Calcium and Facilitation at Two Classes of Crustacean Neuromuscular Synapses

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ABSTRACT   The closer muscle of the crab, *Chionoecetes*, has at least two classes of excitatory neuromuscular synapses. In one class of synapses an action potential depolarizing the synaptic region releases much more transmitter if it has been preceded recently by another action potential. The other class of synapses shows this property, called facilitation, to a far lesser extent. Immediately after one conditioning stimulus the level of facilitation is similar in both classes. The rate of the ensuing decay of the facilitation is the critical factor differentiating the two classes of synapses. The relationship between external Ca^{++} concentration and transmitter release is similar for both classes of synapses. The slope of a double logarithmic plot of this relationship varies from 3.1 between 5 and 10 mM Ca^{++} to 0.9 between 30 and 40 mM Ca^{++}. Facilitation does not significantly change when tested in external Ca^{++} concentrations ranging from 7 to 30 mM. The extracellularly recorded nerve terminal action potential does not increase in amplitude during facilitation. The results suggest that the mechanism of synaptic facilitation is similar for both classes of synapses and occurs after the stage in transmitter release involving Ca^{++}.

INTRODUCTION

The muscles of crustaceans are innervated by only a few axons. This pattern of innervation is fundamentally different from vertebrate muscles where hundreds of axons control the activity of a single muscle. Usually in this latter case graded contractions are produced by the summation of the contractions of varying numbers of motor units. In contrast, in crustaceans the information for graded contractions must be encoded in the pattern of firing of the few motor axons, and mechanisms for transforming this code into graded contractions must be present at the periphery. Part of this integration relies on the properties of the muscle fibers (for review, Atwood, 1967 *a*). However, the properties of the neuromuscular synapses are another important factor. In one class of neuromuscular synapses in the closer muscle of the marine crab, *Chionoecetes*, an action potential depolarizing the synaptic

terminal releases much more transmitter if it has been preceded recently by another action potential. A second class of synapses shows this synaptic facilitation to a far lesser extent. A similar presynaptic differentiation has been found in neuromuscular synapses from the crayfish, *Procambarus*, and the crabs, *Grapsus* and *Pachygrapsus* (Bittner, 1968; Atwood, 1967 *b*; Atwood and Bittner, 1971).

The present study explores the mechanisms underlying this synaptic differentiation by measuring at both classes of synapses the time-course of the decay of facilitation and the effect of the external Ca^{++} concentration on transmitter release and facilitation.

METHODS

Preparation

All experiments were performed with the closer muscle from any of the first three walking legs of the crab, *Chionoecetes bairdi* (Rathbun). This muscle was chosen because, in addition to having synapses which show two types of facilitation, the muscle fibers are large (100–500 μm diameter), loosely bundled, and not enmeshed in extensive connective tissue.

The basic neuromuscular organization of this preparation was investigated in detail by Atwood (1965). The muscle is innervated by one inhibitory axon and two excitatory axons, one excitatory axon termed "fast" and the other "slow." Atwood found that the muscle has two quite different types of muscle fibers (as well as some intermediate fibers). One type has long sarcomeres (10–11 μm), shows no active membrane responses, and contracts in a graded fashion in response to graded depolarizations of the membrane. These long sarcomere muscle fibers are innervated only by the inhibitory axon and the slow axon (SA). The second type of muscle fibers has short sarcomeres (4–5 μm) and, in response to several summated junction potentials (jp's), often exhibits spikes or graded spikes and produces fast twitch-like contractions. These muscle fibers are innervated by the slow axon (SA) and the fast axon (FA). All data in the present study were taken from the short sarcomere muscle fibers at the distal end and interior side of the muscle. In these short sarcomere muscle fibers stimuli applied at 1/s to the SA produce jp's averaging between 0.05 and 4.0 mV. Stimulation of the FA at 1/s produces jp's of approximately 20 mV in the short sarcomere muscle fibers. Consequently, for FA experiments several branches of the nerve bundle were cut within 2–3 mm of the muscle fibers to be used in order to reduce the amplitude of the FA jp's and thus minimize nonlinear summation of unit potentials (Martin, 1955) and "nonspecific" potentials (Katz and Miledi, 1965 *a*). Cutting several of the branches did not alter the relatively uniform depolarization of the muscle fibers used because each branch tended to form endings over most of the length of each muscle fiber and because the length constant of these muscle fibers is about 1 mm (Atwood, 1965). As a result of this cutting of some of the innervation in the FA experiments, the FA jp's were reduced in amplitude to approximately the amplitude of the SA jp's in completely innervated preparations, and the SA jp's became extremely small. In SA experiments the SA jp's were recorded with the innervation intact.

When the frequency of stimulation of the FA is increased from 1/s to 10/s, the FA jp's increase at most to 1.5 times their amplitude at 1/s. In contrast to this, the SA jp's increase in amplitude at least 10 times when the stimulation frequency was increased from 1/s to 10/s. This test operationally identified the two axons. Fig. 1 A shows sample records of FA and SA jp's.

The preparation was composed of from 20 to 50 muscle fibers of both types attached on one end to a piece of the central tendon and on the other end to a strip of exoskeleton. This bundle of muscle fibers was mounted on small movable hooks in a plexiglass groove of 3.0 ml capacity.

The preparation differed from most other crustacean neuromuscular preparations in three aspects. (a) For most experiments the nerve bundle containing the two excitatory axons and the one inhibitory axon was sucked into a single glass capillary suction electrode. The SA usually had the lowest threshold and the inhibitory axon the highest with the FA intermediate. This procedure was only possible because the stimulation of the inhibitory axon could be accurately monitored in the long sarcomere muscle fibers where the inhibitory jp's are large, obvious hyperpolarizations. In any case, the inhibitory innervation of the short sarcomere muscle fibers is at most extremely small (Atwood, 1965) and may not be present at all in the specific fibers used for the present study. Simultaneous stimulation of the inhibitory axon with the excitatory axons produced no observable effects of the jp's either during steady stimulation or with paired stimuli. Stimulation of the SA and FA could be distinguished because of the lower SA threshold and the fact that the SA jp's averaged about 5% of the amplitude of the FA jp's. FA measurements were made with the SA being stimulated simultaneously. Corrections were made for the presence of the SA jp's in the facilitation experiments by subtracting the amplitude of the SA jp's from the FA measurements. However, this had a negligible effect on the results since fibers with unusually low SA innervation were chosen for FA experiments. (b) In FA experiments several branches of the nerve bundle were cut 2–3 mm from the muscle fibers to be used in order to reduce the amplitude of the jp's (see above). (c) The preparation was isolated from the leg and kept in a perfusion system. This proved to be important in maintaining longer-term recording conditions.

Solutions

The standard saline consisted of 422 mM NaCl, 39 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM KCl, and 3 mM $NaHCO_3$. The ionic concentrations are similar to those of the major ions in the local seawater except that Mg^{++} was reduced and there is no SO^{--}. The Mg^{++} concentration was determined from data on the ionic composition of the blood of the closely related *Maia* (Robertson, 1953). By the same measure the Ca^{++} concentration should be 36% higher. However, the lower value of the seawater was chosen mainly because the lower Ca^{++} concentration allows a higher frequency of stimulation to be used without contraction of the muscle. Also, the Ca^{++} activity of the blood may well be lower than its concentration if blood proteins bind significant quantities of Ca^{++}. The Ca^{++} and Mg^{++} concentrations were changed with an isotonic substitution of Na^+. The pH of the saline with HCO_3^- was approximtely 7. In two experiments where the size of the jp's was measured as a function of Ca^{++} concentration, the pH

was more closely controlled with 10 mM TES [*N*-tris(hydroxymethyl)-methyl-2-amino ethanesulfonic acid] buffer adjusted to pH 7.5 with solid NaOH. Data from these experiments are plotted with separate symbols in the results and are similar to those with HCO_3^-.

The experimental solutions were introduced continuously at one end of the plexiglass groove and aspirated from the other end. In experiments in which the Ca^{++} or Mg^{++} concentrations were varied the nerve was stimulated once every 10 s and a perfusion rate chosen so that the jp amplitude reached a new steady level within 5 min after the beginning of the solution change. The jp's were measured starting 8–10 min after the beginning of the solution change.

The solution in the groove was maintained at 8°C by water circulating in the jacket beneath the groove. Before entering the groove, the perfusing solution was cooled to 8°C as it passed through a glass tube coiled inside the cooling jacket.

Recording

The automatic averaging was done with a computer of average transients (Mnemotron, model 400B) kindly loaned to me by Dr. G. Hoyle. Except for the studies with the extracellularly recorded action potential, the number of responses used to obtain an average value with the averaging computer was determined from similar hand-measured experiments. A number of responses considerably in excess of the number required to produce a standard error of 10% or less in the hand calculations was chosen. However, for the experiments in this study the standard error for determining each average produces a false measure of the accuracy of the results since many data sets were collected over a 1–2 h period and the variability of the synaptic response over such an interval was the limiting factor for the accuracy of the results. For this reason either multiple experiments are shown or the range of values used to determine an average are given rather than the standard errors for determining the averages.

In experiments in which the preparation was stimulated with pairs or trains, the pairs or trains were repeated every 10 s, except in experiments in normal Ca^{++} or lower where the pairs were repeated every 5 s. All data presented were taken within 8 h of the dissection. No data were taken before 1 h after the dissection since there was often a slow increase in the size of the jp's during this equilibration period.

Intracellular recordings were made with glass microelectrodes filled with 3 M KCl with resistances of 2–5 MΩ. Extracellular recordings were made with electrodes filled with 5 M NaCl or 3 M KCl with resistances of 1–3 MΩ.

RESULTS

Time-Course of Facilitation in SA and FA Synapses

The time-course of facilitation was tested by placing a test stimulus at various intervals after either a single conditioning stimulus or a short train. In this study facilitation is defined as $V/V_o - 1$ where V is the amplitude of the test junction potential (jp) and V_o is the amplitude of the control jp, here the conditioning jp or the first jp in the conditioning train.

In Fig. 1 B measurements of facilitation are plotted as a function of time after the conditioning stimulus on linear coordinates from one experiment with fast axon (FA) synapses and one with slow axon (SA) synapses. The solid lines represent facilitation after a single conditioning stimulus, and the
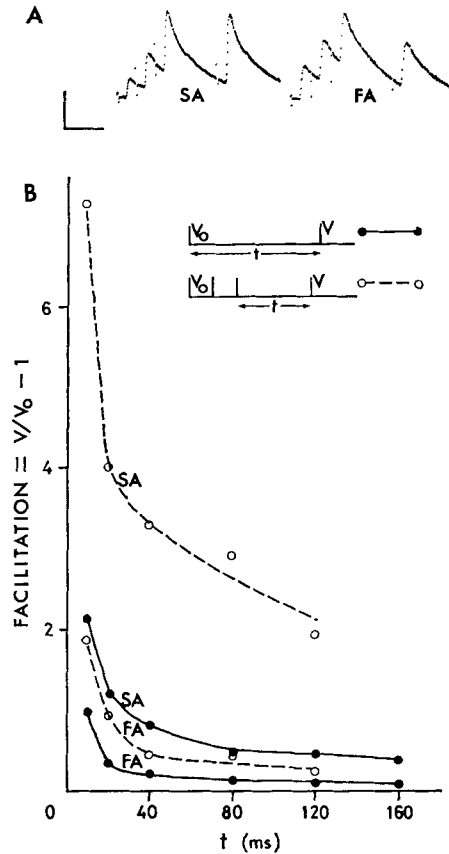

FIGURE 1 A. Sample measurements of facilitation at slow axon (SA) and fast axon (FA) synapses. Calibration, SA: 0.3 mV, 50 ms, FA: 0.6 mV, 50 ms. 50 averages/trace. FIGURE 1 B. Time-course of decay of facilitation. A test stimulus was placed at various times after either a single conditioning stimulus (solid lines) or three stimuli at 40/s (dashed lines). Facilitation is defined as $V/V_o - 1$ where V is the amplitude of the test response and V_o is the amplitude of the control response.

dashed lines facilitation after three stimuli at 40/s. The results chosen for Fig. 1 are representative of the results from five preparations. All experiments are characterized by a rapid decay of facilitation between 10 and 20 ms, followed by a much slower prolonged phase of decay.

Fig. 2 shows the same experiments as those in Fig. 1 plotted on semilogarithmic coordinates. When graphed in this manner the curves for the

FA and SA synapses followed the same general form, but the SA synapses all showed a slower rate of decay of facilitation over the entire time interval tested.

Fig. 2 also shows that the time-course of the decay of facilitation at both
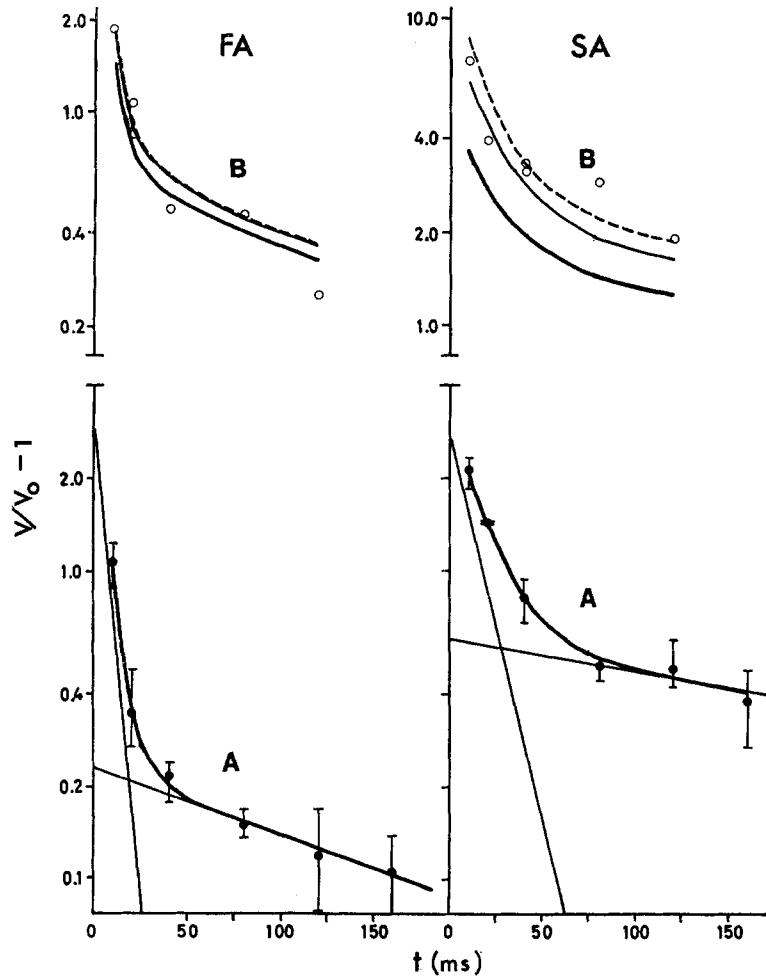

FIGURE 2 A. Time-course of decay of facilitation at SA and FA synapses after a single conditioning stimulus plotted on semi-logarithmic coordinates. The heavy solid line represents the sum of the two exponential functions indicated by the light solid lines. Each point represents the average of at least three measurements. The vertical bars represent the range of values used to determine the average.

FIGURE 2 B. Time-course of decay of facilitation after three stimuli at 40/s. Note the scale is different for SA and FA. The heavy solid line is the prediction if the facilitative effects of single stimuli add linearly. The light solid line is the prediction if a substance accumulates which has a 4th power relationship to transmitter release. The dashed line was calculated assuming facilitative effects of single stimuli multiply (see text).

the FA and SA synapses is described approximately by the sum of two exponential functions. The time constant for the initial rapidly decaying component averaged 12.4 (range 10.0–17.4) ms for the five SA experiments and 8.5 (range 7.5–10.0) ms for the five FA experiments. Values for the slower component averaged at least 350 ms for the SA and less than 200 ms for the FA.

Using the frog neuromuscular junction, Mallart and Martin (1967) described a slower component as beginning after a delay of 60–80 ms, reaching a maximum at about 120 ms, and decaying thereafter with a time constant of about 250 ms, which is intermediate between the values for the FA and SA slower component. The data in the present study were not analyzed assuming the slower component started after a delay because the method used more closely fits most of the data. Fitting the data in the manner of Mallart and Martin would produce a time constant for the faster component in the same range with that for the frog first component for the SA synapses, but not for the FA synapses.

Initial Level of Facilitation

Does facilitation at the SA synapses start at a higher level in addition to decaying more slowly? Extrapolating the fast component to zero time gave an average of 2.9 (range 2.8–3.3) for both classes of synapses in four SA and three FA experiments. One FA experiment and one SA experiment gave values of 2.0 and 1.8, respectively. The 10th experiment gave a much lower value of 0.9 for the FA that may simply have been a result of fatigue.

With the exception of this last experiment, the results suggest that facilitation in the two classes of synapses is differentiated principally by a different rate of decay from a similar initial state. This conclusion is contingent, of course, on the validity of extrapolating the results into a region which could not be tested with the present techniques.

Summation of Facilitation

The heavy solid line in Fig. 2 B represents the prediction for facilitation after three stimuli at 40/s if the facilitative effects of the individual conditioning stimuli are assumed to add linearly. This model (Mallart and Martin, 1967) predicts the facilitation after several stimuli by adding the amounts of facilitation that would be produced by the conditioning stimuli if each conditioning stimulus was applied separately. This model provides a good prediction for facilitation at the frog neuromuscular junction (Mallart and Martin, 1967; Magleby, 1970). However, in the present study the predicted facilitation after a short train was much less than the actual facilitation in the two SA experiments tested. In one FA experiment all data points were within 15% of the prediction. In the second FA experiment (Fig. 2 B) linear summation

predicted values close to the experimental for 40 and 120 ms after the end of the train, but gave values about 20–30% too low for 10 and 20 ms.

One possible explanation for the nonlinear summation is that facilitation relies on the accumulation of some state or substance that has a nonlinear relationship to transmitter release. For instance, Dodge and Rahaminoff (1967) suggested that transmitter release is proportional to the 4th power of the fraction of Ca^{++} receptors combined with Ca^{++}. Assuming that the size of the jp is proportional to the nth power of some state or substance S, then the ratio of the amount of S in the facilitated state to the control state after a single stimulus is:

$$S/S_o = (\mathrm{jp}/\mathrm{jp}_o)^{1/n} = (f + 1)^{1/n}.$$

Assuming that the fractional increment of S adds linearly,

$$(S' - S_o)/S_o = \sum_i (S_i - S_o)/S_o = \sum_i [(f_i + 1)^{1/n} - 1],$$

where S' is the amount of S resulting from i conditioning stimuli and f_i is the facilitation expected from each of the i conditioning stimuli if they were applied separately, then the facilitation f' resulting from the combined effects of the individual stimuli of the conditioning train is given by:

$$f' = (S'/S_o)^n - 1 = \left\{\sum_i [(f_i + 1)^{1/n} - 1] + 1\right\}^n - 1. \tag{1}$$

The thin solid line in Fig. 2 B shows the values calculated using Eq. 1 with n = 4 to predict facilitation after three stimuli at 40/s. The fit for the other two experiments is similar to those shown. A similar analysis assuming that the amplitude of the jp is proportional to exp (S) and that the increment of S adds linearly during a train gives:

$$f' = \exp\left[\sum_i \ln (f_i + 1)\right] - 1. \tag{2}$$

The dashed line in Fig. 2 B shows the values calculated using Eq. 2 to predict facilitation after the short train.

The Relationship between External Ca^{++} Concentration and Transmitter Release

The effect of changing the external Ca^{++} concentration on the average amplitude of the jp is shown in Fig. 3. This figure shows results from four SA and three FA experiments in which the Ca^{++} concentration was varied from 5 to 40 mM. The normal Ca^{++} concentration is 10 mM (see Methods). In all these experiments the amplitude of the jp returned to within 10% of its original value when the preparation was returned to the control Ca^{++}
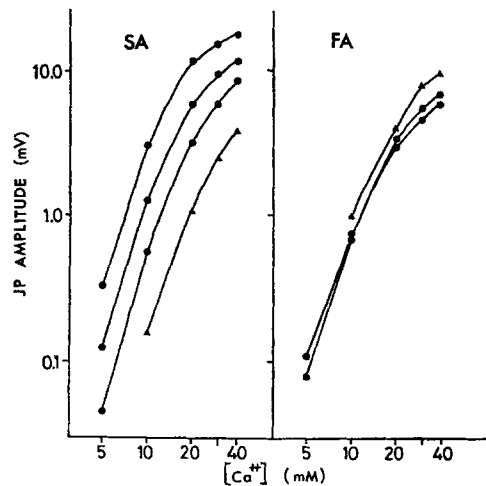
FIGURE 3.   Double logarithmic plots of junction potential (jp) amplitude as a function of external Ca^{++} concentration. Each curve is a separate experiment. The control jp amplitude returned to within 10% of the original value for all experiments. Circles, 3 mM HCO_3^-; triangles, 10 mM TES buffer. Mg^{++} concentration was 39 mM for all experiments. Stimulation rate: 0.1/s.

concentration. Data are presented below which support the conclusion that the size of the jp is proportional to the amount of transmitter released with two qualifications: (a) The input resistance of the muscle fibers was 10% lower in 40 mM Ca^{++}. (b) The jp amplitudes in the higher Ca^{++} concentrations were large enough for the correction for nonlinear summation of unit potentials (Martin, 1955) to be significant in some cases.

The double logarithmic plots in Fig. 3 show that both the FA and SA synapses have a strong nonlinear dependence on the external Ca^{++} concentration. The slopes of the double logarithmic plots between 5 and 10 mM Ca^{++} averaged 3.1 (range 2.8–3.3). The slope decreases for both the FA and SA synapses as the Ca^{++} concentration is raised above 10 mM Ca^{++} until a value below 1 is reached between 30 and 40 mM Ca^{++} (range 0.6–1.4). The slopes over the entire range of Ca^{++} concentrations are similar for both the SA and FA.

The data can be analyzed in terms of the Dodge and Rahaminoff (1967) model. In two FA experiments and one SA experiment, the size of the jp's was measured in 10 and 20 mM Ca^{++} with 39 mM Mg^{++} (normal) and in 5, 10, and 15 mM Ca^{++} with 19.5 mM Mg^{++}. Reducing the Mg^{++} concentration shifted the double logarithmic plot of jp amplitude as a function of Ca^{++} concentration upward without altering its slope for all three experiments. This is consistent with a competitive inhibition of Ca^{++} by Mg^{++}. The value for K_2 in the Dodge and Rahaminoff model for these experiments was 38 mM (SD ± 8 mM). When the data from Fig. 3 were plotted on the Lineweaver-Burke plot used by Dodge and Rahaminoff, a slightly curved line re-

sulted, possibly because there may be insufficient transmitter for the high release levels in 30 and 40 mM Ca^{++}. Values for K_1 were found to range between 7.4 and 12.3 mM if the points in 5, 10, and 20 mM Ca^{++} were used to form a straight line on this plot. The ratio of K_1 to K_2 found here is approximately equal to that found by Dodge and Rahaminoff for the frog, indicating that the relative strength with which Mg^{++} interacts with the nerve terminal compared to Ca^{++} is similar for both the frog and crab.

The Influence of Ca^{++} Concentration on Quantal Effectiveness

As a control, the possibility that the Ca^{++} concentration affects the postsynaptic effect of the transmitter was examined in three ways.

In three experiments the spontaneous miniature potentials (presumably from both the SA and FA) were recorded intracellularly in the short sarcomere muscle fibers in 5, 10, and 40 mM Ca^{++}. Because of the distributed innervation of the muscle fibers, the amplitudes of the intracellularly recorded spontaneous potentials range down into the noise level of the recording system. For this reason an arbitrary amplitude (30–40 μV) was selected which was well above the threshold level for detecting the presence of a spontaneous potential (approximately 10 μV) and only those spontaneous potentials above this level were measured. Neither the average size of the spontaneous potentials nor the largest 20% of the potentials changed systematically anywhere in the range of Ca^{++} concentrations used and in no instance was the change more than 16 and 21%, respectively.

In two experiments the input resistance was measured in 5, 10, and 40 mM Ca^{++} by inserting a second microelectrode filled with 2 M K-citrate into the muscle fiber within 50 μm of the recording electrode, and then passing sufficient current through this electrode to hyperpolarize the muscle fiber about 0.5 mV for 100 ms. The same amount of current was injected after the solution was changed and the size of the hyperpolarization measured. In both experiments no change could be detected between 5 mM Ca^{++} and the 10 mM Ca^{++} control. In both experiments there was a 10% reduction in the size of the potential measured in 40 mM Ca^{++} compared to the control. This means that in 40 mM Ca^{++} a quantum of transmitter interacting with the postsynaptic membrane will produce a potential change slightly less than the same amount of transmitter would in 10 or 5 mM Ca^{++}.

If transmitter is released according to the Poisson distribution, then the size of the jp should be proportional to $1/CV^2$ if the quantal effectiveness remains constant. This should be true regardless of the shape of the distribution of the quantal amplitudes (see Hubbard, Llinas, and Quastel, 1969). The coefficient of variation (CV) is equal to the standard deviation of the jp amplitudes divided by the mean amplitude. Only one SA intracellular experiment produced sufficiently large coefficients of variation to test this over the entire range

of Ca^{++} concentrations. The values for $1/CV^2$ were plotted as a function of Ca^{++} concentration on double logarithmic paper. The slopes were almost identical to those in the plot of average jp amplitude as a function of Ca^{++} concentration (3.9 mV max jp amplitude) except between 30 and 40 mM Ca^{++}. The slope here was 1.0 for the average jp amplitudes and 2.1 for the $1/CV^2$ plot. This is expected because of the lower input resistance in 40 mM Ca^{++} and especially because of the possibility that there may be insufficient transmitter available at such relatively high release levels. Using the crayfish neuromuscular junction, Bracho and Orkand (1970) also found that plots of $1/CV^2$ as a function of Ca^{++} concentration had slopes similar to plots using the average amplitude of the jp.

Finally, it is important to note that the amplitude of the jp's in the higher Ca^{++} concentrations is sufficiently large that the amount of depolarization produced by the individual quanta of the jp will be smaller since the large size of the jp has significantly reduced the difference between the membrane potential and the jp equilibrium potential. In the experiment with the largest jp amplitudes, the slope between 30 and 40 mM Ca^{++} concentrations would be increased from 0.49 to 0.65 if the correction is made for this nonlinear summation of unit potentials (Martin, 1955) assuming an equilibrium potential 60 mV positive to the resting potential (see Taraskevich, 1971). This correction would increase the slope between 20 and 30 mM Ca^{++} from 0.61 to 0.74. Corrections for other experiments result in considerably smaller increases in slope.

The Effects of Ca^{++} Concentration on Facilitation

Fig. 4 shows two FA and two SA experiments in which facilitation after a single conditioning stimulus was tested in Ca^{++} concentrations ranging from 7 to 30 mM. The solid circles represent the multiple controls in 10 mM Ca^{++} and give an indication of the variability of the response over the 1–2 h periods in which the data were collected. Within the limits of this variability the Ca^{++} concentration had no significant effect on facilitation. This is particularly evident in the SA case where the measurements of facilitation were more accurate because of the larger facilitation.

The Ca^{++} concentrations in this section fall within the range where quantal effectiveness was found to be constant in the preceding sections. All experiments in this section had jp's smaller than 2.0 mV except for the experiment shown in the lower left hand corner of Fig. 4 which had a maximum jp amplitude of 8.7 mV. In this experiment the correction of facilitation for nonlinear summation of unit potentials (see above) would increase facilitation at 10 ms in 20 and 30 mM Ca^{++} by 0.06 and 0.11 (17%), respectively. Other values in this experiment would be increased much less by this correction.
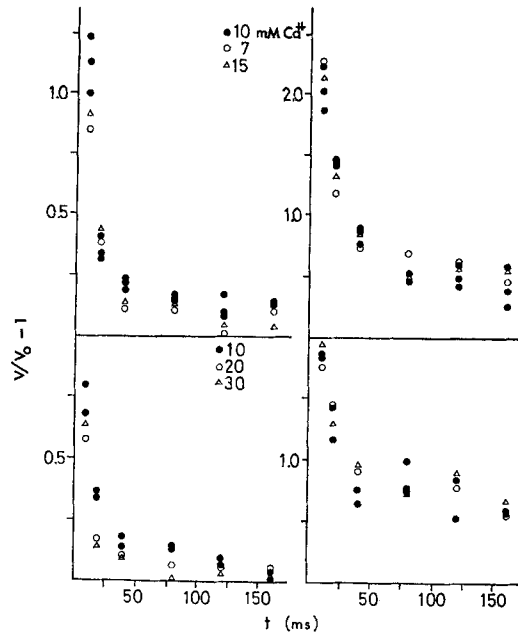
FIGURE 4. Facilitation after a single stimulus tested in Ca^{++} concentrations ranging from 7 to 30 mM. The solid circles are the multiple controls in 10 mM Ca^{++} and approximately indicate the variability of the response. Note scale is different for SA and FA. Each point is the average of 20 intracellular responses except those with SA in 10 and 7 mM Ca^{++} which are the average of 50 responses.

The Extracellularly Recorded Nerve Terminal Action
Potential (erntap)

A study of the erntap's for the SA synapses was undertaken as a control since Dudel (1965) reported for the crayfish neuromuscular junction that the amplitude of the monophasic erntap increased proportionally with the amplitude of the extracellularly recorded junction potential (erjp) as the frequency of stimulation was raised.

The three main categories of the erntap waveforms observed are shown in Fig. 5. The small size of the averaged erjp's in these records during stimulation at 1/s is due to the fact that SA synaptic areas typically have a very low probability of releasing a quantum of transmitter on any given stimulus. In all these records the size of the postsynaptic response to one quantum is many times larger than the averaged response.

By far the most common waveform observed was a small triphasic or diphasic potential (Fig. 5 A), usually too small to be observed without averaging hundreds of responses. The size on some occasions was observed to decrease about 10% during stimulation at 10/s.

At six synaptic areas, large, predominately negative-going erntap's were
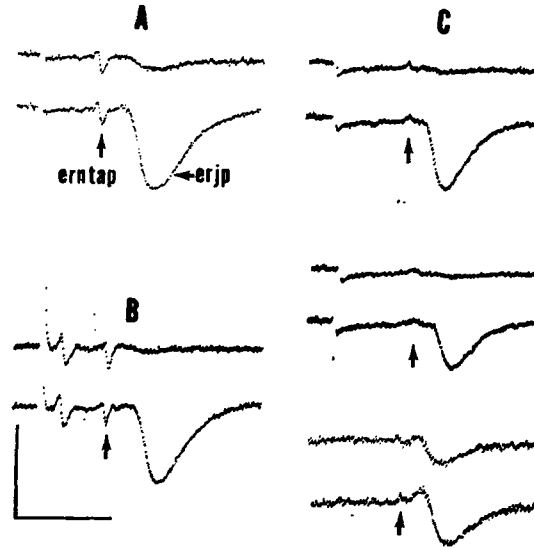
FIGURE 5. Characteristics of extracellularly recorded nerve terminal action potentials (erntap's). Arrows indicate action potentials. The upper trace of each pair is the response at 1/s and the lower trace is the response at 10/s. (A), diphasic potential. (B), predominately negative-going potential (the first diphasic potential is the response in the main nerve bundle which in this case passed close to the electrode). (C), monophasic potentials. All potentials are from SA terminals except the third pair in (C) in which SA and FA were stimulated together. Calibration: 112 μV, 11 ms.

recorded. As Fig. 5 B illustrates, the amplitude of the large negative-going erntap's either remained constant or decreased slightly with facilitation.

Small, positive monophasic erntap's were found only in three experiments. All three are shown in Fig. 5 C. Although the error in estimating the size of these small potentials is considerable, no obvious change in size is evident. The last pair of records shows a monophasic action potential followed by a second positive-going potential that increased roughly in proportion to the increase in size of the erjp. This might have been due to synaptic currents from nearby areas that were activated slightly before the area near the electrode. This potential might have been considered an erntap if the other potential had not been evident.

The three types of waveforms recorded here are similar to those found by Dudel (1965) at the crayfish neuromuscular junction and Katz and Miledi (1965 a) and Braun and Schmidt (1966) for the frog neuromuscular junction. Unlike the crayfish, but similar to the frog, no significant increase in size was noted for any of the waveforms when the frequency of stimulation was increased from 1/s to 10/s. If there was a change in the size of the erntap, it was a small decrease.

DISCUSSION

The time-course of facilitation at fast axon (FA) and slow axon (SA) synapses was measured by placing a test stimulus at various intervals after a conditioning stimulus. Facilitation at both classes of synapses began at a similar level after the conditioning stimulus and then decayed in two phases (Fig. 2 A). Both the initial rapid phase and the more prolonged phase decayed more slowly at SA synapses. An economical interpretation of these results is that both classes of synapses possess a common mechanism for facilitation and that the process governing the rate of decay of facilitation is the critical factor that differentiates them.

The two components of facilitation described here are probably not a complete description of facilitation. For instance, Katz and Miledi (1968) found a very early, intense facilitation that presumably operates during the action potential at the frog neuromuscular junction. Also, a very small, but slowly decaying component could be a significant factor in experiments where facilitation is measured by continuous, repetitive stimulation. However, the second component with a time constant of several hundred milliseconds, coupled with the nonlinear summation of facilitation, might account for a large portion of even the facilitation of 50 observed by Bittner (1968) at the crayfish neuromuscular junction.

Katz and Miledi (1965 *b*) suggested that the initial phase of facilitation is due to a residuum of Ca^{++} left from the conditioning stimulus. This view is supported by the fact that facilitation at the frog neuromuscular junction depends on the presence of Ca^{++} during the conditioning stimulus (Katz and Miledi, 1968) and by the fact that facilitation at about 20 ms or earlier decreases with increasing Ca^{++} concentration (Rahaminoff, 1968). Also, post-tetanic potentiation (PTP) depends on the presence of Ca^{++} during the conditioning train (Rosenthal, 1969; Weinreich, 1971).

The Ca^{++} hypothesis for facilitation suggests a simple mechanism by which the integrative properties of the crab neuromuscular synapses might be controlled. The more rapid decay of facilitation at FA synapses compared to SA synapses could result from a faster removal of a residuum of Ca^{++} left from the conditioning stimulus. In the present study two results are consistent with the Ca^{++} hypothesis for facilitation and one result argues against it.

(*a*) The plot of junction potential (jp) amplitude as a function of Ca^{++} concentration (Fig. 3) is very steep at normal concentrations of Ca^{++}. This means that only a relatively small residuum of Ca^{++} would be necessary to cause a large increase in the amount of transmitter released. The slopes of the double logarithmic plots of jp amplitude as a function of Ca^{++} concentration averaged 3.1 between 5 and 10 mM Ca^{++}. This slope is similar to that found

for the frog neuromuscular junction (Dodge and Rahaminoff, 1967), the squid giant synapse (Katz and Miledi, 1970) and the rat neuromuscular junction (Hubbard, Jones, and Landau, 1968). It is quite different from the linear relationship found at the crayfish neuromuscular junction (Bracho and Orkand, 1970). The slopes over the range of Ca^{++} concentrations tested are similar for both classes of synapses (Fig. 3). This suggests that the interaction of Ca^{++} with the nerve terminal is similar for both the SA and FA, and that the activation of this stage in the process of transmitter release is similar for both classes of synapses.

(b) Facilitation accumulates as if it were based on some state or substance, such as Ca^{++}, that bears a nonlinear relationship to transmitter release. When a short train is used as the conditioning stimulus, the resulting facilitation is much greater than the linear sum of the effects due to the individual conditioning stimuli. Two models can account for this as well as the relatively small deviation for linear summation at FA synapses (Fig. 2 B). In one model transmitter release is assumed to be proportional to the 4th power of the amount of a substance that accumulates during facilitation. In the other model transmitter release is assumed to be exponentially related to the state or substance. Distinguishing between the two models would require a new technique allowing the data to be collected in a much shorter period of time than is allowed with intracellular recording from single muscle fibers.

The results from the experiments in which facilitation was measured in various Ca^{++} concentrations, on the other hand, argue against the Ca^{++} hypothesis for facilitation. The analysis of this data requires an important assumption. There are two Ca^{++} concentrations to be considered: the Ca^{++} concentration in the bath and the "active" Ca^{++} concentration. Only the Ca^{++} concentration in the bath is known and some assumption must be made about the relationship between this concentration and the active Ca^{++} concentration. The simplest assumption would appear to be that the Ca^{++} entering the active pool during the depolarization of the terminal is proportional to or equal to the external Ca^{++} concentrations. This implies that the decreasing slope of the relationship between external Ca^{++} concentration and jp amplitude is mainly due to a saturation of the step where Ca^{++} interacts with its receptors or steps after this process. This assumption is used below.

The relationship found between the Ca^{++} concentration and jp amplitude predicts that if facilitation occurs at the Ca^{++} step or is mediated by it, the magnitude of the facilitation should vary as it is tested over a range where the double logarithmic slopes vary. In other words, while the absolute size of the jp's increases with increasing Ca^{++}, the ratio of the facilitated jp's to the control jp's should decrease since increments of Ca^{++} become decreasingly effective in incrementing transmitter release as the concentration of Ca^{++} increases.

The log-log slope averaged 3.13 between 5 and 10 mM Ca^{++} and 2.22 between 10 and 20 mM Ca^{++}. Facilitation of 1.0 in 7 mM Ca^{++} would imply that the larger release involved approximately 25% more Ca^{++}. The same increment of Ca^{++} should cause facilitation of 0.63 in 15 mM Ca^{++}. Facilitation should be reduced much more in the 20 and 30 mM experiments. Although there is scatter in the data due to the fact that they were collected over 1- to 2-h periods and synaptic properties slowly fluctuate over such a time interval, a change of facilitation of the predicted magnitude would have been detected. However, no change of the expected magnitude was found (Fig. 4). This is similar to the results of Hubbard, Jones, and Landau (1971) who found that facilitation was the same in 2 and 5 mM Ca^{++} at the rat hemidiaphragm preparation.

A change in the presynaptic depolarization might explain facilitation. However, in the chick ciliary ganglion no change in amplitude or configuration of the presynaptic depolarization is found during either facilitation or PTP (Martin and Pilar, 1964) and with the squid giant synapse the amplitude of the presynaptic action potential is not larger during facilitation (Miledi and Slater, 1966; see also, Takeuchi and Takeuchi, 1962). With tetrodotoxin present to prevent active changes in the Na^+ permeability, facilitation can be produced by controlled artificial depolarizations at the squid giant synapse (Katz and Miledi, 1967 a; Bloedel et al., 1966) and the frog neuromuscular junction (Katz and Miledi, 1967 b). Also, PTP similar to that normally observed occurs at the frog neuromuscular junction when the synapse is artificially depolarized (Weinreich, 1971).

In the present study two results indicate that facilitation at *Chionoecetes* neuromuscular synapses, like facilitation in the other preparations just discussed, is not based primarily on variations in the terminal depolarization. First, the three types of extracellularly recorded nerve terminal action potentials (erntap's) recorded did not show an increase in size with increasing frequency of stimulation. The decrease in size of the erntap's with increasing frequency of stimulation can be attributed to depolarizing afterpotentials. Secondly, since an increased depolarization would exert its effect by increasing Ca^{++} entry into the active pool, facilitation in higher Ca^{++} concentrations should be influenced by the decreasing slope of the relationship between Ca^{++} concentration and jp amplitude. This influence was not observed (Fig. 4).

In summary, with the present assumptions the data suggest (a) that the activation of the process of transmitter release is essentially similar for both SA and FA synapses up to and including the Ca^{++} step, and (b) that the mechanism of facilitation is similar for both classes of synapses and occurs after the Ca^{++} step.

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