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BLOCKAGE OF SYNAPTIC RELEASE BY BRIEF HYPERPOLARIZING PULSES IN THE NEUROMUSCULAR JUNCTION OF THE CRAYFISH

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SUMMARY

1. Synaptic currents were evoked at the neuromuscular junction of the deep extensor abdominal muscle of the crayfish by direct depolarization of motor nerve endings.

2. Quantal content and time course of neurotransmitter release were determined from delay histograms of unitary release events recorded with a macropatch clamp technique.

3. Synaptic facilitation was elicited by pairing depolarizing pulses at intervals ranging from 10 to 200 ms. At 14 °C the duration of facilitation was about 50 ms. Reducing activity of the Na_o^+–Ca_i^{2+} exchange by lowering $[Na^+]_o$ by 50% resulted in prolonged facilitation, which lasted approximately 150 ms.

4. Normalized synaptic delay histograms at normal $[Na^+]_o$ and 50% $[Na^+]_o$ were the same for the first and the facilitated second response, indicating that activity of the Na^+–Ca^{2+} exchange does not determine the time course of release.

5. The application of a hyperpolarizing post-pulse after the first depolarizing stimulus reduced release and altered its time course to a similar extent both in normal and in 50% $(Na^+]_o$. However, it did not affect the level and the time course of release of the facilitated response.

6. A hyperpolarizing post-pulse given after the first and second pulses of a pair reduced release to the same extent for the two depolarizing pulses.

7. These results indicate that whereas manipulations thought to increase $[Ca^{2+}]_i$ (i.e. reducing activity of the Na_o^+–Ca_i^{2+} exchange or facilitation) affect the quantal content, they do not influence the time course of release. However, changes of membrane potential do affect the quantal content, and more importantly the time course of release, thus suggesting a contributory role of membrane potential in the control of synaptic release.

INTRODUCTION

The rise of calcium concentration inside presynaptic endings is necessary for evoked transmitter release (Katz, 1969). The 'Ca^{2+} hypothesis' assumes the rise in

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Ca^{2+} concentration in the terminal, after the action potential, to also be sufficient for transmitter release (Augustine, Charlton & Smith, 1987). Consequently, the time course of release is assumed to be controlled by the rapid entry and rapid removal of Ca^{2+} from below the release sites (see review, Augustine *et al.* 1987). However, there is some evidence which casts doubt on this latter assumption. The time course of release is much shorter than the time required for calcium removal from the terminals (cf. review, Parnas, Parnas & Segel, 1990). Furthermore, the time course of release has been found to be invariant under conditions thought to change the intracellular calcium concentration (Barrett & Stevens, 1972; Andreu & Barrett, 1980; Datyner & Gage, 1980). Various hypotheses have been put forward to account for these discrepancies, their basic tenet being that calcium is unevenly distributed inside the terminal and that its overall level, as determined by current methods, cannot be established in specific domains in which calcium channels and releasing sites are in close proximity. In such domains, the calcium turnover might differ from that in the cytosol (Chad & Eckert, 1984; but see Parnas, Hovav & Parnas, 1989 and Delaney, Zucker & Tand, 1989).

Another observation difficult to reconcile with the view of $[Ca^{2+}]_i$ as the only limiting factor for transmitter release is that a brief hyperpolarizing pulse (as short as 0·5 ms), applied either before or after a depolarizing stimulus, does suppress the evoked release of transmitter in crayfish and frog neuromuscular junctions (Dudel, 1984; Parnas, Parnas & Dudel, 1986*a*). This phenomenon has been interpreted in two ways: (a) the hyperpolarization reduces calcium entry and thereby lowers release (Zucker, 1987), and (b) there is a direct influence of membrane potential on release, independent of calcium channel openings (Parnas, Dudel & Parnas, 1986*b*). In this latter view, which was formulated as the 'Ca^{2+}–voltage hypothesis' (Parnas *et al.* 1986*b*; Parnas, Parnas & Dudel, 1986*c*; Parnas & Parnas, 1986, 1988), depolarization promotes release by two separate mechanisms: by allowing Ca^{2+} entry and by rendering the release mechanism sensitive to Ca^{2+}.

Recently it was demonstrated that after injecting nitr-5 into crayfish terminals, and increasing intracellular Ca^{2+} concentration by a flash of light, a depolarizing impulse increased the level of release under conditions which do not support entry of Ca^{2+} (Hochner, Parnas & Parnas, 1989), thus supporting the Ca^{2+}–voltage hypothesis.

Nevertheless, it is possible that the brief hyperpolarizing impulse given immediately after a depolarizing test pulse, in addition to its direct effect on the release mechanism, also affects the entry of Ca^{2+} ions. Here we report experiments that make this second possibility unlikely.

At present, the spatial and temporal resolution necessary to determine the rapid and perhaps minutely discrete changes of calcium concentration at releasing sites is unachievable. Instead, use has been made of indirect approaches, such as estimating the presence of Ca^{2+} in the presynaptic terminal, under the membrane, by monitoring a Ca^{2+}-induced potassium current (Connor, Kretz & Shapiro, 1986), or quantifying an event known to result from the action of Ca^{2+} on the release mechanism itself. For example, facilitation (F) is ascribed to the presence of a residual amount of free Ca^{2+} or residual Ca^{2+} complex inside the terminal after the initial release (Katz & Miledi, 1968). Therefore facilitation, and especially its duration, can be used to estimate the

time course of removal of Ca^{2+} (free or complexed) which is effective for release (Parnas & Segel, 1989).

In the present work, facilitation was used to determine whether the suppression of depolarization-evoked release consequent to a hyperpolarizing stimulus (post-pulse) affects entry of Ca^{2+}.

Three experimental approaches were used. In the first, a brief hyperpolarizing pulse was applied subsequent to a depolarizing pulse of varying amplitude, not just one amplitude as done before (Dudel, 1984; Parnas *et al.* 1986*a*). Following a short interval, a second depolarizing pulse of a constant amplitude was administered. The quantal content and the time course of release of the first depolarizing pulse at varying amplitude were altered by the hyperpolarizing pulse, whereas the quantal content and the time course of release of the second constant test pulse were not affected.

In the second set of experiments the rate of activity of the Na_o^+–Ca_i^{2+} exchange was reduced, and the time course of facilitation was prolonged (Parnas, Parnas & Dudel 1982*a*; Meiri, Zelinger & Rahamimoff, 1986), but the time course of release after the first or second depolarizing pulses remained the same as in controls.

In the third set of experiments brief hyperpolarizing impulses were given after equal first and second depolarizing pulses of a pair. We found that release was reduced by the same extent following both pulses even though the quantal content of the second impulse was higher due to facilitation.

Together these results appear to support the hypothesis that changes in intracellular Ca^{2+} concentration do not affect the time course of release, and support the proposition that changes in membrane potential contribute to the release process in addition to their known effect on the entry of Ca^{2+} ions.

METHODS

Crayfish, *Procambarus clarki*, were obtained from Atchafalaya Co., LA, USA. The neuromuscular system of the deep extensor abdominal muscles of the crayfish was used (Parnas & Atwood, 1966). For long-term recordings it was found preferable to use small animals (4·0–4·5 cm) and to leave the muscles attached to their natural insertions. The abdominal segments were mounted in a small (1 ml) lucite chamber and perfused with van Harreveld solution: NaCl, 220 mM; KCl, 5·4 mM; $MgCl_2$, 3·0 mM, $CaCl_2$, 13 mM; Tris-maleate, 10 mM. In experiments where sodium concentration was reduced to 50%, choline was substituted for sodium to maintain equimolarity. The Ca^{2+} concentration was varied within a range of 3 to 13 mM. The pH was adjusted to 7·4.

The fluid was circulated through the recording chamber at a rate of 1 ml/min and temperature was kept constant with an accuracy of $\pm 0{\cdot}5$ °C, within a range of 5–14 °C. Depolarization of synaptic terminals and recording of currents were done with the macropatch technique (Dudel, 1981). Tetrodoxin (5×10^{-7} M) was used to block excitability. The electrodes had an inner diameter of 18 μm and an outer diameter of 22 μm.

Data were stored on a video cassette recorder connected to a neuro-corder (Neurodata) with 20 μs/address A/D conversion. In parallel, the sweeps were fed on-line through a Nicolet 2090 (20 or 50 μs A/D conversion) to an Olivetti M-28 computer. Using software prepared in our laboratory, the data were converted to delay and peak histograms.

RESULTS

Previous studies using the frog neuromuscular junction and the crayfish opener muscle (Dudel, 1984; Parnas *et al.* 1986*a*) have shown that a brief hyperpolarizing

post-pulse reduces the quantal content induced by a depolarizing pulse. Facilitation is not affected by such hyperpolarization (Dudel, Parnas & Parnas, 1983). Since the magnitude of test pulse facilitation (see below) is thought to reflect residual Ca^{2+} from the first depolarizing pulse, it was concluded that post-pulse hyperpolarization

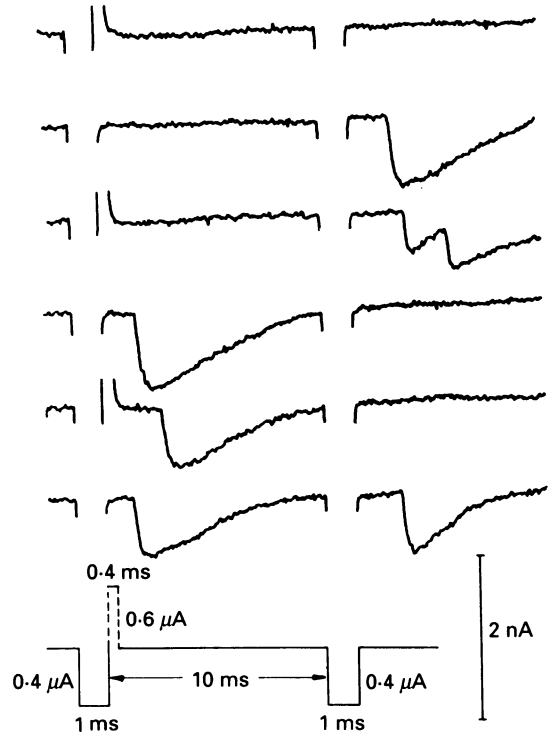

Fig. 1. Sample recordings of synaptic currents taken at a temperature of 6 °C using a stimulus regime as shown diagrammatically below (depolarizing pulse negative deflection) and applicable to the experiment of Fig. 2. The hyperpolarizing post-pulse is shown by dashed lines and is applied after the first pulse in each alternating pair of pulses (first, third and fifth pairs). $[Ca^{2+}]_o$ = 13 mM. Repetition rate 1 Hz.

does not affect Ca^{2+} entry. It was, however, argued that facilitation is not sensitive enough to detect small changes in Ca^{2+} entry (Zucker, 1987).

In the present experiment we extend the test pulse facilitation measurement for different levels of first impulse depolarization. Usually facilitation is measured as the ratio of release produced by a second pulse of a pair to that produced by an equal first pulse. With such an experimental protocol, as the quantal content of the pulses increases, facilitation decreases (Parnas, Dudel & Parnas 1982*b*; Parnas & Segel, 1989). Therefore the level of this type of facilitation cannot be used as a convenient measure for Ca^{2+} entry during a first pulse of variable amplitude. Instead, a different procedure, the constant test pulse facilitation (Dudel *et al.* 1983), was adopted. Here, a constant depolarizing test pulse was given after a variable first pulse, with facilitation being defined as the ratio of release of the constant test pulse when given

after a varying first pulse to the level of release produced by the test pulse when given alone. The level of depolarization of the test pulse is constant, and therefore the same entry of Ca^{2+} is assumed when it is given alone or after a depolarizing first pulse. Hence, facilitation of the constant test pulse might reflect, without contamination, the Ca^{2+} remaining from the first pulse. If the post-pulse hyperpolarization reduces Ca^{2+} entry during the first pulse, test pulse facilitation is expected to be smaller. In order to maximize such an effect, the depolarization levels of the first pulse should be very small or very large, as under these conditions entry of Ca^{2+} during the depolarization pulse is small, and the presumed reduction in the amount of Ca^{2+} entry by the post-pulse hyperpolarization will be significant. For depolarizations at which entry of Ca^{2+} during the first pulse is large, the hyperpolarizing post-pulse effect should be relatively small.

Results of such an experiment are shown in Figs 1 and 2. To improve the control conditions for the post-pulse effect, we alternated a pair of depolarizing pulses, a first pulse and a constant test pulse, with a pair of the same depolarizing pulses but with a post-pulse hyperpolarization after the first pulse (Fig. 1). We thereby eliminated possible artifacts due to run-down of the preparation over time. Such a protocol was repeated at one synapse for several levels of first pulse depolarization.

In the experiment illustrated in Fig. 2, the constant test pulse had an amplitude of $-0{\cdot}5\ \mu A$ and the first pulse varied between $-0{\cdot}5$ and $-1{\cdot}1\ \mu A$. Test pulse facilitation varied, as described before (Dudel *et al.* 1983), as a function of the intensity of the first pulse, increasing in the range of $-0{\cdot}5$ to $-0{\cdot}7\ \mu A$, suggesting an increase of Ca^{2+} entering the terminal as depolarization increased. At $-0{\cdot}9$ and $-1{\cdot}1\ \mu A$, facilitation decreased, suggesting a reduced entry of Ca^{2+} (Fig. 2*B*). As previously found for one level of a depolarizing pulse (Parnas *et al.* 1986*a*), the hyperpolarizing pulse was quite effective in reducing release of the first pulse at all levels used. It reduced the quantal content of the first pulse in the range of 30–50% (Fig. 2*A*). It nevertheless had no effect on the quantal content of the test pulse and facilitation was not altered at any of the first pulse current intensities (Fig. 2*B*). The delay histogram in Fig. 2*C* shows that the time course of the facilitated response was also not altered by the hyperpolarizing post-pulse given following the first pulse.

Effects of slowing Ca^{2+} extrusion

According to the Ca^{2+} hypothesis, any slowing down of the Ca^{2+} removal process should affect not only facilitation but also the time course of release when the level of intracellular Ca^{2+} concentration is higher than normal (G. Hovav, H. Parnas & I. Parnas, unpublished observation). In nerve terminals loaded with the tracer $^{45}Ca^{2+}$, the rate of tracer efflux decreased by a factor of about ten when extracellular sodium concentration was reduced under conditions of low extracellular Ca^{2+} concentration (Blaustein, 1988). Reduction of the rate of activity of the sodium–calcium exchange by lowering $[Na^+]_o$ resulted in prolongation of facilitation (Parnas *et al.* 1982*a*; Meiri *et al.* 1986). Thus, in a series of experiments, $[Na^+]_o$ in the bath was lowered to half its normal value (110 mM). One common consequence of these manipulations was a reduction in amplitude of the unitary responses. This reduction became a useful criterion to evaluate the time for exchange of solution in the bath. As seen in Fig.3, the response amplitude was reduced to less than 50% of its normal size. The traces

at the left are samples taken at 110 mM-Na^+ (upper recordings) and at 220 mM-Na^+ (lower recordings), illustrating various amplitudes in the population of recordings. At the right is shown the amplitude distribution histogram. At the 50% $[Na^+]_o$ concentration the range of amplitudes is from 100 to 750 pA, with a mean at 300 pA,

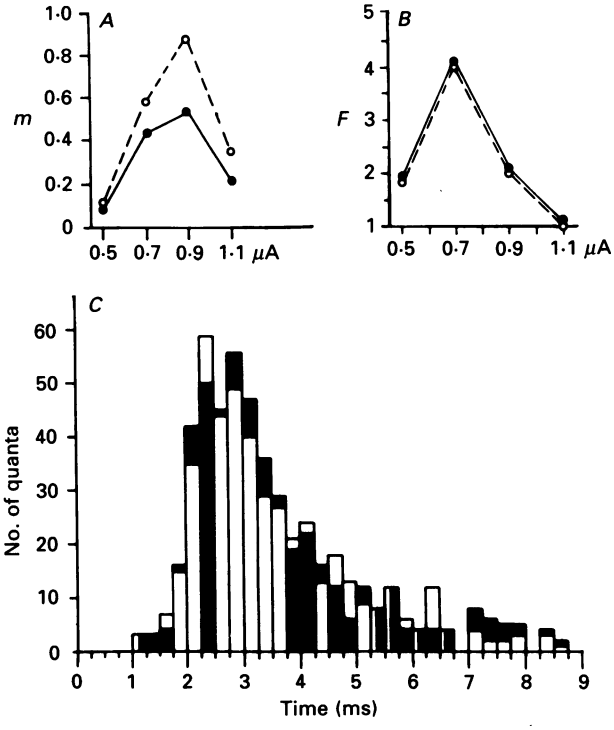

Fig. 2. The effect of a hyperpolarizing post-pulse on the quantal content of the first pulse, on facilitation of a test pulse and on the delay histograms of the test pulse. Stimulus parameters: first pulse 0·7 ms duration, and variable amplitudes as shown in *A*. The hyperpolarizing post-pulse was of 0·7 ms duration and +0·5 μA in amplitude. The test pulse given with an interval of 10 ms was −0·5 μA, 0·7 ms duration. Repetition rate 1 Hz. *A*, quantal content of the first pulse when given alone (○) at the different pulse amplitudes and with the post-pulse hyperpolarization (●). *B*, facilitation of the test pulse (○) was not affected by the post-pulse hyperpolarization (●). *C*, open bars: delay histogram of the test pulse when given after a first pulse of 0·7 μA. Filled bars, the depolarization–hyperpolarization combination. The total number of pulses was 2350 (1175 for each histogram) and the quantal content was 0·41 and 0·40 for the open and filled histograms respectively. Temperature 6 °C. $[Ca^{2+}]_o$ = 13 mM.

whereas at 220 mM the amplitude ranges from 450 pA to 1·5 nA with a mean at 600 pA. The effect was reversible.

It was first necessary to recheck the effects on facilitation of reducing extracellular sodium concentration in the deep abdominal extensor muscles. The duration of facilitation was prolonged in 50% $[Na^+]_o$. In normal saline solution facilitation at 14 °C was over by 40–50 ms, while at 50% $[Na^+]_o$ facilitation was still apparent after 150 ms. In six experiments, after 100 ms, facilitation in 50% $[Na^+]_o$ was $1{\cdot}26 \pm 0{\cdot}04$ (S.D.).

In Fig. 4 the time course of release of the first and second pulses of a pair under conditions of normal and 50% $[Na^+]_o$ are compared. Two pulses were given with an interval of 50 ms. In normal $[Na^+]_o$, 108 quanta, out of the 1000 pulses given, were released after the first pulse (Fig. 4*A*). After the second pulse 109 quanta were

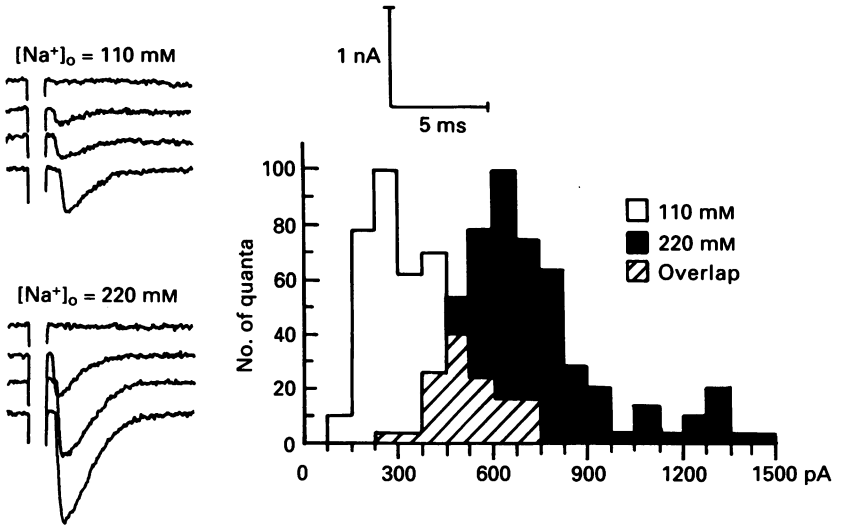

Fig. 3. Amplitude histograms of single quantal events recorded in 110 mM $[Na^+]_o$ (□, ▨) and 220 mM $[Na^+]_o$ (■, ▨). Samples of single quanta showing relative differences in amplitude are shown at the left. The experiment was performed at a temperature of 14 °C. $[Ca^{2+}]_o$ = 3 mM.

released (Fig. 4*A*). Thus there was no facilitation. The median for both delay histograms was 1·95 ms. In 50% $[Na^+]_o$, m_1 (quantal content for first pulse) was 0·13 (Fig. 4*B*) and m_2 (quantal content for second pulse), 0·27, hence facilitation was about 2. The medians of the delay histograms of the first and second pulses were 2·0 and 2·1 ms respectively. Superposition of the delay histograms of the responses at low and high $[Na^+]_o$ solution shows that the time course is virtually the same (Fig. 4, *C* and *D*). This result indicates that the rate of activity of the Na_o^+–Ca_i^{2+} exchange which exerts a significant effect on facilitation does not regulate the time course of evoked release.

Hyperpolarization blocks release under conditions of slowed Ca^{2+} extrusion

Twin impulses separated by 50 ms and 100 ms were given at 50% $[Na^+]_o$. The first pulse was followed by a hyperpolarizing post-pulse in every other pair. Figure 5 depicts the delay histograms of the first and second pulses with and without the hyperpolarizing post-pulse. The histograms are presented in three forms. In Fig. 5*A–C* the actual number of quanta per bin is given. Figure 5*D–F* presents the data as a percentage of peak amplitude (peak normalization), and in Fig. 5*G–I* the histogram is presented as a percentage of total release.

The quantal content of the first pulse was 0·25 (n = 1000) and, with the post-pulse hyperpolarization, 0·07. In addition, following hyperpolarization the peak of the

histogram shifted to the left and blockage of release is most apparent in the time bins immediately following the peak. The median of the control histogram was 2·6 ms, and with the hyperpolarization, 2·2 ms. The effect is more apparent in the normalized histograms. Peak normalization (Fig. 5*D*) shows a clear shift of the peak to the left,

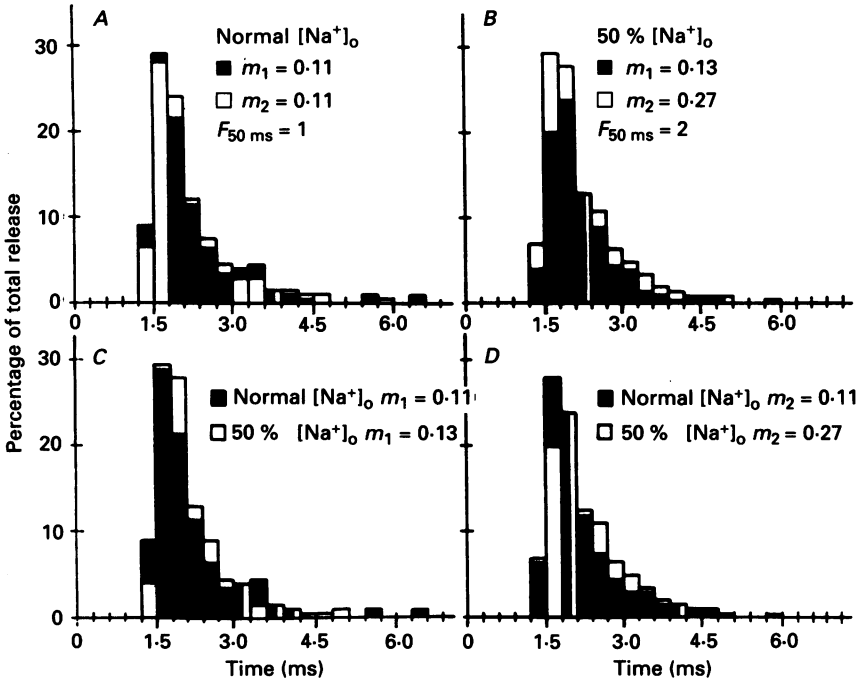

Fig. 4. Delay histograms obtained in normal $[Na^+]_o$ and in 50 % $(Na^+]_o$. Twin depolarizing impulses of 1 ms duration and $-0{\cdot}6$ μA in amplitude were given with an interval of 50 ms. Repetition rate was 1 Hz. For each histogram 1000 pulses were given (2000 alternating pairs). Histograms are given as a percentage of total release. *A*, the histograms of the first and second responses at normal $[Na^+]_o$ are superimposed. The median for both histograms was 1·95 ms. The quantal content of the first pulse m_1 (■) was 0·11, and m_2 (□) was also 0·11. Thus facilitation was over. *B* as in *A*, but in 50 % $[Na^+]_o$. m_1 (■) = 0·13 and m_2 (□) = 0·27. Facilitation is 2. The medians were 2·0 and 2·1 ms respectively. *C* and *D*, superposition of the histograms of the first responses (*C*) and second responses (*D*) at normal and 50 % $(Na^+]_o$ show that their time course is the same. Temperature 13 °C. $[Ca^{2+}]_o$ = 3 mM.

and percentage normalization (Fig. 5*G*) indicates that a much larger fraction of the release occurs at earlier bins. Similar results were obtained in fourteen experiments. The reduction in the quantal content by the hyperpolarizing post-pulse was 51·2 ± 0·13 % (mean ± S.D.), and the median of the histograms shifted to the left by 0·2–0·5 ms. In these fourteen experiments, the reduction of the quantal content by the hyperpolarizing post-pulse in normal $[Na^{2+}]_o$ was the same: 50 ± 0·11 %. The medians of the histograms also shifted to the left, by the same extent as in 50 % $(Na^+]_o$.

The second pulse, in 50 % $[Na^+]_o$, showed facilitation of 1·8 for the 50 ms intervals, and 1·2 for the 100 ms interval. The delay histograms were the same for the releases obtained with and without the hyperpolarizing post-pulse, and the medians were not altered (Fig. 5*B* and *C*). Normalization and superposition shows that the histograms

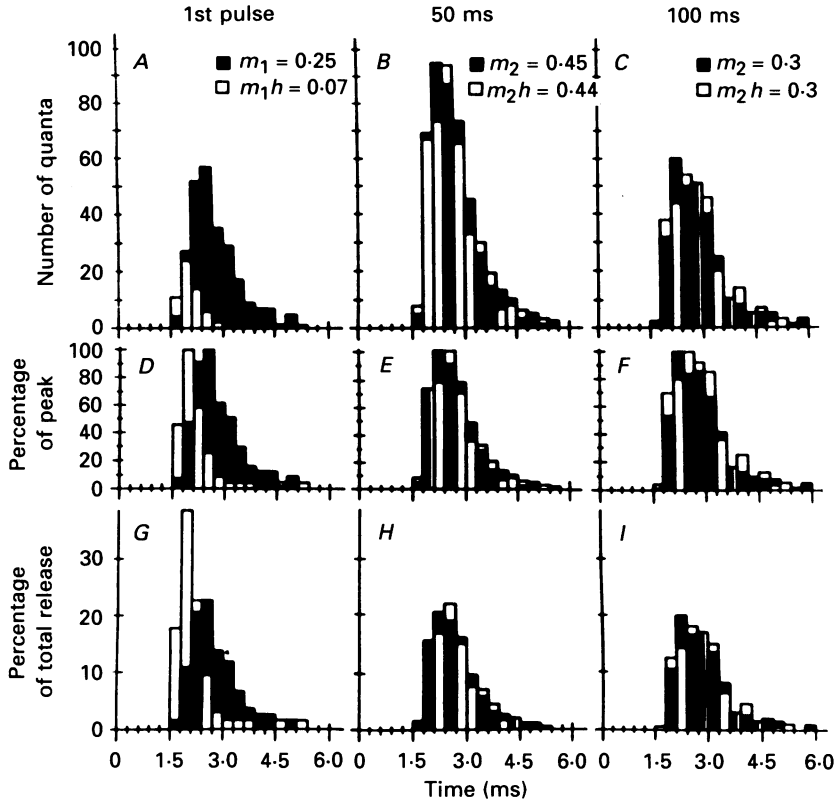

Fig. 5. The effect of a hyperpolarizing post-pulse on release and facilitation in 50 % $[Na^+]_o$. Twin impulses separated by 50 and 100 ms were given at a rate of 1 Hz. Depolarization pulse parameters were: 1 ms duration, −0·8 μA. In each alternate pair the first pulse was followed by a hyperpolarizing post pulse of 0·5 ms duration and +0·6 μA amplitude. The histograms with (open bars) and without (filled bars) the post-pulse hyperpolarization are superimposed. *A*, absolute values; *D*, normalized to peak; *G*, percentage of total release. The quantal content of the first pulse alone, m_1, was 0·25 ($n = 1000$). With the hyperpolarizing post-pulse, m_1h was 0·07. The median of the histograms of the first pulse alone was 2·6 ms, and with the hyperpolarization, 2·2 ms. The histograms of the second pulse, 50 ms interval, are given in *B*, *E* and *H*, and for the 100 ms interval in *C*, *F* and *I*. Quantal contents of the second pulse at the two intervals are given in *B* and *C*. The median for the histograms in *B* for both m_2 values was 2·6 ms, and for the histograms in *C*, 2·7 ms. For further details see text.

are virtually the same (Fig. 5*E*, *F*, *H* and *I*). It should be emphasized that the hyperpolarizing pulse, when given alone, had no effect on the quantal content or on the delay histogram even when given 10 ms before the second pulse.

We conclude that in 50 % $[Na^+]_o$ the hyperpolarizing post-pulse reduces release of

the first pulse to the same extent as in normal $[Na^+]_o$, and that despite the slower extrusion of Ca^{2+}, it had no effect on the time course of the facilitated responses.

Effect of post-pulse hyperpolarization on the facilitated response

Figure 6 (left) shows recordings of an experiment (normal $[Na^+]_o$) in which a brief hyperpolarizing pulse was given after both the first and second depolarizing pulses of a pair (10 ms apart). These alternated with pairs of depolarizing pulses alone. The first and second depolarizing pulses were equal in amplitude, as were the hyperpolarizing pulses. On the right-hand side of Fig. 6 (*A–D*), different combinations of stimulation patterns are given. When only two depolarizing pulses were given (*A*), m_1 and m_2 were obtained. When only the first depolarizing pulse was followed by hyperpolarization, m_1h and $m_2(m_1h)$ were obtained (*B*); $m_2(m_1h)$ designates the quantal content of the second pulse when it followed m_1h. Other combinations are given in *C* and *D*. These combinations are important in order to compare experimental results with predictions from the Ca^{2+} hypothesis and the Ca^{2+}–voltage hypothesis for neurotransmitter release (see Discussion). Table 1 summarizes results obtained at three sites in the same preparation (seven sites in three preparations yielded similar results). The results obtained from site 1 are described here in detail. The quantal content, m_1, of the first depolarizing pulse was 0·84. When followed by the hyperpolarization, the quantal content, m_1h, was 0·2. Thus in this case release was reduced by 76%. The quantal content of the second pulse was 1·24, both after m_1 or after m_1h (see also Fig. 2*B*). When the second pulse was followed by the same hyperpolarizing pulse, the quantal content, m_2h, was 0·33. The values $m_2h(m_1)$ and $m_2h(m_1h)$ were the same (not shown). Similarly to the reduction in the quantal content after the first pulse, the reduction after the second pulse was 74%. The results obtained for sites 2 and 3 show essentially the same trend, namely that hyperpolarizing post-pulses given after the first and second pulses reduce release to the same proportional extent.

DISCUSSION

In this study we again demonstrate that the time course of release is not altered by treatments thought to change intracellular Ca^{2+} concentration (review, Parnas *et al.* 1990). In particular we show that reducing the activity of the Na_o^+–Ca_i^{2+} exchange by lowering $[Na^+]_o$ (Blaustein, 1988) had a significant effect on the duration of facilitation without any effect on the time course of evoked release. It was already assumed that the activity of the Na_o^+–Ca_i^{2+} exchange is not involved in determining the time course of evoked release (Sanchez-Armass & Blaustein, 1987), and we reach the same conclusion after direct measurements. We also found that post-pulse hyperpolarization reduces release to the same extent in normal and in 50% $[Na^+]_o$ solution. The activity of the Na_o^+–Ca_i^{2+} exchange is modulated by changes in membrane potential and it has been suggested that rapid changes in membrane potential may affect the activity of the Na_o^+–Ca_i^{2+} exchanger (Blaustein, 1988). Therefore, it could be argued that the brief (0·5 ms) hyperpolarizing post-pulse increases the activity of the Na_o^+–Ca_i^{2+} exchange, thereby accelerating termination of release. This is unlikely as we demonstrate that the time course of evoked release is not at all affected by the activity of the exchanger.

Another explanation for the more rapid termination of release after a hyperpolarizing post-pulse is the proposition that Ca^{2+} entry is reduced (Zucker, 1987). Again, this is unlikely, as the time course of release is the same with very low and very high levels of Ca^{2+} entry (Andreu & Barret, 1980), and in the present study test

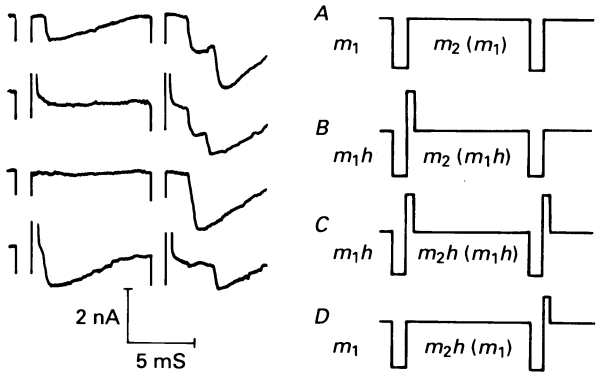

Fig. 6. A facilitation experiment in which post-pulse hyperpolarization was given after the first and second pulses. Paired depolarizing pulses (−0·6 μA amplitude, 1 ms duration, 10 ms interval) alternated with paired depolarizing–hyperpolarizing combinations in which the post-pulse hyperpolarization was 0·3 ms in duration and +0·6 μA in amplitude. Sample records show alternation between pairs. The second and fourth traces incorporate the post-pulse hyperpolarizations. *A–D*, schematic representation of quantal contents for different combinations of stimulation patterns. *A*, m_1, quantal content of first pulse alone. $m_2(m_1)$, quantal content of second pulse when given alone after m_1. *B*, m_1h, quantal content of first pulse with a hyperpolarizing post-pulse. $m_2(m_1h)$, quantal content of second pulse when given after m_1h. *C*, $m_2h(m_1h)$, quantal content of second pulse with a hyperpolarizing post-pulse when given after a first pulse which was followed by hyperpolarization (m_1h). *D*, $m_2h(m_1)$, quantal content of second pulse with a hyperpolarizing post-pulse when given after m_1.

pulse facilitation was not affected by the post-pulse hyperpolarization. At the different quantal contents of the first pulse (and therefore according to the Ca^{2+} hypothesis, at different levels of $[Ca^{2+}]_i$), hyperpolarization reduced release to the same extent. The dependence of release on calcium is sigmoidal to gradual saturation (Dodge & Rahamimoff, 1967). It should therefore be expected that at the high levels of release, small changes in Ca^{2+} would not exert a large effect on release, and this was not found experimentally.

The bulk of the experimental evidence is in favour of the residual Ca^{2+} hypothesis for facilitation (review, Parnas *et al.* 1990). Recent experiments by Tanabe & Kijima (1988, 1989), using Ca^{2+} chelators introduced into the nerve terminals, further support the residual Ca^{2+} hypothesis for twin pulse facilitation. Therefore from the facilitation experiments (especially such as those shown in Fig. 6 and Table 1), it is possible to calculate the putative reduction in the Ca^{2+} entry by the hyperpolarizing pulse, if this is in fact the mechanism. Release was suggested to be related to intracellular Ca^{2+} as follows (Parnas & Segel, 1980):

$$m = \frac{\bar{m}(Ca_{ir} + Y)^4}{(K_L + Ca_{ir} + Y)^4}. \quad (1)$$

In this equation, m is the quantal content, $\bar{m}$ the maximal possible quantal content, $\bar{m}$ the maximal possible quantal content, Ca_{ir} the resting Ca^{2+} concentration (μM), Y the change (in μM) in intracellular Ca^{2+} concentration after the depolarizing pulse, and K_L the Michaelis–Menten constant (μM). The co-operativity was taken as 4 (Dodge & Rahamimoff, 1967; Parnas, Parnas & Segal, 1986*d*). The values of $\bar{m}$, Ca_{ir}

TABLE 1. Quantal contents for twin pulses alone and with a hyperpolarizing post pulse

Site	m_1	m_1h	m_2	m_2h
1	0·84	0·2	1·24	0·33
2	0·52	0·2	0·76	0·28
3	0·45	0·13	0·62	0·18

The quantal contents are given for the same experiment as in Fig. 6. The quantal contents recorded from three release sites in one preparation are shown as four values per site: m_1 and m_2 represent the quantal content of the first and second pulses of a depolarizing pair of pulses alone. m_1h and m_2h represent quantal contents when the first and second pulses were followed by a hyperpolarizing pulse.

and K_L are not known, but can be estimated from experimental findings. In the crayfish opener and deep extensor abdominal neuromuscular systems, maximal measured quantal content for single pulses is less than 10. We took $\bar{m}$ in our calculations to be either 10 or 20. The resting Ca^{2+} concentration measured in several preparations (Cohen, Connor & Kater, 1987; Connor, Wadman, Hockberger & Wong, 1988) is in the range of 0·01–0·1 μM. According to some investigators, K_L can be as low as 1 μM while according to others it is in the range of hundreds of micromolar (cf. review, Smith & Augustine, 1988; Delaney *et al.* 1989). In our calculations K_L varied between 1–100 μM. Although the values taken for $\bar{m}$, Ca_{ir} and K_L extend over a wide range, the differences in calculated results were very small, the reason being that the relative values of both Y and K_L with respect to Ca_{ir} are of importance.

Knowing the quantal content, m, and using eqn (1), Y can be calculated for a certain set of conditions. For example, in Table 1, for site 1, m_1 was 0·84. Taking $\bar{m} = 10$, $Ca_{ir} = 10^{-8}$ M and $K_L = 1$ μM, Y is calculated to be 1·14 μM. Taking the same parameters and $m_2 = 1{\cdot}24$. we find that Y plus the residual Ca^{2+} from the first pulse is 1·45 μM. Assuming the same entry of Ca^{2+} during the second pulse, the residual Ca^{2+} is 0·31 μM. Repeating the calculation for m_1h and m_2h (see Fig. 6 for definitions), $Y_1h = 0{\cdot}6$ μM, and the residual Ca^{2+} is 0·15 μM.

With these values and again using eqn (1), it is possible to calculate what m_2 should have been had the hyperpolarization indeed reduced Ca^{2+} entry, and to compare these predicted values (for the different stimulation configurations) with the experimental results (Table 2). For the sake of clarity, the calculations for site 1 are given in some detail. We first calculate the prediction for $m_2(m_1h)$. The Ca^{2+} available for release during $m_2(m_1h)$ is taken as the sum of Y, the entry during the second depolarizing pulse (1·14 μM), and of the 0·15 μM-residual Ca^{2+}, which is the concentration remaining from m_1h when the second pulse was given. The predicted $m_2(m_1h)$ is only 0·9 and not 1·24 as found experimentally. The ratio of the predicted ($m_2(m_1h)$ to the measured $m_2(m_1h)$ is 0·72. This calculation is repeated for $m_2h(m_1)$. Now, however, the Ca^{2+} concentration for release is taken as 0·6 μM (Y_ih) plus 0·31 μM, the residual Ca^{2+} remaining from the first pulse (m_1 alone) when the second

TABLE 2. Measured and predicted values of quantal contents for different stimulation patterns

Site	Quantal content of	Experimental result: measured m	Predicted m		Predicted m / Measured m	
			Ca hyp.	Ca–V hyp.	Ca hyp.	Ca–V hyp.
1	$m_2(m_1h)$	1·24	0·9	1·24	0·72	1
	$m_2h(m_1)$	0·33	0·5	0·3	1·51	0·91
2	$m_2(m_1h)$	0·76	0·61	0·76	0·80	1
	$m_2h(m_1)$	0·28	0·46	0·29	1·64	1·04
3	$m_2(m_1h)$	0·62	0·51	0·62	0·83	1
	$m_2h(m_1)$	0·18	0·28	0·18	1·55	1

Same experiment as in Fig. 6 and Table 1. $m_2(m_1h)$, $m_2h(m_1)$ as in the schematic representation in Fig. 6. The experimental results for $m_2(m_1h)$ and $m_2h(m_1)$ were taken from Table 1. Predicted m values for these two stimulation configurations were calculated for the Ca^{2+} hypothesis (Ca hyp.), and Ca^{2+}–voltage hypothesis (Ca–V hyp.). For details of calculations see text. The ratio between the predicted values and the measured values for the Ca hyp. and Ca–V hyp. is given in the two right-hand columns.

m_2h was given. The predicted m_2h is 0·5 while the experimental value is 0·33. The ratio of predicted m_2h to measured m_2h is 1·51. Similar calculations were made for sites 2 and 3 (Table 2). The ratios between predicted values and measured values differ appreciably. Such differences could easily have been detected with the techniques used and the number of pulses given.

If, on the other hand, we assume that the effect of the hyperpolarizing pulse is independent of Ca^{2+} entry, as the Ca^{2+} voltage hypothesis suggests, the same ratios of suppression should be obtained after the first and second pulse by the hyperpolarization. The comparison between measured values of m and predicted values of m are also given in Table 2. For example, from Table 1, site 1, m_1 was 0·84, and m_1h, 0·2; m_1h in this case is 24 % of m_1. In this site, m_2 was 1·24, and the predicted $m_2(m_1h)$ is 0·3, while the experimental value is 0·33. The ratio between predicted to measured values is 0·91 (Table 2). For the combination $m_2(m_1h)$, the Ca^{2+} voltage hypothesis predicts that m_2 and $m_2(m_1h)$ should be the same, since the reduction in m_1h is not due to a smaller entry of Ca^{2+}. This was indeed the case, as is also shown in Fig. 2. The ratio between these two values was always 1 or close to 1. Similar calculations for sites 2 and 3 show a very good agreement between the predictions of the Ca^{2+}–voltage hypothesis and the experimental results. The ratio between predicted and measured values is 1 or close to 1.

Thus the predictions from the Ca^{2+}–voltage hypothesis correspond much more closely with the experimental results, and reduction in Ca^{2+} entry cannot explain the reduction in quantal content obtained after a hyperpolarizing pulse. It therefore seems improbable that the reduction in release seen after the hyperpolarizing post-pulse is produced by closure of Ca^{2+} channels, reducing Ca^{2+} entry, and the explanation proposed by the Ca^{2+}–voltage hypothesis appears more plausible.

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