shown in Fig. 5*A*. Before the periods of enhanced quantal release, fewer than 10% of the data sets were fitted better by two distribution functions. Enhanced quantal release when no inhibitor was present did not substantially alter the percentage of the data sets fitted better with two distributions, but when any one of the three inhibitors was included in the solutions there was a marked increase in the percentage fitted better by two distribution functions.

The fractions in the two sub-populations

Figure 5*B* shows the fraction of the ∫MEPP sizes that were estimated to fall into the larger of the two sub-populations after various amounts of quantal release in the presence of the inhibitors of vesicle loading. Only the instances where

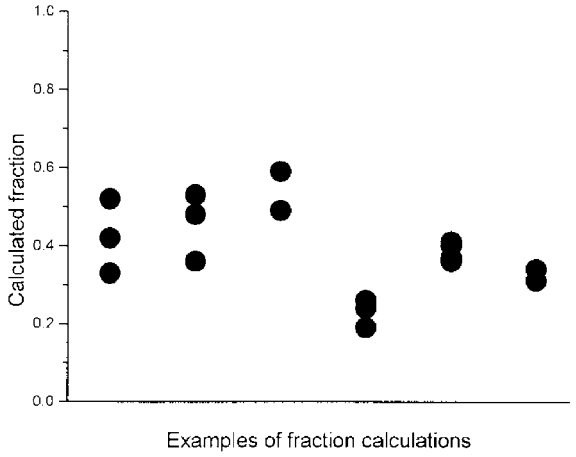

Figure 4. The reproducibility at the same junctions of the determinations of the fraction of the ∫MEPPs that are in the sub-population with the larger mean

Each group of points falling along a vertical shows measurements from the same endplate. The preparation had been exposed to 30 mM K^+ solution containing NH_4^+, VES or HC3 for 15–20 min before the measurements were made in gluconate Ringer solution.

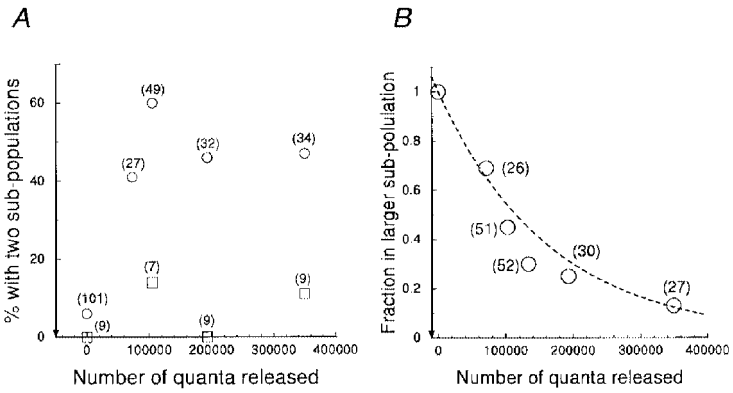

Figure 5. Changes in the sub-populations as quanta are released

A, the percentage of the preparations that have two sub-populations increases as more quanta are released. The numbers in parentheses show the total number of sets of ∫MEPPs analysed. With no inhibitor (□); with HC3, VES or NH_4^+ (○). *B*, the fraction of the ∫MEPPs falling in the larger sub-population decreases exponentially as more quanta are released in the presence of HC3, NH_4^+ or VES. For the exponential curve $R^2 = 0{\cdot}92$. In parentheses are the number of examples in each of the points.

two sub-populations were detected were used for making the figure. The fraction of the ∫MEPPs that belong to the larger sub-population decreases as a function of the number of quanta released. The decrease can be fitted by an exponential curve. This result is expected if the recycled quanta, made smaller because of the presence of the inhibitors of vesicle loading, are mixed along with the vesicles formed before the inhibitors were present into a population of releasable vesicles (see Introduction).

Sizes of ∫MEPPs in the two sub-populations

The ratio of the mean quantal size in the larger sub-population to that in the smaller is shown for all examples in the presence of the inhibitors in which two sub-populations were distinguished (Fig. 6). Overall, the mean size of the ∫MEPPs in the larger sub-population is about three times that in the smaller sub-population. There was no clear differentiation between the effects of the different inhibitors.

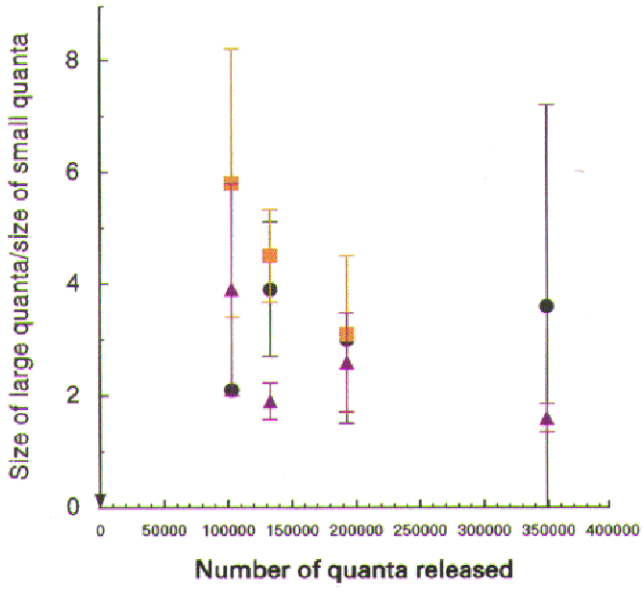

Figure 6. The mean sizes in the larger and smaller distributions change relatively little when more quanta are released

The inhibitors used were HC3 (black circles), NH_4^+ (orange squares), and VES (purple triangles).

To determine whether additional inhibitor would decrease quantal size even more, measurements were made on eight fibres after soaking for 20 min in 30 mM K^+ solution containing 10 μM VES. The ratio of the mean quantal size in the larger sub-population to that in the smaller was $3{\cdot}1 \pm 1{\cdot}45$, not significantly different from the results when the VES concentration was 2 μM.

Predicting the size of the store of releasable quanta

If the recycled vesicles containing less ACh are returned to a releasable pool from which all vesicles have an equal probability of release, then when the fraction of the quanta of normal size being released reaches 1/e, the total number of quanta released is equal to the size of the releasable pool. Using this interpretation, the exponential curve in Fig. 5*B* shows that the releasable pool was about 166 000 quanta. This estimate is well below most of the values in the literature for either the number of releasable quanta in the frog or the number of vesicles seen in the frog nerve terminal (reviewed by Van der Kloot & Molgó, 1994).

Number of quanta released by CCCP

It seemed possible that part of the discrepancy between the literature and our data might be due to differences in the characteristics of the frogs. Therefore, we decided to measure the size of the releasable quantal pool in animals from the batches used in the other experiments. We could not use methods that rely on a steady MEPP size throughout the measurements (Reid *et al.* 1999). Molgó & Pecot-Dechavassine (1988) found that exposure to CCCP produces a period of elevated F_{MEPP}, during which hundreds of thousands of quanta are released. This was followed either by a state in which the F_{MEPP} was very low,

or by a state in which no MEPPs whatsoever were seen. After the period of elevated release the number of synaptic vesicles in motor nerve terminals was greatly reduced, many terminals were almost devoid of vesicles. Apparently CCCP both elevates the rate of quantal release and blocks the formation of recycled vesicles. It is not known how many quanta are released by the CCCP before recycling is blocked, or how recycling is blocked. Nevertheless, estimates of the total number of quanta released by the terminals in the presence of CCCP seemed the best available method to estimate the releasable store.

We measured the total number of quanta released in the presence of 10 μM CCCP. There was substantial variation in the time course for the release of quanta, even from the two preparations from the same animal (Fig. 7*A*). Nonetheless, the store sizes in paired muscles from the same animals were correlated (Fig. 7*B*). The size of the CCCP-releasable store appears to be a characteristic of the individual animal. To illustrate this correlation, the measurements summarized in Fig. 7*B* include a number of experiments performed on different groups of frogs from those used in the inhibitor experiments. These experiments attempted to elucidate the mode of action of CCCP. Since they did not reveal anything about how CCCP acts, they will not be reported in detail. In brief, the F_{MEPP} and number released does not appear to be altered in paired muscles placed in Ca^{2+}-free Ringer solution containing EGTA (Molgó & Pecot-Dechavassine, 1988), or if one muscle is pretreated for 1 h in 30 mM K^+ solution, or for 10 min in 10 μM oligomycin (the reason for using oligomycin will be given in the Discussion). The only treatment that appeared to alter the response to CCCP reliably was pre-treatment of one of the paired muscles with the intracellular Ca^{2+} chelator BAPTA AM (Van der Kloot & Molgó, 1993), which reduced the number of quanta released from the treated preparation (Fig. 7*B*). The results with BAPTA AM were included because they may provide some clue about the chain of events set in motion by CCCP, but this point requires further investigation.

The store of releasable quanta estimated with CCCP, from frogs in the same batches used in the sub-population experiments, is shown in Fig. 8. The figure also shows the estimate of the store calculated from all examples obtained in the presence of an inhibitor in which two sub-populations were detected. (The mean number of quanta released in all of the CCCP experiments summarized in Fig. 7*B*, except those involving BAPTA AM, was 296 000 $\pm$ 44 000. This is higher than the value shown in Fig. 7*B*, which includes only results from the same batches of frogs used in the other experiments.) The assumptions used in calculating the size of the stores were that the recycling vesicles enter the releasable pool in the nerve terminal from which they have an equal probability of release. The estimates from the different methods were indistinguishable. This supports the idea that recycling vesicles and pre-existing vesicles in the pool have similar probabilities of release.

We examined nerve terminals from cutaneous pectoris preparations treated for 1 h in 10 μM CCCP with the electron microscope. In agreement with the results of Molgó & Pecot-Dechavassine (1988), who used 2 μM CCCP for longer times, after the treatment most terminals had few remaining vesicles. An occasional terminal in the same preparation retained a substantial vesicle population.

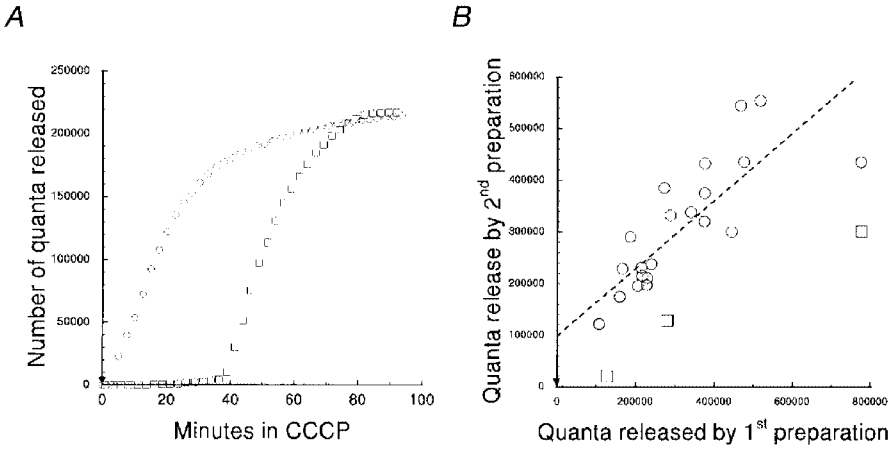

Figure 7. The number of quanta released from preparations soaked in 10 μM CCCP

A, the time course of the release from paired preparations from the same frog. The example shows the most substantial difference in the time course observed in the experiments. *B*, a scatter diagram of the release from the two preparations from the same frogs. Different protocols have been pooled in the figure, because they all illustrate the correlation in the numbers of quanta released by preparations from the same animal, $R^2 = 0{\cdot}68$. Untreated preparations, preparations in which one muscle was kept in 30 mM K^+ solution for 1 h before exposure to CCCP and preparations in which one muscle was in Ca^{2+}-free gluconate Ringer solution containing 1 mM EGTA (○). Experiments in which the second preparation was exposed for 2 h to Ca^{2+}-free gluconate Ringer solution containing 1 mM EGTA and 0·2 mM BAPTA AM before CCCP was added to the same solution. The first preparation was in Ca^{2+}-free gluconate Ringer solution containing 1 mM EGTA throughout (□).

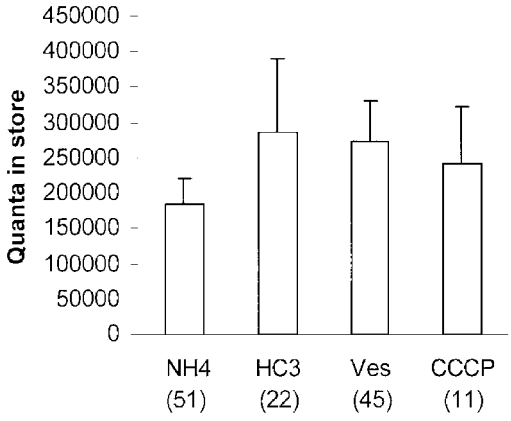

Figure 8. Estimates of the size of the store of releasable quanta

The error bars show the +95% confidence limits. The numbers in parentheses below the the *x*-axis show the number of junctions from which the measurements were made. The values for NH_4^+, HC3 and VES were calculated from data from individual junctions that had been treated in 30 mM K^+ solution + inhibitor and showed two sub-populations. The CCCP values come from estimates of the total number of quanta released in the presence of 10 μM CCCP from the same batches of animals used in the inhibitor studies.

Recovery of quantal size

After quantal size had been reduced, we wondered whether subsequent incubation in the absence of an inhibitor of ACh uptake would increase quantal size back toward initial levels. These experiments were done using NH_4^+ as the inhibitor, since when the bathing solution no longer contained NH_4^+, the inhibitor should readily diffuse back out of the vesicles, so that active ACh uptake would be restored. The protocol was to treat a preparation for 20 or 30 min in 30 mM K^+ solution containing 20 mM NH_4Cl. Then the preparation was returned to gluconate Ringer solution, recording was started at a junction and a set of 300 MEPPs recorded. After an interval, further sets of MEPPs were recorded. The measurements were continued for up to 120 min, if the resting potential did not fall substantially. The result of one of these experiments is shown in Fig. 9. During recovery, the mean ∫MEPPs roughly doubled in size. As the mean quantal size enlarged, the CV of the measurements decreased, which is shown in the figure by the narrowing of the error bars showing the 95% confidence intervals. Just after the treatment in 30 mM $K^+ + NH_4^+$ about 20% of the ∫MEPPs fell into the lognormal probability distribution with the larger mean. Over time, the percentage in the larger distribution increased to about 90%.

The results could be accounted for if after the return to NH_4^+-free solution the nerve terminal once again started to produce quanta of a larger size and therefore the percentage of the quanta in the pool in the larger size distribution increased with time. Similar increases in ∫MEPP size with recovery were obtained in five further experiments using the same protocol. Cumulative plots of the data sets from one of these experiments are shown in Fig. 9*B*. This interpretation requires that there is a relatively small number of vesicles in the immediately releasable pool: otherwise we would not expect to find that most of the vesicles in the pool were recycled due to spontaneous release over an hour or so. We tested this hypothesis by measuring the total number of quanta released by 10 μM CCCP from junctions in a muscle and also from junctions in the paired muscle that had been first soaked for 1 h in 30 mM K^+

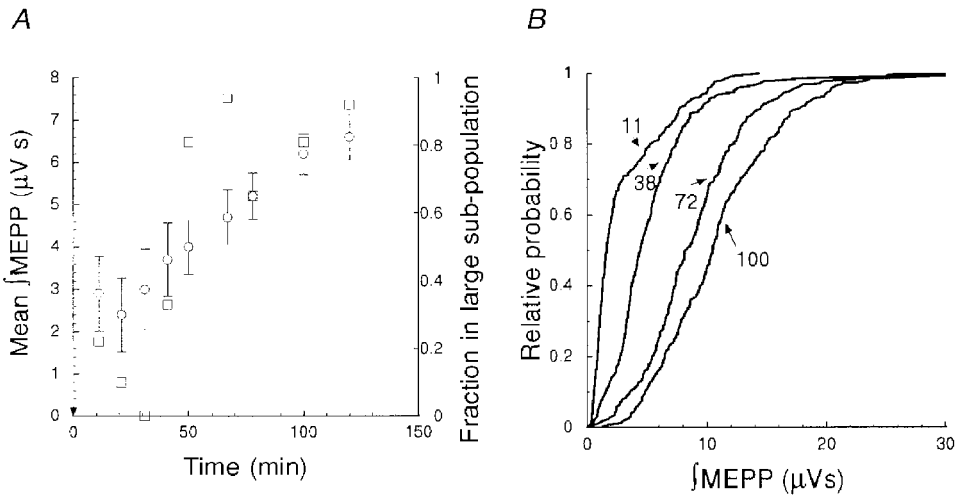

Figure 9. The recovery in ∫MEPP size at junctions in preparations pre-treated in 30 mM K^+ solution and then placed in gluconate Ringer solution without NH_4^+

A, mean ∫MEPP size (○) ±95% confidence limits, with the scale on the left vertical axis, and the fraction in the larger sub-population (□), with the scale on the right vertical axis. *B*, cumulative plots of the data from a second experiment. The time (min) in the NH_4^+-free solution at which recording of the data in each set finished is shown beside the curves.

solution. The paired preparations – untreated and exposed to elevated K^+ – released similar numbers of quanta in CCCP. Therefore, exposure to 30 mM K^+ solution does not appear to produce a detectable decrease in the releasable pool. A possible explanation for the recovery of quantal size in these experiments will be presented in the Discussion.

It follows from our picture of recycling that following the exposure to 30 mM K^+ solution + NH_4^+, a second exposure to the elevated K^+ solution without any inhibitor should substantially increase quantal size, by the formation of recycled vesicles containing a larger amount of ACh. The experiments to test this idea required the use of a different protocol. A preparation was placed in gluconate Ringer solution. One hundred ∫MEPPs were recorded from each of five randomly selected junctions. Then the preparations were exposed to 30 mM K^+ solution + 20 mM NH_4Cl for 20 or 30 min. After return to gluconate Ringer solution, a second data set was obtained. Finally the preparations were exposed to 30 mM K^+ solution (without inhibitor) for 30 min and a final data set was recorded. The exposure to the elevated K^+ solution + NH_4^+ produced a substantial decrease in the mean ∫MEPP (Fig. 10). However, the second exposure to elevated K^+ did not significantly increase the size of the ∫MEPPs. A considerable number of experiments were performed attempting to improve recovery by including substrates in the bathing solution: glucose, choline, lactate and glutamine. Since none of these substances produced a detectable effect, the data will not be reported in detail. To give one example, the ratio of the size of the ∫MEPPs after the treatment to the initial ∫MEPPs in the controls was $0{\cdot}58 \pm 0{\cdot}29$ ($n = 5$), while in those kept in the solutions containing 10 μM choline the ratio was $0{\cdot}51 \pm 0{\cdot}17$ ($n = 3$). The entire matter of recovery following the diminution in quantal size remains moot.

Inhibitors of v-ATPase

Two antibiotics are known inhibitors of the v-ATPase that is responsible for the acidification of the interior of the synaptic vesicles: concanamycin A and balifomycin A (reviewed by Parsons *et al.* 1993). Preparations were soaked for 30 min in gluconate Ringer solution containing 1 μM of one of the inhibitors. Then ∫MEPPs were measured from several junctions in each preparation. Next the muscles were soaked for 20 min in 30 mM K^+ solution + 1 μM antibiotic, returned to gluconate Ringer solution, and ∫MEPPs measured again from several junctions. The controls were indistinguishable from those in the experiments with other inhibitors already reported. The exposure to elevated K^+ solution + antibiotic increased the CVs substantially: CV with balifomycin A = $0{\cdot}56 \pm 0{\cdot}10$ ($n = 17$), CV with concanamycin A = $0{\cdot}72 \pm 0{\cdot}13$ ($n = 18$). Following the treatment with balifomycin A, 29% of the junctions studied appeared to have two sub-populations of quantal sizes; with concanamycin A it was 33%. In those examples that appeared to have two sub-populations, the fraction with the larger mean was $0{\cdot}82 \pm 0{\cdot}09$ for balifomycin A and for concanamycin A it was $0{\cdot}41 \pm 0{\cdot}24$.

DISCUSSION

The hypothesis we set out to test electrophysiologically comes directly from Betz & Bewick (1992, 1993), who followed the movement of cellular membranes with FM1-43. They concluded that recycled vesicular membrane is formed into new vesicles, which mix into a pool of releasable vesicles. All vesicles in the pool appeared to have a similar probability of release.

To test this hypothesis it is essential to estimate the number of vesicles released during the experiments. We elevated F_{MEPP} by depolarizing the terminals in solution containing 30 mM K^+. By measuring F_{MEPP} from many penetrations during the K^+ exposure, we estimated the mean number of quanta released as a function of time. Natural variation from junction to junction means that there is considerable spread in the estimates of the number released. We utilized a method for determining whether sets of ∫MEPPs are fitted best by one or by two lognormal probability distributions. When they are fitted by two, we can test whether this is because the hypothesis that they are made from two is correct, rather than that the fit is improved simply by the addition of more fitting parameters. These methods appear to work well, but again there is statistical scatter when the measurements are repeated at the same junction. To counter the variance just described, we tested the hypothesis by conducting substantial numbers of experiments. The validity of our interpretations relies on the statistical methods employed. The testing of these methods is a major component of this paper. There is considerable natural variation in the extent of the response to 30 mM K^+ solution, the sizes of the ∫MEPPs and the like. Although the data are in accord with the simplest interpretation, as will be explained next, the variability might obscure the operation of more complicated kinetic schemes for vesicle recycling.

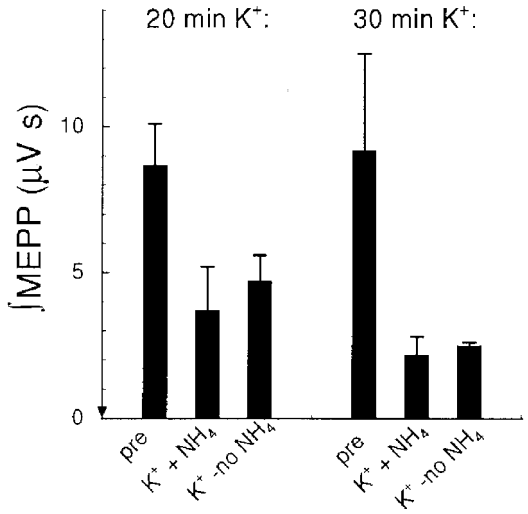

Figure 10. Quantal size does not increase following a second exposure to 30 mM K^+ solution that does not contain NH_4^+ to inhibit ACh accumulation

The results with two different K^+ exposure times are shown. The error bars show the +95% confidence limits.

The results agree with the idea that when vesicles are recycled in the presence of an inhibitor of ACh loading into synaptic vesicles, then quanta of larger and smaller sizes are both released. The inability of earlier workers, summarized in the Introduction, to see the two sizes in the frog is readily understood by looking at the sample distribution in Fig. 1*B*. The separation into two size classes is difficult to detect by eye.

As release and recycling continue the fraction of the quanta falling into the larger size category progressively decreases, probably along an exponential curve. When we used the fit to an exponential to calculate the total number of vesicles in the releasable store, the results were in agreement with the estimates of the size of the store released by CCCP. This suggests that the assumptions made were reasonable. The detection of the two classes of quantal sizes puts the frog in line with the mammal (Searl *et al.* 1991) and the reptile (Prior, 1994), where the sub-populations appear more distinct. In these other vertebrate classes, there are no measurements to indicate whether the smaller, recycled quanta mix into the pool with a similar probability of release. In the mammal, it also seems that the concentrations of VES used in the present experiments can completely block refilling (Searl *et al.* 1991).

CCCP appears to release almost all of the vesicles in the nerve terminal and to block membrane recycling for the formation of new synaptic vesicles (Molgó & Pecot-Dechavassine, 1988), though morphological evidence shows that once again a fraction of the junctions behave differently, retaining substantial numbers of vesicles after an hour in CCCP. The mechanism by which CCCP elevates F_{MEPP} has not been clearly established. By collapsing the potential across the mitochondrial membrane, CCCP releases Ca^{2+} from the mitochondria (David *et al.* 1998). It also reverses the direction of ATP synthase operation, so that the enzyme rapidly transforms ATP into ADP, thereby stopping ATP-driven cellular ion pumps. In neurons, the removal of ATP seems to be responsible for enhanced quantal release (Budd & Nicholls, 1996). The evidence for this conclusion is that enhanced transmitter release is delayed when the preparation is first treated with the ATP synthase inhibitor oligomycin. Inhibiting ATP synthase stimulates ATP production by glycolysis and prevents the enzyme from running in reverse after the proton gradient is diminished. We compared the time course of elevated quantal release in the presence of CCCP in paired preparations, one first exposed for 10 min to oligomycin. There was no consistent difference in the time courses (data not shown). The mechanism by which CCCP enhances F_{MEPP} is worth further investigation. It is relevant for models of vesicular exocytosis that require ATP in a step close to exocytosis. Presumably, the CCCP also collapses the proton gradient required for ACh accumulation in the vesicles, though this remains to be established.

Two antibiotics that block the v-ATPase found in the membrane of the vesicles – concanamycin A and balifomycin A – decrease the amount of ACh incorporated into the recycling quanta. This is just what would be expected from the effects of the other inhibitors. The effect of the antibiotics was much like those of NH_4^+. This suggests that the major action of NH_4^+ is the diminution of the proton gradient across the membrane of the synaptic vesicles. Therefore, any changes that NH_4^+ might produce in other cellular compartments are probably immaterial to the changes in quantal size. Decreases in axoplasmic pH have other effects: intracellular acidification reduces endocytosis at lizard neuromuscular junctions (Lindgren *et al.* 1997).

During exposure to HC3, VES, NH_4^+ or the antibiotics, quanta continue to be formed; they contain 20–30 % of the usual content of ACh. With the HC3 treatment, one might suppose that some ACh synthesis continues with a supply of choline from sources other than that recycled by the high affinity choline transporter. With NH_4^+, it seems possible that the synaptic vesicles still maintain some proton gradient across their membrane, sufficient for reduced ACh accumulation. It is harder to account for the data in VES. The K_i for VES in isolated synaptic vesicles is 50 nM. VES is lipid soluble and should have no difficulty penetrating the nerve terminal. At 10 μM VES is no more effective than at 2 μM. Consequently, it is difficult to believe that the higher concentrations of VES did not block active ACh uptake almost completely. It seems likely therefore, that 20–30 % of the ACh packaged into the quanta is trapped by a mechanism that does not depend on a proton gradient and which is not blocked by VES. The available evidence gives no indication about how this partial filling mechanism works.

If recycling vesicles mix into the releasable pool with an equal probability of release, then the obvious way to promote recovery of quantal size following a period of enhanced F_{MEPP} in the presence of an inhibitor of vesicle loading is to promote a second period of enhanced F_{MEPP} in the absence of the inhibitor. In these experiments, recovery did not occur. Possibly exposure to elevated K^+ for periods of many minutes damages the nerve terminals so that they lose the capacity to form recycled vesicles with normal ACh contents. In experiments on snake junctions exposed to elevated K^+ and elevated Ca^{2+}, prolonged exposure reduced the F_{MEPP} and the turnover of FM1-43 (Calupca *et al.* 1999). The declines are less when oligomycin is present, to stimulate glycolysis, or when glucose is added. In the affected preparations the mitochondria were swollen and distorted. The recovery experiments may be distorted by deterioration of the nerve terminals produced by the experimental protocols. This important question deserves further attention.

Enhancing F_{MEPP} in the presence of the inhibitors of vesicular ACh uptake decreased the size of the ∫MEPPs

substantially, presumably because the recycled vesicles contained substantially less transmitter. According to Jones & Kwanbunbumpen (1970) such treatment employing HC3 as the blocker leads to a marked decrease in the sizes of the synaptic vesicles in the rat motor nerve terminals. This provocative observation should be tested on frog terminals in which quantal content has been diminished by the treatments used in the present paper. Recent work at a glutaminergic synapse shows that altering the size of the vesicles, by manipulating the formation of the clathrin coat, changes their transmitter content (Zang *et al.* 1998).

A popular hypothesis is that there is an equilibrium between the concentrations of ACh in the vesicles and the cytoplasm which establishes the concentration of transmitter in the vesicle (Large & Rang, 1978; Williams, 1997). Experiments with the false transmitter precursor monoethylcholine argued against such equilibrium (Naves *et al.* 1996). The simultaneous existence of two sub-populations of releasable quanta with different ACh content also argues strongly against equilibrium. Some other mechanism must be employed to set the amount of transmitter incorporated into each vesicle.

We are beginning to see how vesicular membrane is recycled, new vesicles are formed and how they acquire their content of ACh. Since, as just discussed, the vesicles in the pool appear to retain their charge of ACh until released, it appears they are loaded at a distinct step before they enter the pool. With glutaminergic synaptic vesicles, there is also evidence for loading with transmitter at an early step in their life history (Prior & Claque, 1997). As pointed out above, a fraction of the loading does not appear to require a proton gradient or to be inhibited by VES. The greater part of the ACh accumulation appears to be mediated by the VES-inhibited ACh transporter and to require the proton gradient. Probably two protons from within the vesicle exchange for each ACh entering (Nguyen *et al.* 1998). As mentioned above, the mechanism that determines when the vesicle is filled is uncertain. Over-expression of the vesicular transporter leads to a substantial increase in quantal size (Song *et al.* 1997).

The filled vesicles enter a pool from which all appear to have at least a roughly equal probability of release. The corollary to this conclusion is that release probability does not depend on quantal size. This conclusion is based on substantial numbers of experiments, which are required to avoid being misled by the idiosyncratic behaviour of some of the individual junctions. Presumably, random movements of the vesicles within the pool determine which of them are available for binding at the active zones on the pathway for release. Once positioned at the active zones, the vesicles can gain additional ACh by a process called second-stage loading (Doherty *et al.* 1984; Van der Kloot, 1991; Van der Kloot & Molgó, 1994; Naves & Van der Kloot, 1996). Second-stage loading may account for the progressive increase in quantal size at resting junctions after the removal of the inhibitor.

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