Physiology in Press; published online on May 3, 2002 as 10.1113/jphysiol.2001.014407

Journal of Physiology (2002), **xxx.x**, *pp.* 000–000
DOI: 10.1113/jphysiol.2001.014407

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Vesicle size and transmitter release at the frog neuromuscular junction when quantal acetylcholine content is increased or decreased

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We investigated whether the synaptic vesicles at the neuromuscular junction change size when their acetylcholine (ACh) content is altered. The size of the miniature endplate potential (MEPP) increased 3- or 4-fold in preparations pre-treated in a hypertonic solution in which the anion was gluconate. We measured the dimensions of synaptic vesicles in such preparations and in controls. The size of the vesicles and size distribution were indistinguishable. Quanta contained about half of the usual amount of ACh in preparations stimulated in the presence of hemicholinium-3, an inhibitor of choline uptake, or in NH_4^+, which diminishes the proton gradient for ACh uptake into the vesicles. Neither treatment changed the size of the synaptic vesicles. ACh content and vesicle size were both decreased in preparations stimulated in (−)-vesamicol, an inhibitor of ACh uptake in vesicles. Since the other inhibitors decreased ACh content by a similar amount without altering vesicle size, (−)-vesamicol may decrease vesicle size by acting on another target. We also found that a hypertonic solution in which the anion was aspartate increased quantal size similar to gluconate. Both anions have high hydration energy and a large volume. When these treatments increased quantal size the mean 20–80 % rise time of MEPPs recorded with an extracellular electrode was 170 μs. In the controls it was 97 μs. Perhaps some of the added ACh is bound within the vesicles, which slows the rise. Our major conclusion is that ACh content can change notably without any change in the size of the synaptic vesicles.

(Received 27 November 2001; accepted after revision 14 February 2002)
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The size of spontaneously generated miniature endplate potentials (MEPPs) or currents (MEPCs) can vary by an order of magnitude depending on experimental treatment. The size decreases transitorily following repetitive stimulation (Naves & Van der Kloot, 2001); the decrease is sustained when the stimulation is performed in the presence of inhibitors of acetylcholine (ACh) uptake into synaptic vesicles (Van der Kloot *et al.* 2000). The size increases roughly 2-fold following treatment with any of a number of hormones, because more ACh is released in the quanta (Van der Kloot & Van der Kloot, 1986; Van der Kloot *et al.* 1998). The common pathway in the action of most of these hormones is the activation of protein kinase A in the motor nerve terminals (Van der Kloot & Branisteanu, 1992).

Size also doubles in preparations that are soaked in a hypertonic solution and then returned to normal Ringer solution for the measurements (Van der Kloot, 1987). The increase in quantal size produced by hypertonic pre-treatment can be blocked by (−)-vesamicol, an inhibitor of the vesicular ACh transporter, hemicholinium-3, an inhibitor of the choline uptake system in the nerve terminal, or by NH_4^+, which reduces the proton gradient for ACh uptake into the vesicles (Van der Kloot, 1987, 1991). The size increases can also be blocked by intracellular bromoacetylcholine, which blocks ACh synthesis by inhibiting choline acetyltransferase, or by intracellular acetylcholinesterase, which presumably depletes the terminal of the ACh that is loaded into the vesicles (Brailoiu & Van der Kloot, 1996). The response of the endplate to applied ACh is unchanged by the pre-treatment, and the density, conductivity and mean open time of the endplate ACh channels are unaltered (Van der Kloot, 1987; Yu & Van der Kloot, 1991). We conclude therefore that quantal size increases because more ACh is released per quantum. In this regard it is worth noting that quantal size in developing *Xenopus* neuromuscular synapses in culture roughly doubles following expression of a putative vesicular ACh transporter (Song *et al.* 1997).

The hypertonic treatment activates protein kinase A. The links connecting hypertonic treatment to enzyme activation and enzyme activation to increased ACh

loading remain to be elucidated (Van der Kloot & Branisteanu, 1992). Quantal size can increase by as much as 4-fold when the hypertonic solution is made with gluconate as the anion (Van der Kloot, 1987). Quantal size can also be altered experimentally in a variety of other preparations including synapses in the CNS (reviewed by Sulzer & Pothos, 2000; Engel *et al.* 2001). It is likely that changes in the size of the quanta contribute to the regulation of synaptic function.

If the size of the synaptic vesicles and their ACh content are correlated, it should be possible to detect an increase in vesicle size when the ACh content is increased substantially. However, preliminary experiments showed no change in vesicle size (Spielhotz & Van der Kloot, 1987). After this work was done it was found that only the vesicles in the releasable pool receive the additional ACh, so simply measuring the size of synaptic vesicles in random sections of motor nerve terminals might result in a subpopulation of enlarged vesicles being missed (Van der Kloot, 1993). Measurement of the dimensions of large numbers of synaptic vesicles in all parts of the motor nerve terminal is the crux of the present paper. We detected no change in synaptic vesicle size despite the increase in ACh content. We discuss how the ACh might be stored in the vesicles and how this might account for the lengthening of the rise times of the enlarged quanta. We also measured the size of small clear synaptic vesicles in preparations stimulated in the presence of hemicholinium-3, NH_4^+ or (−)-vesamicol. Vesicle size was decreased only by (−)-vesamicol, therefore quantal size can also be reduced without a change in vesicle size.

We also include data on two other points. Using an extracellular electrode, we measured the rise times of the spontaneously generated endplate currents after the enlargement in size; this had not been accurately measured in our previous work due to inadequate compensation for the electrode capacitance (Van der Kloot, 1995; Van der Kloot & Naves, 1996; Stiles *et al.* 1998). When the quanta contained more ACh the rise time was lengthened. In passing we report that when aspartate was used as the anion in the hypertonic pre-treatment it acted like gluconate. The two anions share physical properties that may be related to their action on the neuromuscular junction.

METHODS

Frogs (*Rana pipiens*) were killed by double pithing, as specified by the State University of New York at Stony Brook Animal User's Committee. Sartorius or cutaneous pectoris nerve–muscle preparations were dissected in Ringer solution containing (mM): NaCl, 120; KCl, 2.0; $CaCl_2$, 2.5; *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid/NaOH (Tes) 4.0 (pH 7.4). Hypertonic solutions contained 200 mM sodium gluconate or 200 mM sodium aspartate in place of NaCl with the other constituents unaltered. In previous experiments involving treatments that diminish MEPP size we used gluconate Ringer solution in which Cl^- was replaced by gluconate$^-$, because an impermeant anion increases MEPP size by increasing the input resistance of the muscle fibre (Van der Kloot *et al.* 2000). This solution contained (mM): sodium gluconate, 120; KCl, 2.0; $CaCl_2$, 2.5; Tes, 4.0; it was used for some of the morphological work in this study. High potassium gluconate solution contained (mM): sodium gluconate, 92; potassium gluconate, 30; $CaCl_2$, 2.5; Tes, 4.0.

Voltage–time integrals of the MEPPs ($\int$MEPPs) were measured in Ringer solution supplemented with 3 μM neostigmine methylsulfate and 0.03 μM tetrodotoxin to block spontaneous contractions. Extracellular MEPPs were recorded in the same solution but without neostigmine.

For morphology with enlarged quantal size cutaneous pectoris muscles were soaked for 2 h in hypertonic gluconate solution and then returned to Ringer solution for 5 min. They were then 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. Control muscles were kept in Ringer solution for the entire period until fixation. For observations under conditions in which quantal size decreased, one of the paired muscles was kept in gluconate Ringer solution while the other was soaked in high potassium gluconate solution containing hemicholinium-3, (−)-vesamicol or NH_4^+ at the concentrations given in the text. Hemicholinium-3 and (−)-vesamicol were obtained from RBI (Natick, MA, USA). After 1 h the muscles were fixed as described above. 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 using a Hitachi H-500 electron microscope. Electron micrographs of neuromuscular junctions (× 20 000) were digitized using an AGFA Duoscan T1200 scanner. Vesicle size was measured using NIH Image software. Many of the vesicles appeared to be elliptical rather than circular. Therefore the operator measured the longest diameter (d_1) and the diameter at right angles (d_2). After measurement of the second diameter, the program marked the vesicle so that it would not be re-measured. The calculation used was:

$$\text{circumference} = 2\pi[(d_1^2 + d_2^2)/2]^{0.5}.$$

The *x*–*y* coordinates of each vesicle in the terminal were also recorded. We did not correct the measurements to take into account how the thickness of the section determines the probability of where a vesicle will be cut through (Feuerverger *et al.* 2000). The corrections are relatively small and our goal was to compare the size of the vesicles in the control with that in the experimental preparations. Moreover, it turns out that in almost every instance both would have been corrected to the same extent.

To test whether the size of nearby vesicles was correlated, as would be expected if those in the releasable pool were large and near one another while those in the reserve pool were small and in different parts of the terminal, we used the following version of nearest neighbour analysis. Starting with the first vesicle (vesicle$_1$) measured in the terminal, two sets of calculations were done. The distance of vesicle$_1$ from all other vesicles in the section (vesicle$_n$) was calculated from the *x*–*y* coordinates. The differences-squared between the size of each of the pairs of vesicles were calculated as (circumference vesicle$_1$ − circumference vesicle$_n$)^2. The set of differences-squared measurements was then sorted into an array in which the index was determined by the distance between the vesicles, so that the closest vesicle was at index = 1 and the furthest

vesicle at index = n. The calculations were repeated in turn with each of the vesicles in the section and the resulting sorted differences-squared values were added to those calculated previously. The procedure was then continued on the next section. To determine whether there was any correlation between the mean summed differences-squared and the relative distance from the starting vesicle, we calculated the Spearman rank-order correlation coefficient, which is a non-parametric method for assessing correlation.

MEPP size was measured from paired muscles from the same frog using a protocol that has been tested extensively previously and shown to be reliable (Van der Kloot, 1987). MEPPs were recorded using bevelled intracellular glass microelectrodes filled with 3 M KCl with DC resistances between 2 and 4 MΩ. The pipette was connected to an Axoclamp-2A amplifier (Axon Instruments, Union City, CA, USA), the signal from which was passed to an Axon Instruments CyberAmp 300 signal conditioner set to amplify over a bandwidth of 0.1–1000 Hz. The signal emerging from the CyberAmp was split, one branch going to an Axon Instruments AI 2020 event detector, and the other to a ComputerBoards DAS-16 330 A/D converter (Mansfield, MA, USA), which stored a pre-determined number of data points before and after the occurrence of the trigger. When a MEPP passed a threshold set just above the noise level, the event detector delivered a transistor–transistor logic (TTL) pulse to the A/D board, which digitized the pulse at 10 kHz. The signal was observed by the operator, who rejected any with overlapping MEPPs or contaminated with electrical noise. The ∫MEPPs were measured by the computer and corrected to a standard resting potential of −90 mV (Katz & Thesleff, 1957). For each muscle about 100 MEPPs were measured from each of five randomly selected endplates. The statistical significance of the difference between the data sets from the paired muscles was determined by ANOVA on the logarithms of the ∫MEPPs (Van der Kloot, 1987).

Previously reported measurements of the 20–80 % rise times of externally recorded MEPPs (Van der Kloot, 1995; Van der Kloot & Naves, 1996) were inaccurate because of inadequate compensation for the capacity between the pipette and solution (Stiles *et al.* 1996, 1998). In the present experiments, recordings were made with a microelectrode of 0.5–2 MΩ DC resistance filled with 3 M NaCl. MEPPs were located by trial and error, by moving the electrode over the endplate region. The pipette and the bath electrodes were each connected to a separate WP 705 electrometer amplifier (World Precision Instruments, Sarasota FL, USA), which were used as differential inputs. The capacity compensation of both amplifiers was adjusted to a level just below oscillation. The output of the differential head stage was led to a CyberAmp 300 signal conditioner, which amplified the output over a bandwidth of 0.1–6000 Hz. The signals were detected as described above, but with the event detector set so that only the largest signals were above threshold. These were digitized at 10 kHz as described above. The 200 points before the TTL pulse and the 800 points after the pulse were stored by the computer. Later, each signal was observed so that noise pulses or overlapping MEPPs could be eliminated prior to estimation by the computer of the 20–80 % rise time of the signal.

For ∫MEPP size and vesicle circumference the confidence limits and the significance of differences between means were estimated by resampling (Van der Kloot, 1996). Differences are termed significant when $P < 0.05$.

RESULTS

Size of synaptic vesicles with enlarged quanta

The overall appearance of the electron micrographs of motor nerve terminals from control muscles and from paired muscles pre-treated in hypertonic gluconate solution was indistinguishable (Fig. 1). The distribution of the mitochondria and synaptic vesicles in the axoplasm was similar. The mitochondria in nerve terminals pre-treated in hypertonic gluconate solution appeared normal. The mean nerve terminal perimeter values were similar:

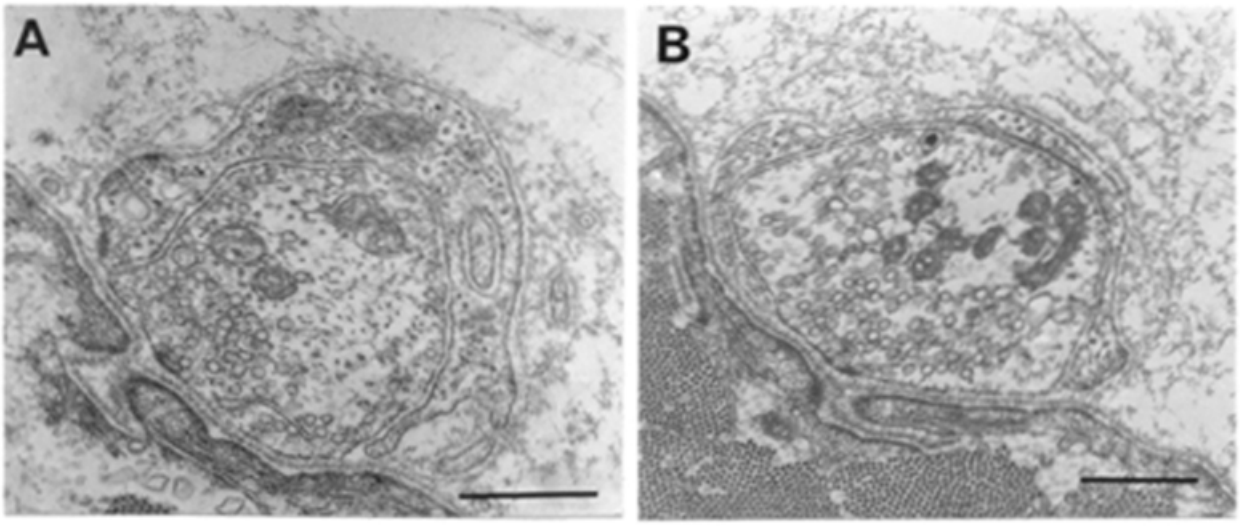

Figure 1. Electron micrographs of motor nerve terminals from control and experimental preparations

A, preparation soaked for 2 h in Ringer solution before fixation. *B*, preparation from the paired muscle from the same frog soaked for 2 h in hypertonic gluconate solution and then returned to Ringer solution before fixation. Calibration bars, 0.5 μm.

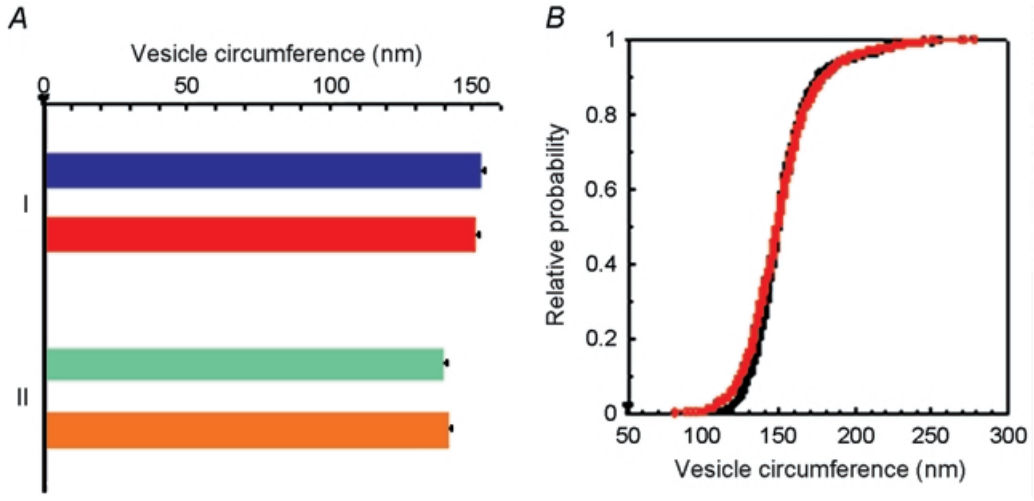

Figure 2. Two examples of the effects of pre-treatment for 2 h in hypertonic gluconate solution on the size of synaptic vesicles in the motor nerve terminal, compared to paired muscle that was untreated

A, mean circumferences; the error bars show the +95 % confidence limits. Preparation I: purple, untreated ($n = 510$); red, treated ($n = 870$). Preparation II: green, untreated ($n = 965$); orange, treated (n = 834). *B*, cumulative distribution of vesicle circumferences in preparation II. Black, untreated; red, treated.

5.62 ± 1.6 μm (95 % confidence interval) from 15 control profiles, and 5.38 ± 2.3 μm from 15 profiles from preparations pre-treated in hypertonic gluconate solution. The number of synaptic vesicles in the same profiles was also very similar: 161 ± 38 (95 % confidence interval) in the controls and 166 ± 26 in those pre-treated in hypertonic gluconate solution.

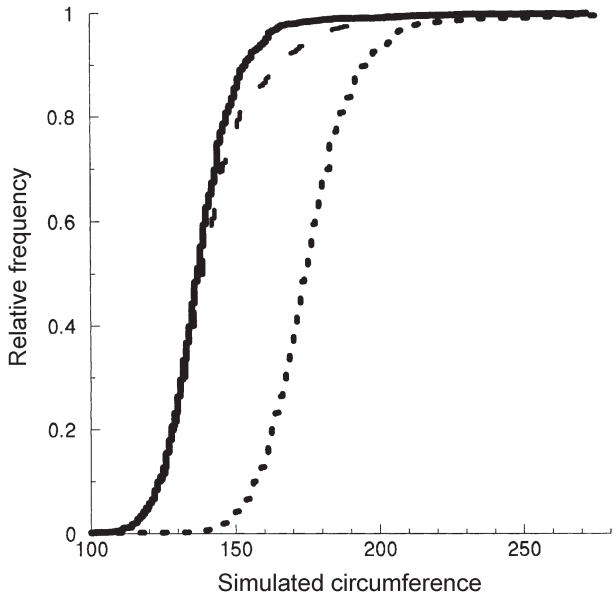

Figure 3. Examples of the simulated data sets

The continuous line shows the measurements of vesicle diameters from a preparation pre-treated in hypertonic gluconate solution. Dotted line, size enlarged by a factor of 1.27; dashed line, sizes of 10 % of the vesicles enlarged by a factor of 1.27.

We measured the size and position of synaptic vesicles in these preparations. We did not find any significant difference in the mean size of the vesicles (Fig. 2*A*). Plots of the cumulative distributions did not give any indication of a subpopulation of larger vesicles in the preparations with increased quantal size, as would be expected if the vesicles in the releasable pool were larger (Fig. 2*B*). The tests for a correlation between the size of vesicles with that of their near neighbours were also negative, and are not reported in detail here. In short, the increase in quantal ACh content produced by the pre-treatment in hypertonic gluconate solution was not accompanied by any change we could detect in the size of the synaptic vesicles.

Naturally we wanted to be sure that our measurements would detect differences in the size of the vesicles should they have occurred. We were especially concerned when the increase in ACh content in the vesicles was less than 4-fold and when only a fraction of the vesicles had an increased ACh content. To determine this we took the measurements of the circumferences of 965 synaptic vesicles from a control preparation. We used the computer to generate a second data set in which each synaptic vesicle was enlarged by a factor of 1.27 ($2.6^{0.33}$; the reason for choosing 2.6 is given later in Results). Examples of the altered data sets are shown in Fig. 3. Then we generated 1000 resampled data sets, each containing 965 values randomly picked from the control set and the same number from the enlarged data set. In each of the 1000 calculations, the mean of the enlarged data set was greater than the mean of the control set. We redid the sequence of calculations using smaller values of vesicle circumference and enlarging only a fraction of the number of vesicles in

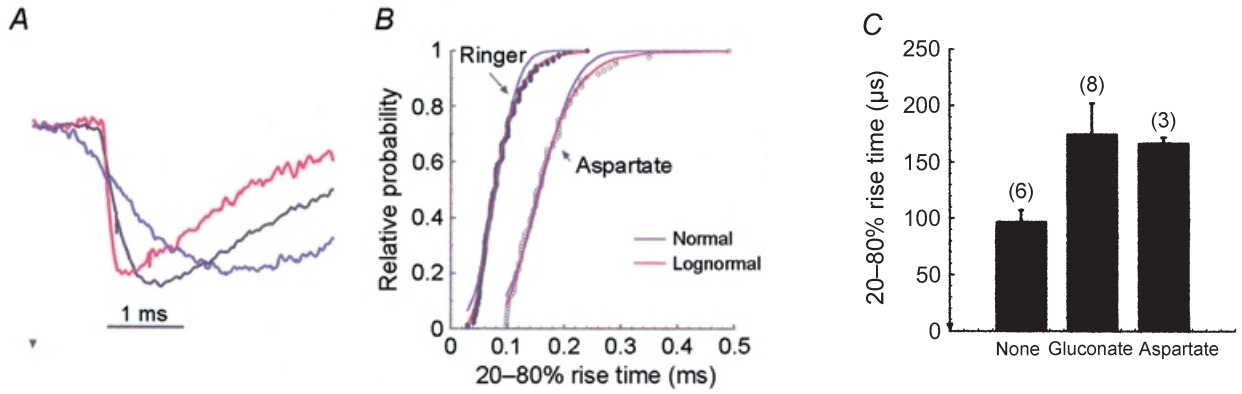

Figure 4. Pre-treatment in hypertonic gluconate or aspartate solutions increases the rise times of MEPPs recorded with an extracellular electrode

A, examples of MEPPs recorded from a preparation that had been soaked for 2 h in hypertonic aspartate solution and then returned to Ringer solution for the recordings. The MEPPs were normalized. Colours are used to help distinguish the traces. *B*, cumulative plots of the distribution of the 20–80 % rise times from paired muscles from the same frog. One muscle received no treatment (Ringer solution) while the other was kept for 2 h in hypertonic aspartate solution and then returned to Ringer solution for the recording. Purple curve: the best-fitting normal probability distribution function. Red curve: the best-fitting log normal probability distribution function. *C*, summary of the mean 20–80 % rise times in all of the experiments. The pre-treatments given are indicated below the bars. Error bars show the +95 % confidence intervals. The number of preparations studied is shown in parentheses above each bar.

the enlarged set. For instance with 250 vesicles in each of the two sets and with only 10 % of the vesicles in the second set enlarged, the mean of the enlarged set was greater than that of the controls in 999 instances out of 1000. These calculations strongly suggest that our measurements would have detected the postulated changes in vesicle size had they occurred.

Externally recorded MEPP rise times

When MEPPs were recorded with an extracellular electrode, which measures voltage changes caused by the inflow of current through the opened endplate channels (Fatt & Katz, 1952), there was considerable variation in the rise times of the signals, as shown by selected examples (Fig. 4*A*) and also in cumulative plots of all of the 20–80 % rise times from a junction (Fig. 4*B*). The mean 20–80 % rise time from junctions in untreated preparations was about 100 μs (Fig. 4*C*). The mean value reported by Stiles *et al.* (1998) was about 120 μs. Pre-treatment with either hypertonic gluconate solution or hypertonic aspartate solution significantly lengthened the rise time by about 70 % (Fig. 4*C*).

Incidentally, Fig. 4*B* also shows that the distribution of the 20–80 % rise times was better fitted by a lognormal probability distribution function than by a normal function. This was also true for the distribution of the $\int$MEPP sizes (Van der Kloot, 1987, 1991).

Size of synaptic vesicles with smaller quanta

The results we obtained with treatments that reduce quantal size are summarized in Fig. 5. Preparations were placed in high K^+ gluconate solution to enhance the rate of quantal release and an inhibitor of ACh accumulation in vesicles was added. These treatments have been shown to decrease $\int$MEPP size by about 50 % (Van der Kloot *et al.* 2000). In every instance, there was a significant decrease in vesicle size when (−)-vesamicol was used as the inhibitor. With hemicholinium-3, there was a slight but statistically significant decrease in one example and no detectable change in the other. NH_4^+ did not reduce vesicle size significantly, even though it was equally effective in

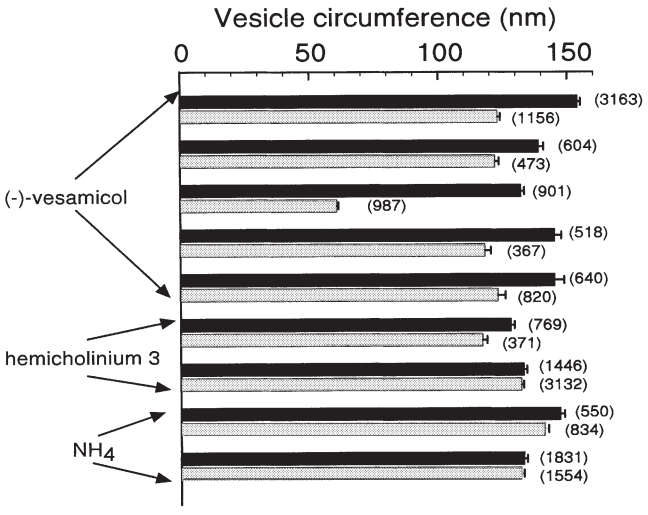

Figure 5. The size of synaptic vesicles in preparations treated so that the quanta contained less ACh

In each case the upper filled bar shows the control and the lower shaded bar shows the treated preparation. Error bars show the +95 % confidence intervals. The number of vesicles measured is given in parentheses. The concentrations of the inhibitors used were: 2 μM (−)-vesamicol, 1 μM hemicholinium-3 and 30 mM NH_4^+.

reducing ∫MEPP size.

Aspartate and gluconate

The reason why hypertonic solutions made with gluconate as the anion are so much more effective than solutions of the same tonicity made with any of a number of other anions is baffling (Van der Kloot, 1991). Smith *et al.* (1999), in quite a different context, plotted for 22 anions the absolute value of their hydration energies as a function of their equivalent sphere radii. Gluconate was at the extreme end of the distribution, with a high hydration energy coupled to a large equivalent sphere radius. Its nearest neighbour was aspartate, which to the best of our knowledge has never been tested for its effect on quantal size. In four experiments, we compared ∫MEPPs from muscles pre-treated for 2 h in 200 mM sodium aspartate solution with those from the paired muscle kept in Ringer solution throughout. After the aspartate treatment some of the MEPPs were strikingly large (Fig. 6*A*). The treatment shifted the distribution of ∫MEPP size to the right (Fig. 6*B*), and the increases in ∫MEPP size were statistically significant in all four instances.

The mean increase in ∫MEPP size compared to the controls was 2.7-fold (range 2.1- to 3.5-fold; note that we used a value of 2.6-fold for the increase in the calculations of the ability of our methods to detect comparable changes in vesicle size). This increase was less than the almost 4-fold increase in ∫MEPP size initially reported following pre-treatment in hypertonic gluconate solution (Van der Kloot, 1987). However, subsequent work revealed variations in the extent of the response to the hypertonic gluconate pre-treatment, which may be due to seasonal changes in the frogs (Van der Kloot & Branisteanu, 1992). To compare the effects of the two anions on quantal size, in six experiments we compared ∫MEPP size in paired muscles from the same frogs, each treated for 2 h in hypertonic solution, one made with gluconate and the other with aspartate and then returned to Ringer solution for recording. In none of the experiments was there a significant difference in the quantal size following the treatments (∫MEPP after gluconate/∫MEPP after aspartate = 1.1 ± 0.15 [95 % confidence interval]).

DISCUSSION

The major finding of this study was that the size of the synaptic vesicles is unchanged by a treatment that enlarges mean quantal size substantially. We know that this is not simply because fixing and embedding of the tissue for the electron microscope could result in vesicles of a uniform size, because vesicle size was reliably decreased by treatment with (−)-vesamicol. This result raises another question as vesicle size was not substantially decreased with either hemicholinium-3 or NH_4^+. The lack of effect with NH_4^+ is ambiguous, even if vesicles are smaller when they contain fewer molecules. The vesicles probably accumulate NH_4^+ in place of ACh^+, so NH_4^+ treatment may not substantially decrease the number of molecules within the vesicles (Van der Kloot, 1991; Van der Kloot & Molgó, 1994; Nguyen & Parsons, 1995; Nguyen *et al.* 1998). The results with hemicholinium-3 demonstrate that quantal ACh content can decrease substantially without altering vesicle size. It is possible that the effect of (−)-vesamicol on

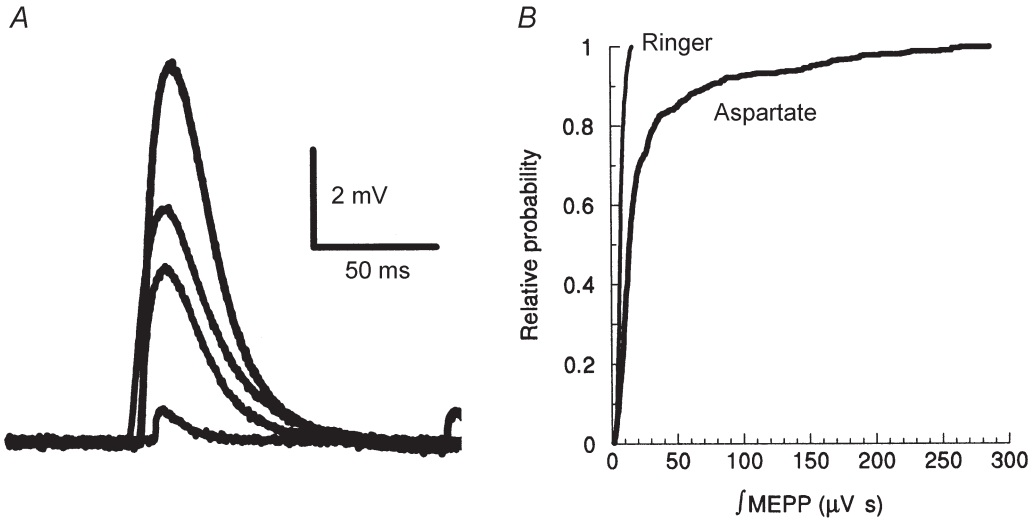

Figure 6. Pre-treatment in hypertonic aspartate solution increases the size of MEPPs recorded with an intracellular electrode

A, examples of MEPPs recorded from a preparation that had been soaked for 2 h in hypertonic aspartate solution and then returned to Ringer solution for the recordings. *B*, cumulative plots of the distribution of ∫MEPPs from paired muscles from the same frog. The distribution on the left is from the muscle kept in Ringer solution throughout; the distribution on the right is from the muscle pre-treated in hypertonic aspartate solution.

vesicle size occurs because it has a target in the motor nerve terminal other than the ACh transporter in the vesicle membrane. In this regard Jones & Kwanbunbumpen (1970) decreased vesicle size at the rat neuromuscular junction with repetitive nerve stimulation in the presence of hemicholinium-3, using concentrations of the drug that were substantially higher than that required to block choline uptake. These results again suggest the possibility of a second target of action by the drug.

Estimates of the number of ACh molecules in a quantum at the neuromuscular junction range from 8000 to 10000 (Van der Kloot & Molgó, 1994). Taking the size of the vesicles measured here and assuming that the membrane surrounding the vesicles is about 5 nm thick, we estimate that the ACh concentration in the vesicle interior, if it were in free solution, would be about 0.5 M.

Pre-treatment with hypertonic gluconate or aspartate solutions increases ∫MEPP size substantially, by increasing the ACh content of the quanta. We do not know enough about the relation between the size of the endplate response and the quantity of ACh released to assert that after treatments that increase the mean ∫MEPP 4-fold, the concentration of ACh if it were in free solution would be 2 M, but it must be well above the 0.5 M normally present.

The vesicles are large enough to contain such quantities of ACh (reviewed by Van der Kloot, 1991). Some of the anion that must be present could come from negative charges on the phospholipids on the inner face of the vesicular membrane, from glycosaminoglycans held in the vesicle, and from the ATP known to be contained in the vesicles; however, for cholinergic vesicles other than those from *Torpedo* electric organ the 'balance sheet' has not been worked out in detail (reviewed by Van der Kloot & Molgó, 1994). We do not know what physical state ACh is in within the synaptic vesicles at the frog neuromuscular junction.

The treatments that enlarge quantal size also extend the rise times of the miniature endplate currents. Their durations are also lengthened, although there is no lengthening of the mean open time of the ACh-operated endplate channels (Van der Kloot, 1987, 1991; Van der Kloot & Naves, 1996). It is by no means obvious how this occurs. The usual picture is that a fusion pore opens between the vesicle interior and the synaptic cleft, and the ACh diffuses out down its concentration gradient (Stiles *et al.* 1996; reviewed by Van der Kloot & Molgó, 1994). If the concentration inside is increased, the rate of diffusion out through the pore will increase proportionally, and there should be little change in the rise time of the endplate current (Stiles *et al.* 1996). If vesicle size increased with the rise in ACh content, so that the concentration of ACh remained constant, then release through the pore would take longer and the endplate current rise time would be lengthened (Van der Kloot & Naves, 1996). Since vesicle size does not increase when there is more ACh within the vesicle, this explanation for the lengthening of the rise time appears untenable. One possibility is that a substantial fraction of the ACh within the vesicle is bound to non-diffusible anions. When the pore opens the free ACh will diffuse out. As it leaves, it would be replenished by ACh released from the bound form; thereby the concentration of ACh would remain relatively constant during the release period. Accordingly, with more ACh initially present release would be prolonged and the rise time lengthened. Binding might also eliminate differences in the osmotic pressure between the vesicle interior and the axoplasm, though it has not been established that osmotic differences would actually create any problem in a structure the size of a vesicle (Johnson & Buttress, 1973; reviewed by Van der Kloot, 1991). Secretory vesicles contain complexes that bind the substances released by exocytosis (Nanavai & Fernandez, 1992), but there is no evidence for such binding in small clear vesicles. The storage of ACh in the vesicles requires further investigation.

Another unsolved problem raised by the results is what determines the amount of ACh stored in a vesicle The simplest idea is that ACh accumulates until its concentration reaches a set level (Williams, 1997). Such a picture now seems unlikely for these cholinergic vesicles because quantal size increases without any increase in vesicle diameter, therefore it appears that the concentration of ACh in the vesicles is elevated.

The discovery that the effect of hypertonic aspartate solution on quantal size is similar to that of hypertonic gluconate solution provides a clue about the physical properties of the anions that underlie their exceptional effects. However, the identity of the receptor for hypertonicity, and its link to protein kinase A, is still unknown, so this is only a first step in solving the puzzle.

Our results suggest that the relation between transmitter content and vesicle size is quite different in cholinergic nerve terminals from that in other systems leading to the release of other neurotransmitters. For instance, in a glutamatergic system, Zhang *et al.* (1998, 1999) studied a mutation in *Drosophila* that eliminates an accessory protein (LAP, an AP180 homologue) involved in the formation of the clathrin coat used in the endocytosis and reformation of synaptic vesicles. The mutants have larger synaptic vesicles as well as a larger quantal size. Bruns *et al.* (2000) used amperometry to measure the quantal release of serotonin from leech Retzius neurons. There are two classes of release, which differ in their size, rate and site. There are also two distinct classes of synaptic vesicles: clear vesicles and larger dense core vesicles. These authors calculated that the two types of vesicles contain similar concentrations of serotonin, and concluded that the differences in the amount of transmitter released are

determined by the size of the vesicles. In rat pheochromocytoma (PC12) cells L-DOPA increases the number of catecholamine molecules in the quanta, but not their concentration (Kozminski *et al.* 1998).

The first demonstration that the amount of transmitter in a vesicle can alter its volume was recently reported by Colliver *et al.* (2000), who showed that L-DOPA increases the size of the dense core vesicles in PC12 cells, while reserpine reduces transmitter content and decreases the size. The picture at motor nerve terminals appears to be quite different, since increases in quantal size did not statistically change the size of the clear synaptic vesicles. There is some evidence that small vesicles do not respond to changes in osmotic pressure in the same way as large vesicles do, but this is a subject that requires more investigation (Johnson & Buttress, 1973; reviewed by Van der Kloot, 1991).

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Acknowledgements
This study was supported by grant 10320 from the National Institute of Neurological Disorders and Stroke, CONICIT-6-97003895-Venezela and by a travel grant from NATO.