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INCREASING QUANTAL SIZE AT THE MOUSE NEUROMUSCULAR JUNCTION AND THE ROLE OF CHOLINE

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SUMMARY

1. In frog a variety of treatments have been shown to increase quantal size at the neuromuscular junction (NMJ), apparently by releasing more acetylcholine (ACh) per quantum. The present experiments were undertaken to see whether similar changes occur at the mouse NMJ.

2. None of the hormones tested, adrenaline, insulin or corticosteroids, significantly increased quantal size at the mouse NMJ.

3. Soaking diaphragms in hypertonic solution (about 475 mosmol/kg) for 15–30 min roughly doubled the size of miniature endplate potentials (MEPPs), miniature endplate currents (MEPCs), and uniquantal endplate potentials (EPPs). We will refer to these as 'large quanta'. Note that all of the measurements were made after returning the preparation to normal (Tyrode) solution.

4. In frog hypertonic solution made with sodium gluconate replacing NaCl increases quantal size four- rather than two-fold. In mouse there was little difference in the effects of solutions made with the two anions. In Cl^--free hypertonic solution, made with sodium gluconate and SO_4^{2-} solutions, quantal size increase is somewhat less, so there may be a role for Cl^- in enlarging quantal size.

5. After hypertonic treatment, quantal size remained elevated for at least 1 h and then gradually declined back to usual levels. The data suggest a gradual decrease in mean quantal size, rather than the appearance of a new subpopulation of smaller quanta.

6. Hypertonic treatment did not change the endplate depolarization in response to ionophoretically applied ACh. This suggests that the increased quantal size is not due to a postsynaptic change. Large MEPP's disappear in the presence of tubocurarine and reappear when the drug is washed away.

7. Vesamicol is an inhibitor of active ACh uptake into isolated synaptic vesicles. 5 μM-vesamicol has no detectable postjunctional effect. However, when vesamicol was included in the Tyrode and the hypertonic solutions the increase in quantal size was blocked. This is further evidence that the large quanta are produced by the release of more ACh per quantum.

8. Even when added after the hypertonic treatment, vesamicol soon decreased quantal size back to the normal level. Two other inhibitors of active ACh uptake into vesicles, tetraphenylboron (TPB) and hexanitrodiphenylamine (HNPA) also de-

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creased quantal size after hypertonic treatment, apparently by a presynaptic action. This suggests that the additional ACh that produces large miniatures may be incorporated into the quanta shortly before release.

9. Experiments were undertaken to test the hypothesis that the additional ACh added to the quanta is newly synthesized transmitter, produced by the acetylation of choline brought into the terminal by the high-affinity choline transport system (HAChT). The formation of large quanta was blocked when the solutions contained hemicholinium-3 (HC-3), an inhibitor of HAChT. At the concentrations used, HC-3 had no notable effect on the postjunctional acetylcholine receptor (AChR). After quantal size was increased, HC-3 decreased quantal size, after a delay of about 15 min.

10. The formation of large quanta was blocked when the solutions contained the anticholinesterase neostigmine, which would prevent the hydrolysis of ACh and therefore cut off the supply of choline, and when the solutions were made with Li^+ in place of Na^+. Na^+ is required for high-affinity choline transport into neurons.

11. The formation of large quanta was blocked by treatments that would raise the intracellular [Na^+], like dihydroouabain, K^+-free solution and aconitine. Size increase was also blocked by elevated K^+-solution, even when the solution did not contain Ca^{2+}. The depolarization of the terminal membrane by the elevated K^+ solution would decrease the Na^+ gradient. The inhibition by K^+-free solution or dihydroouabain was partially overcome by adding choline to the extracellular solution.

12. When monoethylcholine (MECh) was included in the solutions, there was less increase in quantal size, and after treatment the MEPCs decayed more rapidly. It is known that MECh is taken into nerve terminals and made into acetylmonoethylcholine (AMECh); the AMECh opens the AChR channels for a shorter time than ACh, so the presence of the false transmitter can be detected by the shortening of the MEPCs.

13. It is concluded that hypertonic treatment probably causes the addition of ACh to quanta shortly before they are released, by a still unknown mechanism. This additional ACh is recently synthesized from choline that has been brought into the terminal by the HAChT. It is clear that in mouse as in frog quantal size can be experimentally shifted over an appreciable range. We suggest that such changes in quantal size are likely to play a role in the operation of the nervous system.

INTRODUCTION

Quantal transmission is likely to prove the rule at chemical synapses, and many of the plastic changes in the functioning of the nervous system are undoubtedly due to the regulation of the number of quanta released by presynaptic stimulation. There is also the possibility that some plastic changes might be produced by alterations in the size of the individual quanta, but this concept is just beginning to be explored. The neuromuscular junction (NMJ) is an especially favourable preparation for studying variations in quantal size, just as it is for studies on the regulation of quantal output (Katz, 1969).

At the NMJ two extremes of quantal size are well known: large miniature endplate potentials (giant MEPPs) and tiny sub-MEPPs (for giants see review by Thesleff,

1989; for sub-MEPPs see review by Kriebel & Erxleben, 1986). However, it is improbable that these extremes play a role in synaptic transmission, because neither are released by nerve stimulation (reviewed by Van der Kloot, 1990).

Increases by a factor of two or more in the size of quanta that are released by stimulation have been described at frog NMJ. Size increases are produced by prolonged tetanic *in situ* motor nerve stimulation, soaking in hypertonic solution for 2 h, and by adrenaline, adrenocorticotrophic hormone and insulin (Van der Kloot & Van der Kloot, 1986; Van der Kloot, 1987). The increases appear to be due primarily to the release of more ACh per quantum, because they occur without marked change in the endplate depolarization produced by carbachol, the reversal potential, the endplate ACh noise spectrum, or the amount of α-bungarotoxin bound to the preparations. Moreover, the size increases are blocked by inhibitors of active ACh uptake into isolated synaptic vesicles: vesamicol (AH5183), tetraphenylboron (TPB) and hexanitrodiphenylamine (HNPA).

The present study was undertaken to see whether the same variables would increase quantal size at the mouse neuromuscular junction. There is some evidence that at the mammalian NMJ quantal size is variable. Tetanic *in vivo* nerve stimulation increases quantal size in cat, apparently by a postjunctional mechanism (Vrbová & Wareham, 1976). Corticosteroids such as prednisolone and dexamethasone increase quantal size by about 30% in rat diaphragm (Van Wilgenburg, 1979; Veldsema-Currie, Van Wilgenburg, Labruyere & Langemeijer, 1984). In undertaking this study we had the advantage that in previous work with frog adequate statistical methods were developed for detecting significant changes in quantal size, despite the normal variations from endplate to endplate.

METHODS

Animals

Experiments were performed on healthy, male and female Swiss Webster mice, weighing 18–24 g. They were killed by cervical dislocation and the diaphragm was dissected within 5 min. Right and left hemidiaphragms were separated. Preparations were pinned to the silicone rubber base of a plastic chamber containing about 10 ml of Tyrode solution. In some experiments, one hemidiaphragm was used as a control for the other. There were no significant differences in amplitude, duration, size or frequency of the spontaneous miniatures between the two sides.

Solutions

Tyrode solution contained (in mM): NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; NaH_2PO_4, 1; $NaHCO_3$, 15; glucose, 11. Sometimes we used HEPES buffer instead of NaH_2PO_4–$NaHCO_3$. A gas mixture of 95% O_2 and 5% CO_2 continuously bubbled through the solution; pH was 7·2–7·4 unless otherwise indicated. The solutions flowed into the experimental chamber at about 2 ml/min. The temperature of solutions was 35 ± 1 °C unless otherwise mentioned. Osmolarities were measured by freezing point (Precision Systems, Natick, MA, USA).

For most experiments, the solutions contained 0·1 μg/ml tetrodotoxin (TTX) to eliminate spontaneous twitches. Hypertonic solution contained 235 mM-NaCl or 235 mM-sodium gluconate (Chemical Dynamics, Plainfield, NJ, USA) or 135 mM-NaCl and 200 mM-sucrose, with the remainder of the components unchanged. For the even higher osmolarities used in a few experiments, additional NaCl was added.

For experiments on stimulated release, the solution contained (in mM): NaCl, 127; $CaCl_2$, 0·2–0·4; $MgCl_2$, 10; while the other constituents remained as above. Calcium-free–EGTA solution was made with 1 mM-ethyleneglycol-bis(β-aminoethylether)-*N*,*N*'-tetracetic acid (EGTA) and without adding Ca^{2+}. Potassium-free solution contained all Tyrode solution constituents except K^+. When concentrations of components were changed, the osmotic pressure was kept constant by adjusting

the concentration of NaCl. For experiments in Na^+-free solution, NaCl was replaced by LiCl. A few experiments were done with hypertonic Cl^--free solution containing (in mM): sodium gluconate, 235; K_2SO_4, 2·5; $CaSO_4$, 2·0; and $MgSO_4$, 1·0.

Drugs were obtained from Sigma (St Louis, MO, USA) except for vesamicol (2-(4-phenylpiperidino)cyclohexanol) obtained from Glaxo (London, UK) and 2,2′4,4′6,6′-hexanitrodiphenylamine (HNPA) and tetraphenylboron (TPB) from Aldrich Chemical Co. (Milwaukee, WI, USA).

Electrical recording methods

Microelectrodes for intracellular recording and current passing were filled with 3 M-KCl and bevelled to 5–8 MΩ DC resistance. We routinely recorded about 100 miniatures from each of five fibres before and after experimental treatments; in a few experiments MEPPs were recorded from ten fibres, but this did not substantially improve the statistical analysis. Recordings were always made in Tyrode solution unless indicated. Endplate regions were located by looking for the tiny phrenic nerve endings under magnifications of ×20–50, using either an inverted or dissecting microscope. The microelectrode was then inserted into the first layer of muscle fibres near the nerve terminals. The position of the electrode was judged by the rise phase of the MEPPs; if they reached peak in less than 1 ms the electrode was considered close enough to the endplate region. Recordings were filtered between 0·01 and 3000 Hz. The membrane potential was recorded along with the MEPPs and they were corrected later to a standing resting potential of −90 mV (Katz & Thesleff, 1957).

Voltage clamping was done by the two-electrode method using a 8500 system (Dagan, Minneapolis, MN, USA) or Axoclamp-2A (Axon Instruments, Burlingame, CA, USA). Usually the membrane potential was held at −70 mV.

The data were recorded and analysed by computer; the method for catching the entire time course of the miniatures was described by Van der Kloot (1987). Sampling was at 100 μs per point. The amplitude, area (voltage or current–time integrals) and time courses (20–80 % rise time, 50 and 75 % decay time) of miniatures or EPPs were calculated. The processed signals were viewed for artifacts or overlaps before accepting the measurement.

Areas (voltage or current–time integrals) were used as the measurement of quantal size. They record changes both in amplitude and duration of miniatures, and are directly proportional to the total charge flowing in through the endplate (Fatt & Katz, 1951).

Miniature frequency was measured from chart records (Gould Brush 220, Cleveland, OH, USA). For high-frequency counting ($> 100\ s^{-1}$), a Gould digital storage oscilloscope was used. Miniature endplate currents (MEPCs) were recorded because their faster time course favours the better quantification of frequency. A sweep at 20 or 50 ms/cm was frozen on the screen, the number of spikes then was counted.

Ionophoresis of ACh

The sensitivity of postsynaptic receptors to ACh was tested by measuring the responses to locally applied ACh (ACh potential). Microelectrodes were filled with 10^{-5} M-ACh; their DC resistances were 10–15 MΩ. A constant current generator (WPI, New Haven, CT, USA) was used to control ACh efflux. A holding current of 20 μA was applied between ejection pulses. The pulses were about 50 μA in amplitude; the experimenter controlled the duration, applying ACh until the response was close to an asymptote. The recording electrode and ACh electrode were located close to the same endplate.

Statistical methods

It has been shown that the logarithms of miniature sizes recorded from a single junction fit a normal distribution in frog (Van der Kloot, 1987). In mouse, we found that the logarithms of miniature size also fit to a normal distribution. Therefore, the statistical significance of the differences between data sets recorded before and after treatment can be tested by analysis of variance. Control experiments showed that in most instances the analysis of variance did not detect any significant difference between sets of recordings made from preparations from the same animal that received the same treatment.

Plots with a probability scale on the abscissa were used to determine whether data sets fit a normal probability distribution function or if there were two or more subpopulations or

multiquantal sizes (Van der Kloot, 1989). A set of miniature sizes from a single junction, sorted into ascending order, were plotted on this abscissa. A line was fitted to the points by a method that places an equal number of points above and below (Van der Kloot, 1987). From the pattern in the residuals between the points and the line, any systemic deviations of the points from the line could be detected.

Drugs were obtained from Sigma (St Louis, MO, USA), except for monoethylcholine (MECh, hydroxyethyldimethylethylammonium iodide), which was prepared for us by the Chemical Synthesis Laboratory of the National Institute of Mental Health.

Series of experiments were summarized by calculating the ratio of the MEPP, MEPC or EPP sizes in the experimental preparation to that in the control (experimental/control). The means of these ratios are given along with their standard errors (S.E.M.). The statistical significance of differences between means was tested by Student's t test.

RESULTS

Miniature endplate potentials or miniature endplate currents recorded from normal mouse diaphragm preparations in Tyrode solution had mean amplitudes of 1·4 mV or 2·5 nA, reached their peaks in less than 1 ms and appeared at a frequency of 0·9 s^{-1}. The amplitude, frequency and duration of miniatures remained unchanged when preparations were kept in Tyrode solution as long as 120 min.

Effects of hormones

Corticosteroids, like prednisolone and dexamethasone, increase MEPP size in rat diaphragm (Van Wilgenburg, 1979; Veldsema-Currie *et al.* 1984). In mouse diaphragm, 30–60 min treatment in 20–50 μM-prednisolone or dexamethasone increased sizes in some fibres, but the increases were not always seen, were not related to concentration, and the overall results were not statistically significant. The ratios of quantal size before and after hormone treatments were $1{\cdot}5 \pm 0{\cdot}42$ for prednisolone ($n = 9$, $P > 0{\cdot}05$) and $1{\cdot}4 \pm 0{\cdot}31$ for dexamethasone ($n = 10$, $P > 0{\cdot}05$).

Insulin and adrenaline increased quantal size in frog (Van der Kloot & Van der Kloot, 1986). We tested the effects of 30–120 min pre-treatment in 1–100 mU/ml insulin or 10–20 μM-adrenaline on MEPP size in mouse diaphragm. The ratio of quantal sizes after and before insulin was $0{\cdot}8 \pm 0{\cdot}05$ ($n = 5$; $P > 0{\cdot}05$). For adrenaline, the ratio was $1{\cdot}1 \pm 0{\cdot}11$ ($n = 6$; $P > 0{\cdot}05$). These changes were not statistically significant, so these hormones did not alter quantal size in mouse.

Effects of hypertonic solutions

Soaking preparations in hypertonic solution made with 235 mM-NaCl increased the frequency of miniatures. During the first 5 min, the frequency reached $25{\cdot}6 \pm 4{\cdot}50$ s^{-1} ($n = 15$), then it declined to $16{\cdot}3 \pm 10{\cdot}51$ s^{-1} ($n = 15$) for the next 25 min. When returned to Tyrode solution after 30 min in hypertonic solution, in most fibres the frequency declined to about normal level. In some fibres the frequency remained somewhat elevated for 10–30 min.

The amplitudes and durations of MEPPs and MEPCs were increased when recorded in Tyrode solution following 30 min in 235 mM-NaCl solution (Table 1; Fig. 1, Fig. 2). MEPP sizes were increased 2·2-fold and MEPC sizes were increased 2·0-fold. Moreover, the range of miniature sizes increased, there were more giants, and MEPPs took longer to rise (Fig. 2).

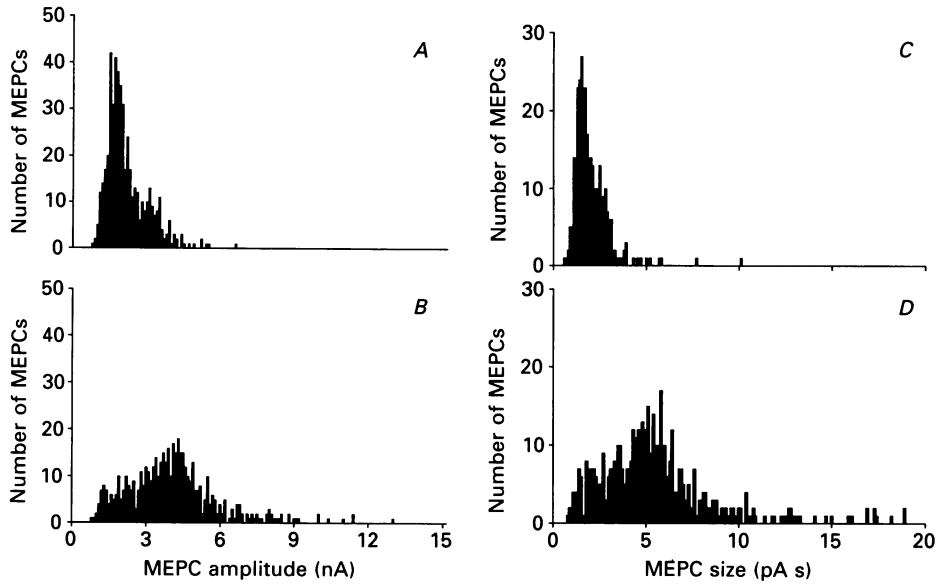

Fig. 1. Effects of hypertonic treatment on MEPCs. *A*, amplitudes from an untreated preparation in Tyrode solution. *B*, same preparation after 30 min in hypertonic NaCl solution, again recording in Tyrode solution. About 500 MEPCs were recorded from five fibres for each histogram. After hypertonic treatment the mean value is shifted to the right and some very large MEPCs appeared. *C*, effects of hypertonic treatment on MEPC size (the current–time integral). Untreated preparation in Tyrode solution, *D*, after 30 min in hypertonic solution, recorded in Tyrode solution.

TABLE 1. MEPP and MEPC at normal- and hypertonic-treated mouse neuromuscular junctions

Data	Treatment	Amplitude	50% decay	Size	*n*
MEPP	Control	1·4 ± 0·17 mV	0·91 ± 0·06 ms	1·8 ± 0·34 μV s	15
MEPP	Hypertonic	2·8 ± 0·26 mV	1·12 ± 0·15 ms	4·1 ± 0·29 μV s	15
Ratio	H/C	2·0 ± 0·11	1·23 ± 0·07	2·2 ± 0·16	
MEPC	Control	2·5 ± 0·21 nA	0·52 ± 0·02 ms	2·5 ± 0·41 pA s	20
MEPC	Hypertonic	4·8 ± 0·32 nA	0·58 ± 0·02 ms	4·9 ± 0·35 pA s	20
Ratio	H/C	1·9 ± 0·12	1·12 ± 0·01	2·0 ± 0·14	

Mean ± S.E.M.; $P < 0·05$ for each pair of data. Control, normal preparation in Tyrode solution. Hypertonic treatment, 235 mM-NaCl solution for 30 min. H/C, after hypertonic treatment/control; the mean of the ratios of each of the paired individual data and the S.E.M.

* First 5 min in hypertonic solution. *n*, number of experiments.

In frog, soaking muscles in hypertonic sodium gluconate solution, replacing the NaCl, produces a roughly 4-fold increase in quantal size (Van der Kloot & Van der Kloot, 1985; Van der Kloot, 1987). The reason for the enhancement by gluconate is not known. Soaking mouse preparations for 30 min in hypertonic solution made with 235 mM-sodium gluconate in place of NaCl increased MEPP size 2·6 ± 0·09-fold (n = 20) (Fig. 2*D*) and MEPC size 2·3 ± 0·13-fold (n = 4). Thus in mouse the size increases produced by hypertonic chloride or gluconate solution were almost identical.

The quantal size increase is related, at least in part, to the high osmotic pressure rather than to the high concentration of Na^+. When the hypertonic solution was

made by adding sucrose to Tyrode solution, MEPC size was increased 1·68 ± 0·23-fold ($n = 3$), this increase was not significantly different ($P > 0·05$) from the controls in hypertonic solution made with NaCl.

Time course of the size increases

Fifteen minutes treatment in 235 mM-sodium gluconate solution doubled MEPP size. Maximum MEPP size was reached by 30 min; there was no significant further

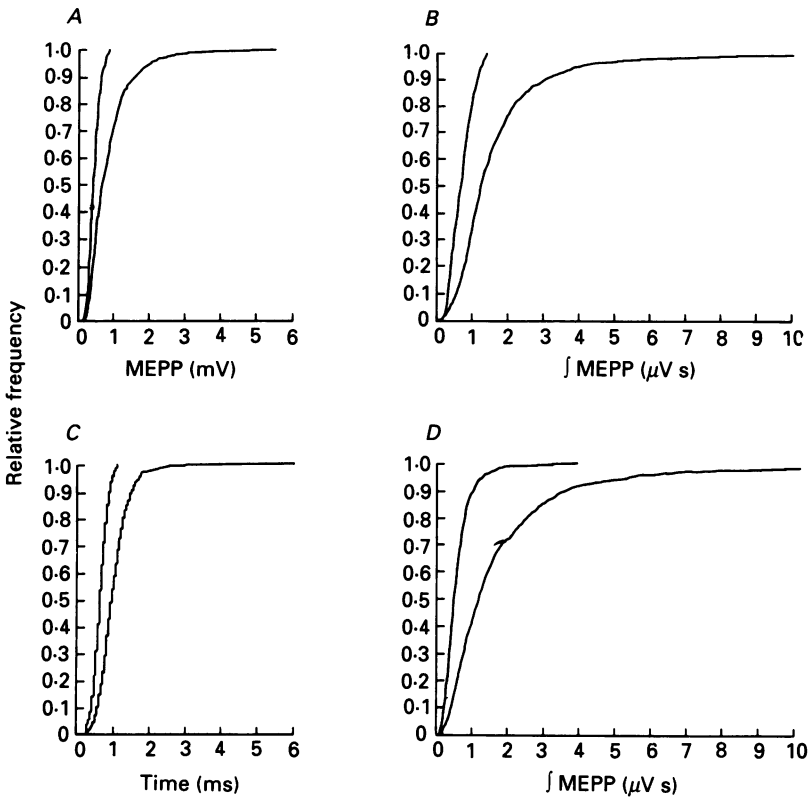

Fig. 2. The effects of treatment in hypertonic solutions on the amplitudes, 20–80 % rise times and areas of MEPPs. All recordings were in Tyrode solution. In *A–C* the preparation was treated for 30 min in hypertonic NaCl solution. *A*, MEPP amplitude before (left curve) and after (right curve) treatment ($n = 15$). *B*, MEPP size (voltage–time integral) before (left curve) and after (right curve) treatment. *C*, the 20–80 % rise time before (left curve) and after (right curve) treatment. All of the above were from the same experiment. *D*, MEPP size before (left curve) and after (right curve) 30 min in 235 mM-sodium gluconate solution ($n = 15$). The differences between control and experimental data were statistically significant.

increase after soaking in hypertonic solution for 120 min (Fig. 3). In most subsequent experiments we kept the preparations in hypertonic solution for 30 min, in contrast to the 120 min treatment used for frog muscle (Van der Kloot, 1987).

Distribution of miniature sizes

To determine whether the large miniatures observed after pre-treatment in hypertonic solution were a separate category or the upper end of a continuous distribution function, we plotted the logarithms of MEPP or MEPC size on a

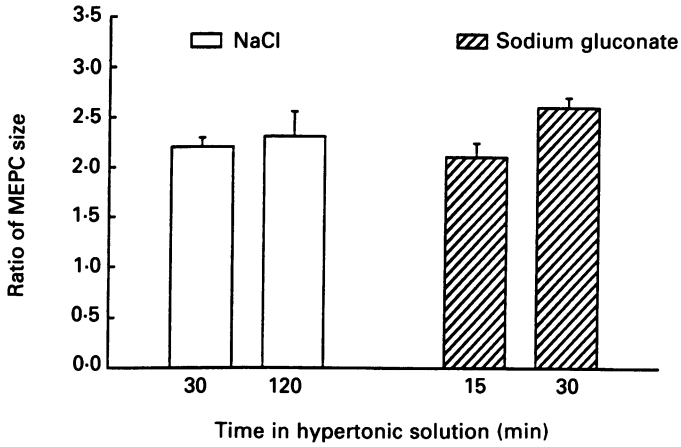

Fig. 3. Effect of duration of soaking in hypertonic solution on the quantal size increase. The preparations were treated in hypertonic NaCl (□) or sodium gluconate (▨) solution from 15 to 120 min. The recordings were made in Tyrode solution before and after treatment. The error bars show S.E.M.

probability abscissa (see Methods). On such a plot if the points fall along a line they fit to a normal probability distribution function. Figure 4*A* shows such plots for data from a preparation before and after treatment in hypertonic solution. The points from the untreated muscle lay close to a straight line except for a few points at the upper end, due to a few of the largest MEPPs. After soaking in 235 mM-NaCl solution and returning to Tyrode solution, the entire line was elevated. Most points still lay on a straight line except for a somewhat higher fraction of the largest MEPPs. The residuals between the points and the calculated straight lines show no obvious trends (Fig. 4*B* and *C*) except at the right end of Fig. 4*C* due to the group of extremely large MEPPs. These results are typical in that most of our data fitted satisfactorily to log–normal probability distributions. Therefore statistical tests based on the assumption that data follows a normal distribution function can be used on the logarithms of miniature size.

These probability plots strongly suggest that the size of the entire population was increased by the hypertonic treatment, rather than that the treatment added a subpopulation of giant miniatures to the usual population. Hereafter we will refer to the population of increased size as 'large miniatures', or 'large quanta' to distinguish them from 'giant MEPPs'.

Reversibility of quantal size increases

The large miniatures produced by hypertonic solution were quite stable; in diaphragms kept in Tyrode solution after treatment, the miniatures stayed large for

at least 1 h. Within 2–3 h the amplitude and size of miniatures declined gradually to the control level. Figure 5*A* shows that MEPP size decreased progressively over 3 h. During reversal there appeared to be a general decrease in MEPP size, not the appearance of a new, smaller subpopulation (Fig. 5*B*).

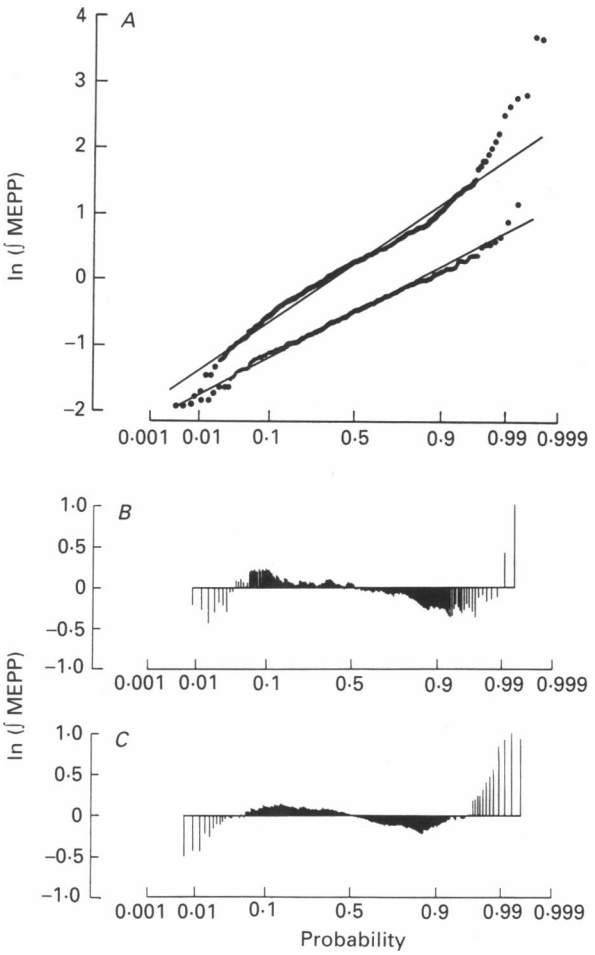

Fig. 4. Probability plots of MEPPs before and after hypertonic treatment. All recordings in Tyrode solution. *A*, the lower line is a plot of MEPP sizes before hypertonic treatment. The upper line shows the MEPP sizes after 30 min in hypertonic NaCl solution. The residuals of MEPP sizes before and after hypertonic treatment are shown in *B* and *C*.

Release by nerve stimulation

Uni-quantal EPPs were recorded in low Ca^{2+}–high Mg^{2+} solution. The $CaCl_2$ concentration was adjusted so that the probability that stimulation released more than a single quantum was low. The phrenic nerve was stimulated at 1·0 Hz and the responses to 150 stimuli were recorded from each of six fibres. We estimated the mean number of quanta released by stimulation, m_0, by the method of failures (del Castillo & Katz, 1954). The mean m_0 was 0·35 ($n = 5$). Therefore, the probability that a stimulus would release more than one quantum was about 0·05. Consistently, in these

preparations there was no significant difference ($P > 0.05$) between the size of MEPPs (1.8 ± 0.34 μV s, $n = 15$) and the size of EPPs (2.1 ± 0.32 μV s, $n = 5$). After 30 min hypertonic treatment uni-quantal EPP size was increased by a factor of 2.1 ± 0.24 ($n = 5$). The increased mean EPP size was almost identical to the 2·2-fold

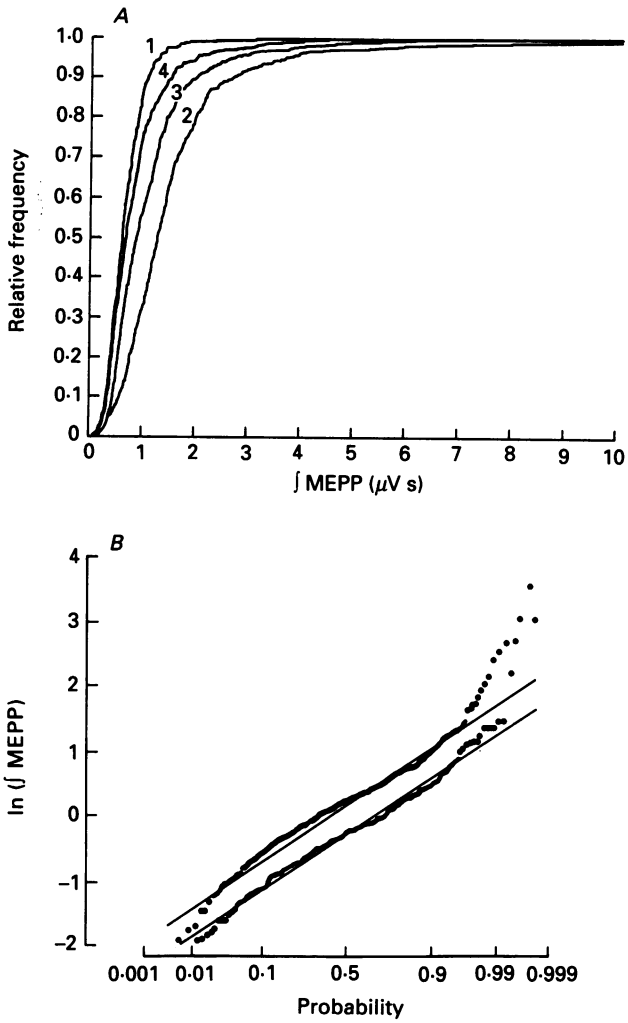

Fig. 5. Reversibility of the increase in MEPP size caused by hypertonic treatment. *A*, cumulative frequency distributions of MEPP size. (1) Recorded in Tyrode solution before hypertonic treatment. (2) Immediately after 30 min in hypertonic NaCl solution, recorded in Tyrode solution. (3) After 2 h back in Tyrode solution. (4) After 3 h in normal Tyrode solution. *B*, probability plots. The upper line shows the MEPPs recorded immediately after hypertonic treatment and the lower line shows those recorded 3 h later.

increase of MEPP size. The large miniatures are released by nerve stimulation as well as spontaneously.

Is the effect pre- or postsynaptic?

Effects of curare

To determine whether the large miniatures were generated by ACh, we applied 10 μM-*d*-tubocurarine (*d*-TC) after hypertonic treatment; all MEPPs were promptly

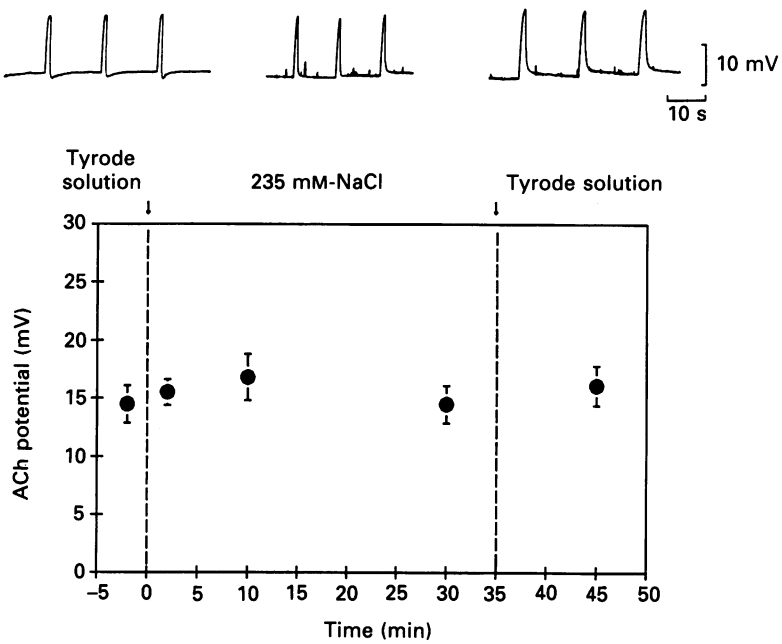

Fig. 6. ACh potentials before, during and after hypertonic treatment. Initially each preparation was in Tyrode solution and ACh potentials were recorded. At time zero, the preparations were put in hypertonic NaCl solution. The next recordings were made in the hypertonic solution. The right dashed vertical line shows the time when the muscles were returned to Tyrode solution. About 10 min later, ACh potentials were measured again. Each point in the graph shows the mean and S.D. from at least nine ACh potential measurements in three preparations.

abolished. When the preparations were returned to drug-free solution the MEPPs gradually appeared again. Apparently, all miniatures were produced by ACh. This does not eliminate the possibility that large miniatures are generated by a co-transmitter which may potentiate the effect of ACh or by a post-junctional change which potentiates the response to ACh.

ACh potentials

To see whether miniature size increased because of a rise in the sensitivity or quantity of ACh receptors on the postsynaptic membrane, we recorded the responses of endplates to ionophoretically applied ACh (ACh potential, Fig. 6). Three ACh pulses were given before any treatment, some pulses were applied while the preparation was in 235 mM-NaCl hypertonic solution, and after the preparations

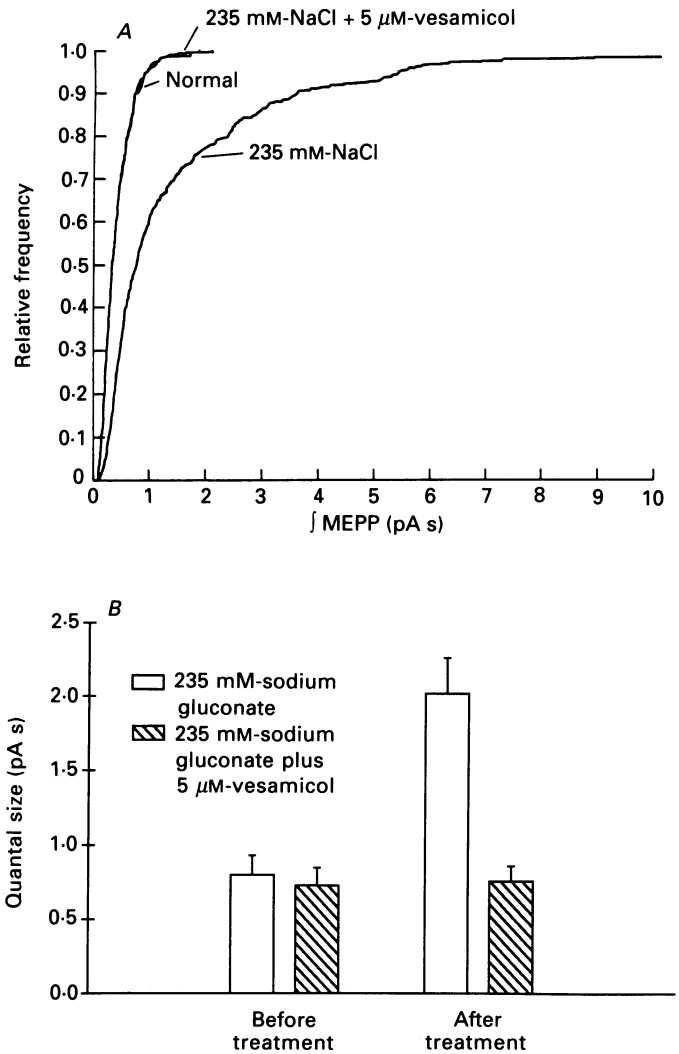

Fig. 7. Effect of vesamicol on the quantal size increase produced by hypertonic solution. *A*, two hemidiaphragms from same mouse were treated for 30 min either in hypertonic NaCl solution or hypertonic NaCl solution plus 5 μM-vesamicol. MEPCs were recorded in Tyrode solution before and after the treatment. The vesamicol-free hypertonic solution increased MEPC size, shifting the cumulative curve to the right. In the preparation exposed to vesamicol, hypertonic pre-treatment failed to increase MEPC size, the cumulative curve is almost identical to that recorded before the treatment. *B*, a summary of five experiments on the effects of vesamicol on the formation of large quanta. Methods as in *A*, except that hypertonic sodium gluconate solution was used. The sizes of the MEPCs are shown before and after hypertonic treatment.

were returned to Tyrode solution three further ACh potentials were recorded. The amplitudes of the ACh potentials were not significantly changed during and after the hypertonic treatment, but as the result of the hypertonic treatment large MEPPs appeared in the records (Fig. 6). The measurements were made when the ACh potentials had reached an asymptote. Since the currents at the asymptote were

essentially the same before and after treatment, this means that the product of the rate of channel opening by the mean channel conductance and the mean channel duration was unchanged (see Cohen, Van der Kloot & Attwell, 1981). The lack of overall change in the properties of the postsynaptic ACh receptors suggests that the increase in quantal size is presynaptic. Further evidence on this point came from the next experiments.

Effects of ACh transport inhibitors

Vesamicol blocks the active transport of ACh into synaptic vesicles (Anderson, King & Parsons, 1983*a*, *b*: Bahr & Parsons, 1986). We used vesamicol to test the hypothesis that as a result of hypertonic treatment more ACh was loaded into quanta.

In control experiments, 5 μM-vesamicol in Tyrode solution for more than 30 min had no effect on quantal size. The MEPC size ratio was $1{\cdot}0 \pm 0{\cdot}42$ (vesamicol/drug free; $P > 0{\cdot}05$, $n = 3$), suggesting that the drug does not affect postsynaptic membrane or AChE at this concentration. Figure 7 shows data from an experiment in which we compared two hemidiaphragms from the same mouse. Both were treated for 30 min in hypertonic solution, but the solutions (235 mM-NaCl and Tyrode) bathing one of the two hemidiaphragms included 5 μM-vesamicol. As expected, hypertonic treatment increased quantal size in the drug-free hemidiaphragm. However, in the hemidiaphragm exposed to vesamicol miniature size was unchanged, so that the cumulative curve was identical to the control curve (Fig. 7*A*). Figure 7*B* summarizes five similar experiments.

In experiments on stimulated release, 5 μM-vesamicol in hypertonic solution blocked the increase in size of uni-quantal EPPs. The EPP size ratio of after/before treatment was $1{\cdot}10 \pm 0{\cdot}21$ ($n = 3$).

Effects of transport inhibitors after hypertonic treatment

Further experiments were done to determine the effects of uptake inhibitors on large quanta after hypertonic treatment. Diaphragms were pre-treated in hypertonic solution; after the treatment 5 μM-vesamicol was added to the Tyrode solution. Within 3–5 min quantal size declined toward the control level (Fig. 8). When the preparations were returned to drug-free solution, quantal size increased once again.

Two other ACh transport inhibitors, 4 μM-HPNA and 5 μM-TPB (Anderson *et al.* 1983*a*, *b*) were tested. They also both decreased the size of large quanta produced by hypertonic treatment (Fig. 8). One explanation of these results is that large quanta are formed by the addition of ACh shortly before they are released.

Other variables

Effects of Ca^{2+} and Mg^{2+}

Experiments were done to see whether changes in extracellular Ca^{2+} affect quantal size increase. The preparations were kept in Ca^{2+} free–EGTA solution for about 45 min to wash Ca^{2+} from the synaptic cleft. They then were placed in Ca^{2+} free–EGTA hypertonic solution for 30 min. MEPP size was increased 2·4-fold after the treatment (Table 2). Elevated concentrations of Ca^{2+} (6 mM) or Mg^{2+} (5 mM) in

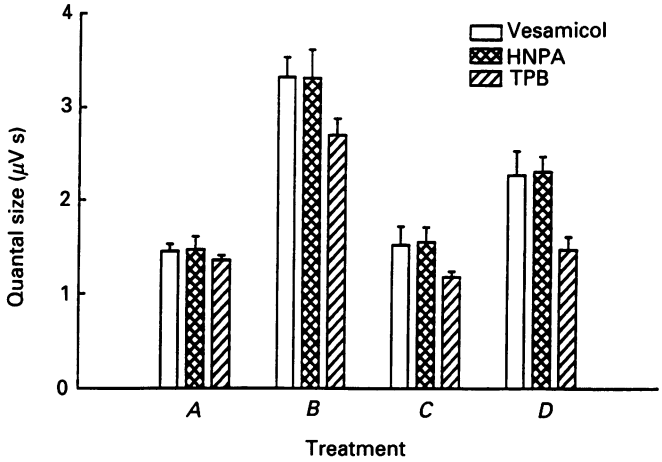

Fig. 8. Effects of vesamicol and related drugs on the large quanta produced by hypertonic treatment. *A*, MEPP sizes recorded in Tyrode solution. *B*, MEPP sizes after hypertonic treatment. The hypertonic solutions used were NaCl for the vesamicol experiment (n = 5), and sodium gluconate for the HNPA (n = 10) and the TPB (n = 3) experiments. *C*, MEPP sizes after 3–5 min in the drugs. *D*, after 30 min in drug-free Tyrode solution. The concentrations used were 5 μM for vesamicol and TPB, 4 μM for HNPA. Means ± S.E.M. All recordings in Tyrode solution.

TABLE 2. Effects of ions and temperature on quantal size increase

Hypertonic solution	Quantal sizes		Ratio: after treatment/ before treatment	n
	Before treatment	After treatment		
Ca^{2+}-free–EGTA	1·6 ± 0·20 μV s	3·8 ± 0·21 μV s	2·4 ± 0·09*	3
6 mM-Ca^{2+}	1·5 ± 0·25 μV s	3·5 ± 0·36 μV s	2·4 ± 0·47*	3
5 mM-Mg^{2+}	1·8 ± 0·17 μV s	3·9 ± 0·25 μV s	2·2 ± 0·29*	4
Cl^- free	2·5 ± 0·20 pA s	4·0 ± 0·25 pA s	1·6 ± 0·05*	3
At 25 °C	2·7 ± 0·30 pA s	3·1 ± 0·27 pA s	1·2 ± 0·15**	3

* $P < 0.05$. ** $P > 0.05$.

hypertonic solutions did not affect the quantal size increase either: MEPP size increased 2·4- and 2·2-fold respectively (Table 2).

The role of chloride

In frog sartorius muscle after exposure to a 42 mM-K^+ solution in which Cl^- was replaced by propionate, or with the Cl^- concentration reduced from 120 to 60 mM, the frequency of giant MEPPs was substantially decreased compared to the controls in Cl^- solution (Molenaar, Oen & Polak, 1987). They concluded that Cl^- was necessary for producing giants.

Choline uptake is partially dependent on extracellular Cl^- (Ducis & Whittaker, 1985). In experiments on mouse diaphragm, quantal size increased after soaking either in hypertonic gluconate or NaCl solution, so Cl^- did not seem to be a critical factor. However, in the sodium gluconate solution there was still about 8 mM-Cl^- from KCl, $CaCl_2$ and $MgCl_2$. To further test the role of Cl^- in quantal size increase, we used a 235 mM-sodium gluconate, Cl^--free solution (see Methods). After soaking

in this solution for 30 min, the quantal size was increased 1·6-fold (Table 2). Obviously, hypertonic treatment increased quantal size without chloride, although the increase probably was less than with chloride.

Effects of pH

At pH 6·4, hypertonic solution did not increase MEPP size significantly (four experiments). However, hypertonic solution at pH 8·2 significantly increased MEPP size ($2·5 \pm 0·47$-fold increase, $n = 3$).

Effects of temperature

The experiments were usually done at 35 °C. When the temperature was lowered to 25 °C for all procedures, quantal size was not increased after the hypertonic treatment. The ratio of after/before treatment was $1·2 \pm 0·16$ ($n = 3$; $P > 0·05$). Further experiments were carried out in which the temperature was lowered to 25 °C only during the hypertonic treatment. The recordings before and after the treatment were both done at 35 °C in Tyrode solution. Quantal size was about the same before and after the treatment (Table 2). The ratio was 1·2.

MEPP frequency and size increase

In mouse diaphragm, the increase in MEPP frequency produced by hypertonic solution is not as great as in frog muscle and it is transitory (Hubbard, Jones & Landau, 1968). In our experiments, the MEPP mean frequency increased from 0·9 s^{-1} in normal solution to 25·6 s^{-1} during the first 5 min in 235 mM-NaCl hypertonic solution, and then averaged 16·3 s^{-1} during the next 25 min ($n = 25$). In 400 mM-NaCl solution MEPP frequency reached $37·8 \pm 13·05$ s^{-1} ($n = 4$), and in 600 mM it was $48·4 \pm 12·12$ s^{-1} for the first 5 min ($n = 3$), while the quantal sizes were increased about 2-fold ($1·8 \pm 0·10$, after 400 mM-NaCl; $1·8 \pm 0·12$, after 600 mM-NaCl). There does not appear to be a direct relation between the increase in frequency while in a hypertonic solution and the increase in quantal size detected after the hypertonic treatment.

Choline uptake and size increase

Hemicholinium-3

Hemicholinium-3 (HC-3) is a potent inhibitor of high-affinity choline transport (HAChT; Yamamura & Snyder, 1973). One to two micromolar HC-3 in Tyrode solution for about 30 min did not affect quantal size. The preparations were then soaked in 235 mM hypertonic solution containing 1 μM-HC-3 for 30 min. Recorded in Tyrode solution plus 1 μM-HC-3 after the treatment, quantal size was about the same as before the treatment. Quantal size was $2·35 \pm 0·37$ pA s ($n = 3$) before treatment and $2·56 \pm 0·52$ pA s ($n = 3$) after treatment. The ratio of after/before hypertonic treatment was $1·08 \pm 0·06$ ($P > 0·05$; $n = 3$).

Adding 40 μM-choline along with HC-3 during the hypertonic treatment did not lead to the appearance of large quanta. The ratio was $1·12 \pm 0·12$ ($P > 0·05$; $n = 3$). Higher choline were not tested because it produces postsynaptic effects.

Additional experiments were done to see what effect 2 μM-HC-3 might have when applied after hypertonic treatment. For 5–10 min after introducing HC-3 there was no obvious change in MEPC size. After about 15 min, MEPCs were smaller, but still

larger than normal (Fig. 9). After an additional 15 min, there was no further decrease in quantal size. Some large miniatures were still observed (Fig. 9).

Hemicholinium-3 has a direct postsynaptic inhibitory action on rat diaphragm at concentrations of 4 μM or higher (Elmqvist & Quastel, 1965). On normal resting

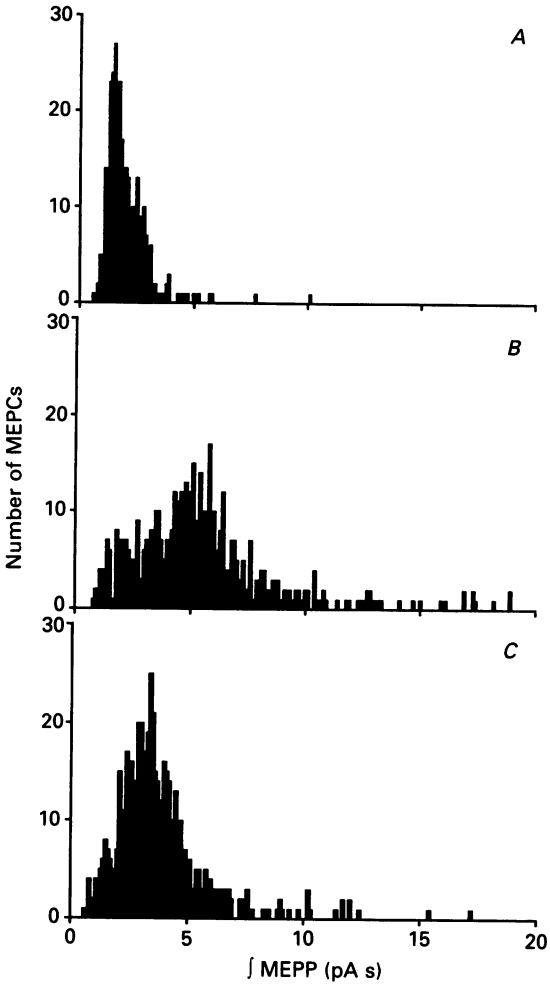

Fig. 9. Histograms showing the effect of HC-3 on large MEPCs after hypertonic solution. *A*, control. *B*, after 235 mM-NaCl. *C*, 10–30 min after adding HC-3. Note the difference in the peak positions in *A*, *B* and *C*, and the large MEPCs that persist in *C*.

mouse diaphragm, 1–2 μM-HC-3 did not decrease miniature size. So the effect of HC-3 on large quanta is probably due primarily to block of HAChT rather than to postsynaptic depression.

Extracellular Na^+

High-affinity choline transport depends on extracellular Na^+. Lithium does not substitute for Na^+ in choline transport (Chao, Pomfret & Zeisel, 1988). When we replaced the NaCl in the hypertonic solution with 300 mM-LiCl after 30 min there was no increase in quantal size (before treatment: $2{\cdot}3 \pm 0{\cdot}16$ pA s, $n = 3$; after

hypertonic treatment: 2·2 ± 1·20 pA s, $n = 3$). The quantal size ratio after/before treatment was 1·01 ± 0·06.

In frog the permeability of endplate channels to Li^+ is 87 % of that to Na^+ (Adams, Dwyer & Hille, 1980). To determine how large the effect of Li^+ might be on endplate channels in mouse, we compared the amplitudes, decay times and sizes of MEPCs recorded in NaCl Tyrode and LiCl solutions. Differences were not significant

TABLE 3. Effects of neostigmine on quantal size increase

Treatment	Amplitude (nA)	50 % decay (ms)	Size (pA s)	n
Control	2·60 ± 0·20	0·56 ± 0·02	2·70 ± 0·30	3
Neostigmine	3·18 ± 0·31	0·76 ± 0·02	4·66 ± 0·87	3
Hypertonic–neostigmine treatment	3·30 ± 0·19	0·67 ± 0·04	4·28 ± 0·29	3
Hypertonic–neostigmine–choline treatment	3·28 ± 0·35	0·72 ± 0·02	4·58 ± 0·29	3

Neostigmine, 0·5 μM; choline, 20 μM. The control data were recorded in drug-free Tyrode solution; neostigmine data were recorded 20 min after adding 0·5 μM-neostigmine; hypertonic data were recorded in Tyrode solution plus 0·5 μM-neostigmine after treatment. There were significant differences ($P < 0·05$) between control group and neostigmine group, control group and hypertonic–neostigmine group. No difference ($P > 0·05$) in MEPC sizes between neostigmine, hypertonic–neostigmine and hypertonic–neostigmine–choline groups.

(Tyrode solution: 2·5 ± 0·21 nA, 0·52 ± 0·02 ms, 2·5 ± 0·41 pA s, $n = 20$. Li^+ solution: 2·4 ± 0·52 nA, 0·53 ± 0·02 ms, 2·4 ± 0·66 pA s, $n = 3$). The failure of miniature size to increase is unlikely to be due to the lower conductance of endplate channels to Li^+.

Acetylcholinesterase inhibition

Inhibition of acetylcholinesterase (AChE) will increase the [ACh] in the cleft but reduce the choline available for the uptake. If the quantal size increase depends on choline uptake, inhibition of AChE should diminish the size increase.

Neostigmine (0·5 μM) was added to Tyrode solution and hypertonic solution to inhibit AChE. The amplitude and duration of MEPCs were larger in Tyrode solution containing neostigmine due to the prolonged action of ACh (Table 3). After 30 min in hypertonic solution containing neostigmine, quantal size remained unchanged. The ratio of after/before hypertonic treatment with AChE inhibitor was 0·98 ± 0·11 (Table 3). There were no statistically significant changes in amplitude or size of MEPCs after hypertonic treatment in solution containing neostigmine. Adding 20–40 μM-choline to the solutions did not antagonize the effect of neostigmine (Table 3).

Intracellular [Na^+]

Inhibition of the Na^+–K^+ exchange pump allows Na^+ to accumulate in neurons and thereby inhibit choline uptake (O'Regan, Vyas & Meunier, 1984). In frog, quantal size increases are blocked by treatments that elevate $[Na^+]_i$ (reviewed by Van der Kloot, 1990).

Ouabain inhibits the Na^+–K^+ exchange pump (reviewed by Clausen, 1986). Preparations were incubated in Tyrode solution containing 40 μM-dihydroouabain for 60 min to allow Na^+ to accumulate in nerve terminals. The muscles were then put in hypertonic solution containing 40 μM-dihydroouabain. In the presence of dihydroouabain, hypertonic treatment induced less increase in quantal size (Fig. 10), 1·4-fold compared to 2·2-fold without dihydroouabain.

When applied after the hypertonic treatment 40 μM-dihydroouabain had no effect on quantal size during the 30 min of observation. The ratio of after/before dihydroouabain was $0{\cdot}98 \pm 0{\cdot}02$ ($n = 3$). This suggests that the reduction in quantal size increase is unlikely to be caused by a postjunctional effect of dihydroouabain.

Aconitine selectively opens membrane Na^+ channels at normal resting potentials,

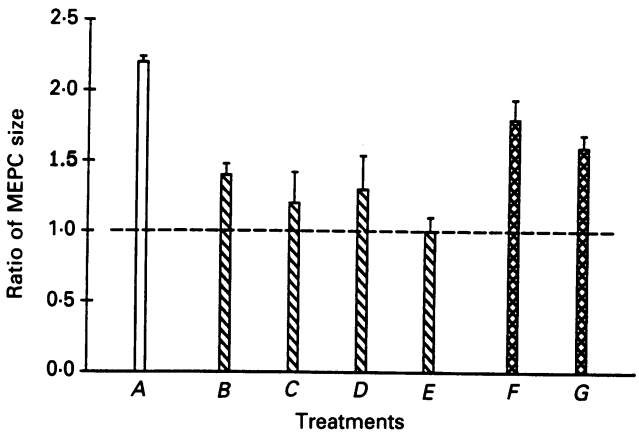

Fig. 10. Effects of treatments that elevate intracellular [Na^+] on quantal size increase and the antagonistic action of choline. The dashed horizontal line shows the control level before hypertonic treatment. All the preparations were treated in hypertonic solution for 30 min. All recordings were in Tyrode solution. The values are means $\pm$ S.E.M. of the ratio of MEPC sizes after to those before hypertonic treatment. The treatments were: *A*, soaking in 235 mM-NaCl solution for 30 min ($n = 15$); *B*, hypertonic solution containing 40 mM-dihydroouabain ($n = 4$); *C*, K^+-free hypertonic solution ($n = 4$); *D*, hypertonic treatment at 7 °C ($n = 3$); *E*, hypertonic solution containing 10 μM-aconitine ($n = 3$); *F*, hypertonic solution containing 40 μM-dihydroouabain and 20 μM-choline ($n = 5$); *G*, K^+-free hypertonic solution containing 20 μM-choline. The differences between the data sets with and without choline are statistically significant ($P < 0{\cdot}05$).

resulting in the accumulation of intracellular Na^+ (Schmidt & Schmitt, 1974). We repeated the above experiments with 10 μM-aconitine. One hour exposure of untreated preparations to aconitine did not affect quantal size. Aconitine in the hypertonic solution inhibited the increase in quantal size (Fig. 10). Two other procedures that inhibit the Na^+ pump were checked for affects on quantal size: K^+-free solution and low temperature (see Clausen, 1988). After recording quantal size in Tyrode solution, the preparations were pre-treated for 45–60 min in K^+-free solution or at 7 °C to allow Na^+ to accumulate in the terminal. Then they were soaked in hypertonic solution under the same conditions and returned to Tyrode solution for recording. Both treatments antagonized the increase of quantal size (Fig. 10).

When 20 μM-choline was added either to the hypertonic solution containing dihydroouabain or to the K^+-free hypertonic solution the quantal size increased $1{\cdot}8 \pm 0{\cdot}14$-fold ($n = 6$) and $1{\cdot}6 \pm 0{\cdot}09$-fold ($n = 3$) respectively (Fig. 10). The quanta were significantly larger than without choline.

Elevated extracellular K^+

Elevated concentrations of K^+ depolarize cell membranes. The depolarization might reduce the driving force for Na^+ entry and decrease choline transport.

Glavinovic (1988) reported that after 20 min in 20 mM-K^+ solution the time course of MEPCs was unchanged while the amplitude was slightly reduced. In our experiments, after recording MEPCs in Tyrode solution the preparations were soaked in 20 mM-K^+ solution for 60 min and returned to Tyrode solution for MEPC recording. There was no significant change in time course or amplitude of MEPCs after such treatment (Fig. 11).

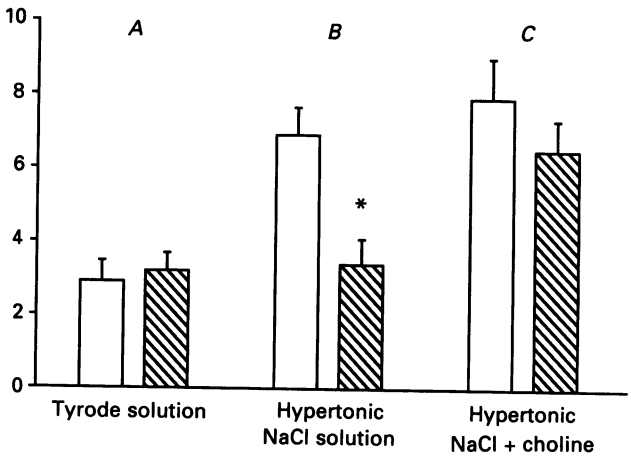

Fig. 11. Effects of high K^+ and choline on large quanta. *A*, recorded in Tyrode solution before any treatment (□, $n = 3$), after 20 min in elevated K^+ solution (▨, $n = 3$). *B*, after 30 min in hypertonic NaCl solution (□, $n = 3$), then the muscle was placed in 20 mM-K^+ solution for 20 min and MEPCs were again recorded in Tyrode solution (▨, $n = 3$). *C*, after treatment in hypertonic NaCl solution (□, $n = 4$), then the muscle was placed in 20 mM-K^+ solution containing 20 μM-choline for 20 min and MEPCs were again-recorded in Tyrode solution (▨, $n = 4$). * indicates $P < 0{\cdot}05$. Means and S.E.M.

When the 20 mM-K^+ solution was used after hypertonic treatment, about 20 min later MEPCs recorded either in the K^+ or Tyrode solutions were reduced to the control size (Fig. 11). This reduction took place either in the presence or absence of Ca^{2+}. Without Ca^{2+} there was no dramatic increase in MEPC frequency, so the reduced quantal size was not solely the result of depletion of large quanta.

When 20 μM-choline was added to the K^+-solution, the large quanta produced by hypertonic treatment did not decline or were only slightly reduced (Fig. 11) and were significantly larger than without choline. This is further evidence that choline uptake and consequent ACh synthesis are closely related to the quantal size increase.

Monoethylcholine

Colquhoun, Large & Rang (1977) and Large & Rang (1978*a*, *b*) placed the false transmitter precursor MECh in the solution and enhanced quantal release by nerve stimulation or K^+ solution. MEPPs or MEPCs gradually became smaller and shorter. Acetylmonoethylcholine (AMECh) opens endplate channels for a shorter time than ACh, so it seems that AMECh is formed in the terminals, transported into vesicles and released. For example, soaking rat diaphragms in 100 μM-MECh and 40 mM-KCl for 60–90 min produced about a 40% reduction in decay time of MEPCs and a 30% reduction in amplitude. They interpreted these changes as due to the total replacement of ACh by AMECh.

In experiments on mouse diphragm, we kept the solutions at 30 °C (instead of 35 °C) to make it easier to detect changes in decay time. Without stimulation, MECh (100 μM) had no effect on normal MEPCs in Tyrode solution for 30–60 min ($n = 3$) (Table 4). Incubation in 100 μM-MECh and 40 mM-KCl solution for 90 min decreased MEPC half-decay time about 13% and amplitude about 22% (Table 4). During the first 5 min in high-K^+ solution, the MEPC frequency was estimated to be 309 ± 177 s^{-1}

TABLE 4. Effects of MECh on amplitude, 50% decay and size of MEPCs

Treatment	Amplitude (nA)	50% decay (ms)	Size (pA s)	n
Control (normal tyrode solution)	2·6 ± 0·24	0·56 ± 0·02	2·7 ± 0·29	3
100 μM-MECh	2·7 ± 0·31	0·56 ± 0·03	2·7 ± 0·33	3
Ratio	1·04 ± 0·10*	1·00 ± 0·02*	1·01 ± 0·15*	
Control (normal tyrode solution)	2·7 ± 0·12	0·56 ± 0·02	2·7 ± 0·15	4
100 μM-MECh + 40 mM-KCl	2·1 ± 0·42	0·49 ± 0·03	1·7 ± 0·42	4
Ratio	0·78 ± 0·10	0·88 ± 0·04	0·63 ± 0·11	
Control (235 mM-NaCl)	4·3 ± 0·29	0·67 ± 0·05	4·9 ± 0·27	10
235 mM-NaCl + 100 μM-MECh	3·0 ± 0·36	0·58 ± 0·03	3·1 ± 0·44	10
Ratio	0·70 ± 0·15	0·87 ± 0·03	0·63 ± 0·16	
Control (235 mM-NaCl)	4·0 ± 0·34	0·65 ± 0·08	4·6 ± 0·52	10
100 μM-MECh after control	3·2 ± 0·36	0·57 ± 0·05	3·2 ± 0·58	10
Ratio	0·80 ± 0·12	0·88 ± 0·07	0·69 ± 0·14	

Ratio: experimental control; P values for ratios are at least $< 0·05$ except the data in the first group marked by *.

(mean ± S.D., $n = 16$). After about 5 min the frequency declined to a lower level, during the rest of the time in high-K^+ solution the frequency was 79 ± 75 s^{-1} ($n = 13$). The average number of quanta released in 90 min therefore was about 500000, which is at least twice the estimated number (130000–270000) of quanta releasable by mammalian motor nerve terminals without replenishment (Elmqvist & Quastel, 1965; Large & Rang, 1978*a*, *b*). For K^+-evoked release in rat, Large & Rang (1978*b*) estimated that about 700000 quanta needed to be released before the quanta contained only AMECh. Whatever the number of quanta that must be released to deplete the terminal may be, it is clear that MECh entered nerve terminals in mouse diaphragm and was converted into AMECh which replaced a substantial fraction of the ACh in each quantum. Therefore we used MECh to determine whether false transmitter was loaded into vesicles during the hypertonic treatment, judging by changes in time course and amplitude of MEPCs.

Monoethylcholine (100 μM) was added to the hypertonic solution. After 30 min treatment the preparations were placed in Tyrode solution containing 100 μM-MECh for MEPC recordings. Although the amplitude was increased, the magnitude of the increase was about 30% less and the half-decay time was 13% shorter than without MECh (Table 4). The quantal size was increased only 1·3-fold. The MEPC frequency during the first 5 min in hypertonic solution was 128 ± 64 s^{-1} ($n = 20$). For the remaining 25 min the frequency was about 17 ± 19 s^{-1} ($n = 36$). The number of quanta released during this period was about 64000. Presumably, only 25–50% of the releasable quanta were released. AMECh apparently was not incorporated into quanta due to vesicle depletion.

When 100 μM-MECh was added to Tyrode solution after hypertonic treatment, the half-decay time of the MEPCs decreased about 12%, while their amplitude was

reduced by 20% (Table 4). Apparently after hypertonic treatment some newly synthesized transmitter is added to quanta before release. This conclusion is consistent with the experiments showing that inhibitors of active ACh transport decreased quantal size when used after hypertonic treatment. It also is in accord with the data that inhibition of choline uptake after hypertonic treatment reduced the quantal size increase.

DISCUSSION

The experiments were undertaken to determine whether treatments that increase quantal size in frog also work on mouse. Quantal size can be experimentally increased in mouse, but, not surprisingly, there are notable differences in time course and in the effectiveness of different treatments. The mouse experiments have been especially useful because they suggest a clearer picture of part of the mechanism by which quantal size is increased.

Fortunately, the methods developed on frog (Van der Kloot, 1987) for reliable statistical tests of the differences between miniatures before and after treatment, taking into account the variability from fibre to fibre, proved applicable to mouse. Such proven methods are essential to advance studies of quantal size beyond the anecdotal level.

The response to hypertonic solution

Hypertonic treatment doubles quantal size at the mouse NMJ, just as in frog (Van der Kloot, 1987). However, in mouse the increase was substantially complete within 15–30 min, while in frog it took at least 60 min. This difference may merely reflect the roughly 13 °C difference in the temperatures at which the experiments were conducted, but this possibility was not explored further.

In frog, it has been proposed that the trigger for the increase in quantal size is a period of enhanced quantal release, because size is increased by other treatments that release many quanta (Van der Kloot & Van der Kloot, 1985). In frog extremely hypertonic solutions did not increase MEPP frequency markedly, nor did they increase quantal size (Van der Kloot, 1987). Either the elevated quantal release triggers the increase in quantal size, or an increase in tonicity increases quantal size and, in parallel, increases the rate of spontaneous release. We do not have the evidence to decide whether size increase is or is not a consequence of elevated release rate in the mouse, though it is clear that there is no direct relation between number of quanta released and the size increase.

It is unclear how a rise in tonicity stimulates spontaneous release. One hypothesis is that the trigger is a rise in intracellular [Ca^{2+}] (Shimoni, Alnaes & Rahamimoff, 1977). Another possibility is that a rise in intracellular ionic strength is a direct trigger: it is thought that intracellular ionic strength serves as a second messenger in other systems (Higgins, Cairney, Stirling, Sutherland & Booth, 1987). The steps by which the increase in tonicity promotes the quantal size increase also remain unknown.

The origin of large quanta

An increase in the input resistance of the muscle membrane would enable a current of usual size to produce a larger potential change. Hypertonic treatment increased

MEPP and MEPC size to the same extent, so the large miniatures did not appear to be due to an increase in membrane resistance.

The amplitudes of responses to ionophoretically applied ACh stayed about the same during and after hypertonic treatment (Fig. 6), which suggests that the ACh receptors in the endplate membrane were not substantially altered. The large quanta are not likely to be triggered by local action potentials in terminals or repetitive antidromic impulses in the motor nerves since they occurred when Ca^{2+} was omitted or in the presence of TTX.

The increase in quantal size occurred gradually, the entire population appeared to increase in size, there was no suggestion that a new subpopulation of large quanta was formed (Fig. 4). These observations also argue against the hypothesis that large quanta are formed by the fusion of two or more usual quanta. If there is multiquantal release, the increase and reduction of quantal size should occur in steps, with some miniatures produced by single quanta, others by two, and so forth, but this did not seem to be the case. After hypertonic treatment, quantal size in Tyrode solution gradually decreased over several hours. On cumulative plots, the curves shifted to the left smoothly in time with no evidence of subpopulations, nor were subpopulations detected with probability plots. Apparently, the ACh content of each quantum increased during and/or after hypertonic treatment and then gradually decreased sometime after the treatment. The effects of agents that alter ACh synthesis and packaging, which will be discussed shortly, also suggest that the major cause for the size increase is the incorporation of more ACh into quanta, and therefore also argue against the multiquantal interpretation.

Hormones

We could find no hormone that increases quantal size in mouse, while several do in frog. Further experiments might confirm an increase by steroids, as reported in rat (Van Wilgenburg, 1979; Veldsema-Currie *et al.* 1984). We measured about the same increase in quantal size, but with our rigorous statistical test the increases were not significant. There is no evidence whether the action of steroids is pre- or postjunctional.

Large quanta and giant miniatures

The large quanta produced by hypertonic treatment share some common characteristics with the giant MEPPs reviewed by Thesleff (1989). First, both have higher amplitude and a prolonged time course. Second, quantal size increase and occurrence of giant MEPPs are temperature- and pH-dependent. For example, hypertonic treatment did not increase quantal size at 25 °C as it did at 35 °C. At rat neuromuscular junction more giants appeared at 35 °C (5·8 %) than at 16 °C (0·61 %) (Heinonen, Jansson & Tolppanen, 1982). Acid Tyrode solution inhibited both the quantal size increase by hypertonic treatment and the higher frequency of giants produced by emetine (Alkadhi, 1989). Third, both the large miniatures and the giant MEPPs (Lupa, Tabti, Thesleff, Vyskocil & Yu, 1986) are reduced in size a few minutes after exposure to vesamicol.

None the less, these two categories of miniatures are not the same. Probability plots suggested that after hypertonic treatment there was an increase in size of the

whole population of MEPPs or MEPCs, but the giant MEPPs are always described as a subpopulation of MEPPs, with most of the MEPPs retaining usual size. Even after hypertonic treatment there often was a small number of very large miniatures, seen as a separate group at the upper end of probability plots, which may correspond to giant MEPPs.

The critical difference between large quanta and giant MEPPs is stimulated release. The large quanta were released by nerve stimulation, but giant MEPPs are not (Thesleff, 1989). After hypertonic treatment we found no statistically significant differences between the sizes of MEPPs and uni-quantal EPPs. Nevertheless, there may be a small number of very large quanta which are released spontaneously but not by stimulation, corresponding to the giant MEPPs (Fig. 7). Even if this is so, the principal point is that the large miniatures are not the same as the giant miniatures.

Inhibition of ACh transport

Vesamicol, TPB and HNPA inhibit the active transport of ACh into isolated synaptic vesicles from *Torpedo* electroplax (Anderson *et al.* 1983*a*, *b*). They appear to act as non-competitive transport inhibitors (Bahr & Parsons, 1986); there are 3–4 vesamicol receptors per synaptic vesicle (Kaufman, Rogers, Fehlmann & Parsons, 1989).

When low concentrations, 0·5–5 μM, of vesamicol were applied to frog or mammalian neuromuscular preparations, there was no change in MEPP or MEPC size for 1 h or more (Van der Kloot, 1986; Whitton, Marshall & Parsons, 1986). When preparations soaked in vesamicol or other ACh uptake inhibitors released large numbers of quanta in response to nerve stimulation or other treatments, quantal size gradually decreased (Van der Kloot, 1986; Whitton, *et al.* 1986). Apparently in intact preparations the major action of low concentrations of vesamicol is to block ACh uptake into synaptic vesicles as they are formed and loaded.

When vesamicol was added to the hypertonic solution, there was no increase nor decrease in quantal size. This fits with the interpretation that the large quanta are formed by adding additional ACh to existing quanta.

The most striking result was that after hypertonic treatment vesamicol produces a prompt and partially reversible decrease in quantal size. Similar effects were obtained with two other ACh uptake inhibitors, HNPA and TPB. The obvious interpretation is that the additional ACh that transforms quanta from usual size to large is added shortly before release.

Effect of anticholinesterase

In mouse, neostigmine blocked the increase in quantal size. This was a surprise because most frog experiments on quantal size increase were done in the presence of neostigmine. Three interpretations came to mind: (i) that in mouse, neostigmine also blocked ACh uptake into vesicles, (ii) that the effect of hypertonic treatment was to depress the activity of AChE, so quantal size could not increase above the level seen in preparations exposed to anti-AChE, and (iii) that the choline produced by ACh hydrolysis was required for the synthesis of new ACh that was added to the vesicles to produce large quanta. Neostigmine does not readily enter cells, so an action on the vesicle seems improbable. The best way experimentally to distinguish between the

other two alternatives was by using other methods to promote or block choline uptake.

The first step was to add choline to the hypertonic solution containing neostigmine. Quantal size still did not increase. However, this experiment must be interpreted with caution. ACh is a potent choline uptake inhibitor. Among the drugs tested by Yamamura & Snyder (1973), ACh was second to HC-3 as the most effective inhibitor of HAChT. One micromolar ACh decreased the uptake of 0·5 μM-[^{3}H]choline by 70%. Even if the concentration of the applied [^{3}H]choline was as high as 100 μM, 1 μM-ACh still inhibited part of its uptake. AChE inhibitors like neostigmine may also affect choline uptake directly (Yamamura & Snyder, 1973), but it is difficult to decide whether the effect is due to accumulating ACh or the AChE inhibitor itself.

Inhibition of high affinity choline transport

Another approach was to use known inhibitors of the HAChT such as HC-3 (Elmqvist & Quastel, 1965; Yamamura & Snyder, 1973). Five micromolar HC-3 did not alter MEPP amplitude in normal rat diaphragm, showing that it does not have notable postsynaptic effects at this concentration (Elmqvist & Quastel, 1965; Lupa *et al.* 1986). HC-3 also inhibits ACh transport into synaptic vesicles, but only at higher concentrations ($IC_{50} = 300$; Anderson *et al.* 1983*a*, *b*). The K_i of HC-3 for choline uptake inhibition is 25 nM (Ducis & Whittaker, 1985). The concentrations (1–2 μM) used in our experiments did not affect amplitude or size of MEPCs in unstimulated mouse preparations. HC-3 did block the increase in quantal size produced by hypertonic treatment, presumably by selectively inhibiting HAChT. The Li^+ results also fit this hypothesis.

Replacement of Na^+ by Li^+ in the hypertonic solution blocked the quantal size increase. Since Na^+ is required for HAChT, this result fits the hypothesis that choline uptake is required for quantal size increase.

Elevated intracellular Na^+

In frog, treatments that should elevate intracellular [Na^+] inhibited the quantal size increase in response to hypertonic treatment or hormones (Van der Kloot, 1990). HAChT depends on the Na^+ gradient across the cell membrane, which provides energy for uptake (O'Regan *et al.* 1984; Ducis & Whittaker, 1985). An increase in intracellular [Na^+] reduces choline uptake (Ducis & Whittaker, 1985). However, in frog the effect of intracellular [Na^+] elevation was not antagonized by adding choline and quantal size increased in the presence of anti-AChE. The increase in quantal size occurred in hypertonic solutions in which all the Na^+ was replaced with sucrose. So there is no convincing evidence in frog that the block by a rise in $[Na^+]_{in}$ was due to inhibition of choline uptake.

In mouse ouabain, dihydroouabain, K^+-free solution, and aconitine inhibited the quantal size increase in response to hypertonic treatment (Fig. 3). The possibility that a rise in intracellular [Na^+] inhibited choline uptake was tested by adding choline to the solutions; with 20–40 μM-choline there were significant increases in quantal size (Fig. 3). This suggests that a supply of choline and choline uptake is necessary for the formation of large quanta in mouse. After entering nerve terminals, choline will be incorporated into ACh. The demand for choline implies that the synthesis of ACh is required for the quantal size increase.

Monoethylcholine

We tested the idea that newly synthesized ACh is loaded into large quanta by using the false transmitter precursor, MECh (Colquhoun *et al.* 1977; Large & Rang, 1978*a*, *b*). MECh had no effect on resting preparations, so it seems to have little postsynaptic effect. When MECh was added to hypertonic solution, the large quanta produced by the treatment had lower amplitudes and decayed more rapidly. This effect was observed even when MECh was added after hypertonic treatment. The decreased amplitude and more rapid decay would be expected if AMECh was released as part of the quanta, because it opens endplate channels for a shorter time.

This suggests a model in which hypertonic treatment promotes the incorporation of additional newly synthesized ACh from the cytoplasm into quanta that already contain the usual complement of ACh. The resulting fall in cytoplasmic [ACh] might stimulate choline uptake to maintain ACh synthesis.

Inhibitors used after treatment

Further evidence for this model of quantal size increase came from experiments in which vesamicol was applied after hypertonic treatment; within minutes size was reduced to pre-treatment levels. There seems to be two explanations for this vesamicol effect. First, that it blocks the uptake of the additional ACh that produces large quanta, so once the available large quanta are released they are not replaced. The second explanation follows from the work of Kaufman, *et al.* (1989); they allowed *Torpedo* vesicles to accumulate labelled ACh and then added vesamicol. Vesamicol released the labelled ACh from the vesicles, but did not decrease their content of previously accumulated, non-labelled ACh. It seems that a single vesamicol molecule binding to the vesicle membrane, by an indirect mechanism, causes the loss of the recently accumulated ACh. Therefore, we might suppose that hypertonic treatment makes all of the quanta in the terminal large, and then vesamicol causes them to dump enough ACh to return to normal size.

This second explanation is not easily reconciled with the MECh results or finding that the HC-3 also decreased quantal size when applied after the hypertonic treatment. HC-3 acted after about a 10 min delay and did not decrease size as much (Figs 1 and 2). The time difference might occur because vesamicol promptly blocks the addition of ACh to quanta, while HC-3 has an effect only after the pre-existing cytoplasmic ACh supply is used up.

Whatever the precise explanation in mouse, it is clear that there are appreciable differences with frog, where the large quanta released after hypertonic or hormone treatment are not diminished by subsequent exposure to vesamicol or other ACh uptake inhibitors.

Models for quantal size up- and down-regulations

To summarize, the data on the effects of hypertonic treatment at the mouse neuromuscular junction appear to fit the following model. In the terminal there is a large number of quanta containing the usual supply of ACh. The increase in tonicity promotes the addition of extra newly synthesized ACh to quanta, so their release produces larger MEPPs, MEPCs and uni-quantal EPPs. The increase can be prevented by inhibitors of ACh transport into synaptic vesicles and by treatments that depress choline uptake.

The size of large quanta can be decreased quite promptly by vesamicol or after a delay by blockers of HAChT, this suggests that hypertonic treatment produces a condition in which additional ACh is added to quanta that are close to release (Fig. 12*A*). The additional ACh loaded into the vesicles is probably newly synthesized

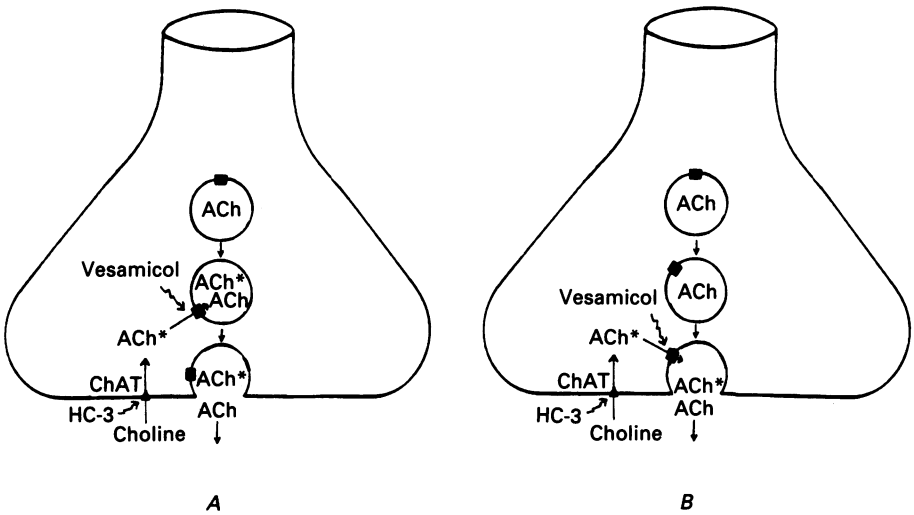

Fig. 12. Alternative models for the up- and down-regulation of quantal size. *A*, vesicles are formed in the nerve terminal with their usual ACh content. Hypertonic treatment stimulates the additional loading of newly synthesized ACh (ACh*) into synaptic vesicles. The high loaded vesicles are released and generate miniatures. Vesamicol blocks the loading of ACh*. *B*, the vesicle contains only its usual complement of ACh, but, due to the hypertonic treatment, the ACh transporters in the vesicle membrane remain active during exocytosis. ACh* is pumped directly from cyotplasm to synaptic cleft. Vesamicol blocks the ACh transporters. HC-3 inhibits HAChT, so the store of ACh* in the cytoplasm is decreased. Therefore the quantal size increase is blocked in all of the models.

ACh, since when MECh is in the extracellular fluid AMECh soon appears in the large quanta. Choline acetyltransferase (CAT) has long been described as a cytoplasmic enzyme, but recent work shows that some is membrane bound, and there is a possibility that choline uptake, synthesis to ACh and packaging into quanta are closely coupled (Rylett, 1989).

A second possibility, which we cannot reject, is that the additional ACh is added to the quantum after the vesicle releases its contents by exocytosis, ACh flowing directly from cytoplasm to synaptic cleft via transporters inserted into the membrane by fusion of the vesicle into the terminal (Fig. 12*B*). This seems unlikely because of the enormous flux of ACh required through a small patch of membrane. It is more attractive to imagine that the additional ACh is added to the vesicles when they are docked at a release site, and that the entire contents are then released by exocytosis. Of course both mechanisms illustrated in Fig. 12 might operate side by side, or other more complicated models are possible.

Conclusion

One of the fascinating properties of chemical synapses is that they are not static. It is well known that the number of quanta released by stimulation is regulated by

past history (Katz, 1969). The present experiments show that the basic unit of release, the quantum, is also regulated. The extent to which changes in quantal size play a role in the operations of the nervous system remains unknown. Quantal size is increased following stimulation at the neuromuscular junction (Vrbová & Wareham, 1976; Van der Kloot & Van der Kloot, 1985), in autonomic ganglia (Koyano, Kuba & Minota, 1985), and in hippocampus (Hess, Kuhnt & Voronin, 1987). The possible functional role of alterations in quantal size awaits exploration.

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