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Facilitation of Transmission at the Frog Neuromuscular Junction at 0°C Is Not Maximal at Time Zero

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Facilitation of quantal release has been thought to be maximal immediately after the first action potential in the presynaptic terminal. However, at the frog neuromuscular junction at 0°C no facilitation was observed in response to direct nerve depolarization when the interval between stimulus pairs was less than 10 msec (Dudel, 1986), while at intervals of 20 msec and beyond, facilitation was increased (Molgó and Van der Kloot, 1991). In the present experiments, facilitation to pairs of nerve action potentials was measured both by the method of failures and by comparing the total inward postsynaptic current generated by the first and second action potentials. Facilitation was observed at intervals as short as 7 msec, but 20–30 msec was required for facilitation to reach a maximum. This suggests that facilitation requires a second messenger or the action of Ca^{2+} at a site other than that eliciting exocytosis.

[Key words: neuromuscular junction, quantum, facilitation, synapse, endplate potential, frog]

Two-pulse facilitation of neurosecretion is observed when the motor nerve is stimulated twice; the second endplate potential (EPP) or endplate current (EPC) is larger because more quanta are released (del Castillo and Katz, 1954). The time course of facilitation is usually described as being maximal after the first stimulus and declining along a time course that follows two exponentials (Mallart and Martin, 1967, 1968; Martin, 1977; Zengel and Magleby, 1982; Zucker, 1989).

Dudel (1986) studied facilitation at 0°C using a single extracellular patch electrode to depolarize a section of nerve terminal and also to record quantal releases. Working in low Ca^{2+}/high Mg^{2+} solution containing tetrodotoxin, he found that at 0°C there was no facilitation. Facilitation appeared at the same site when the temperature was raised to 10°C. Molgó and Van der Kloot (1991) studied facilitation in response to nerve action potentials with an intracellular electrode. They reported that at 0°C facilitation was enhanced and prolonged compared to room temperature (Van der Kloot and Molgó, 1993).

These two studies might seem incompatible, but there was a major difference in the experimental protocols. Dudel (1986) studied the responses to pulses separated by 5 or 10 msec. Molgó and Van der Kloot (1991) used intervals of 20–2000 msec. Possibly at 0°C facilitation requires more than 10 msec to develop.

The present experiments were designed to test this possibility by studying facilitation in response to nerve stimulation at 0°C at intervals shorter than 20 msec, measuring quantal output either by the method of failures (del Castillo and Katz, 1954; Martin, 1966) or as the time integrals of EPPs or EPCs (∫EPP or ∫EPC) (Van der Kloot, 1987, 1991). The integrals are useful because they are proportional to the total current that enters the endplate membrane (Fatt and Katz, 1951), and therefore to the number of acetylcholine receptor channels opened.

Materials and Methods

Details of the recording methods have been described (Molgó and Van der Kloot, 1991). The isolated sciatic nerve–sartorius muscle preparation from *Rana pipiens* was placed in a chamber above a Peltier plate, which lowered the bath temperature to 0°C. The nerve was dissected to the urostyle; the proximal end was placed over platinum stimulating electrodes held in a holder filled with mineral oil. The nerve was stimulated with a pulse of 100 V amplitude and 0.1 msec duration.

The bathing solution contained (in mM) 120 NaCl, 2 KCl, 2.5 $CaCl_2$, 4 *N*-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) at pH 7.4. The low Ca^{2+} solution contained 2.5 mM $MgCl_2$ and 0.35–0.45 mM $CaCl_2$.

EPPs or EPCs were detected with an Axon voltage clamp and were amplified over a bandwidth from DC to 1000 Hz using an Axon Subtracter. The gain of the clamp was adjusted so that the holding membrane potential changed by less than 1.5% the driving potential during an EPC. The responses were stored in the computer and then analyzed at a later time. For experiments using the method of failures, the computer operator determined whether or not a response had occurred during the release period following the first or second stimulus, and also whether a spontaneous MEPP occurred in a comparable time interval before the first stimulus. The data were corrected by subtracting the spontaneous rate before it was used to calculate the quantal output, m_0, following the first and second stimuli, using the equations given by Martin (1966). At each interval between stimuli, the responses to 100–200 stimulus pairs were measured. Plots of m_0 as a function of time during the experiment suggested that the data were stationary. The pairs of stimuli were delivered every 3 sec.

At short intervals, the second ∫EPPs or ∫EPCs used for calculating facilitation will be contaminated with the stimulus artifact, which must be removed (Fig. 1*a*). For experiments in which ∫EPP or ∫EPC were measured, the nerve was given pairs of stimuli every 3–10 sec, and the responses recorded on disk. Later, the operator determined the data point following the first stimulus artifact at which integration should begin and the point following the second response at which integration should end. The operator determined when the artifact began and when it ended. The computer program removed these points and replaced them by an interpolation produced by cubic splines, as shown in Figure 1*b* (Press et al., 1989). The corrected signal was then integrated and also averaged. During each experiment, responses to single stimuli were also integrated and averaged. These responses were used for the controls. Controls also were calculated by interpolating them just as if a stimulus artifact was being removed. For each interval between stimuli, facili-

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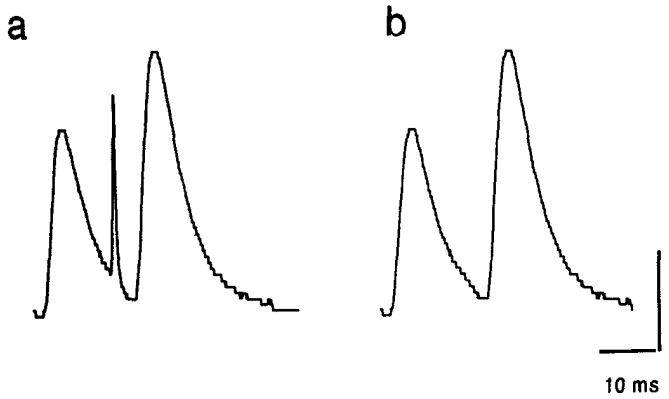

Figure 1. An example of the use of cubic splines to remove the stimulus artifact from a recording of EPPs. *a*, The original record. *b*, The same record after using cubic splines to correct for the artifact.

tation, f, was calculated as the ratio of the mean integral of the pairs of responses (with artifact removed), p', divided by the sum of the mean integral of the single response, s, and the mean integral of the single response corrected as if there were an artifact, s':

$$f = p'/(s + s') \quad (1)$$

At each stimulus interval from 20 to 100 responses were measured, depending on the solution in which the measurements were made: fewer responses were recorded when quantal output was relatively high. In such cases, pairs of stimuli were given every 10 sec, to avoid problems with depression.

Another method for dealing with the stimulus artifact problem was to set all of the data points in which the artifact contaminated EPPs or EPCs to zero. The same segment of the control EPPs or EPCs was set to zero. Then the signals were integrated. Facilitation was calculated by Equation 1. These measurements are not given in the results because they produced conclusions identical to the smoothing method: in eight experiments the mean facilitation at 15 msec by the zero method was 1.02 ± 0.039% of the facilitation calculated by the spline method.

Results

Figure 2 shows the results of three of the nine experiments done by the method of failures. The shortest interval tested was 15 msec, because the refractory period was usually between 7 and 11 msec. In all of the examples, facilitation increased as the interval between the paired stimuli was increased, reaching a maximum at intervals ranging from 18 to 30 msec. In all nine of the experiments, facilitation at the shortest interval tested was less than the maximum.

A question about these experiments is whether occasionally at 15 msec the second action potential fails to reach the nerve terminal. This would be recorded as a failure when in fact it was not a valid test, and such a miscatagorization would decrease the measured facilitation. To access this possibility of error, after the failure measurements the [Ca^{2+}] in the extracellular solution was raised to 1 mM and 3 μM *d*-tubocurarine was added, so that quanta were released following each stimulus. In these controls, in hundreds of stimulus pairs with 15 msec interval, a response was always observed following the second stimulus. Later, experiments in solutions with 1 mM Ca^{2+} and *d*-tubocurarine, in which the second stimulus always elicited a response, showed that in many preparations the second response could be observed with stimulus pairs as close as 7 msec apart.

To confirm these results, facilitation was measured on ∫MEPPs or ∫MEPPs in solutions in which the Ca^{2+} was elevated to levels at which almost every stimulus was followed by a quantal release. The artifact from the second stimulus was compensated

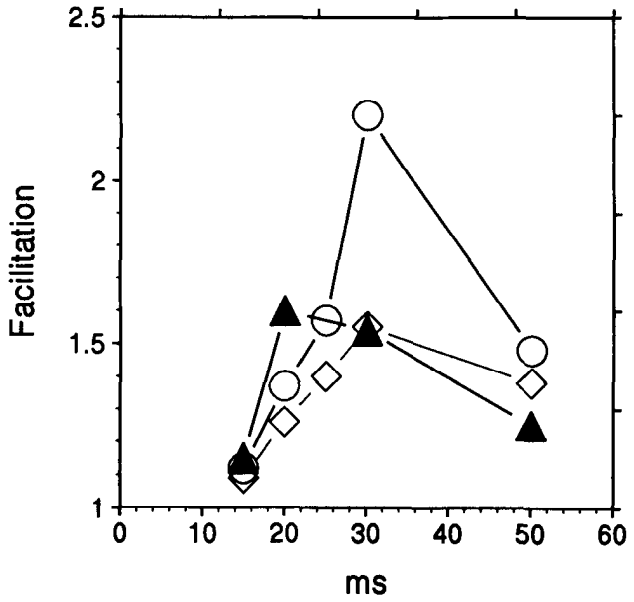

Figure 2. Facilitation at 0°C measured by the method of failures. Three examples are shown, which are typical of nine experiments.

for as described in Methods and Materials (Fig. 1). Any record in which there was no response to the first or second stimulus was rejected, but such rejections were infrequent and in most experiments nonexistent. Figure 3 shows results from three of the nine experiments on ∫MEPPs. In each example there was less facilitation at the shortest interval than at the maximum, which was reached at intervals between 20 and 40 msec.

∫EPCs were studied last, because I anticipated problems with instrumental noise, larger artifacts, and with the interpolation procedure when the signals were changing more rapidly. In practice, none of these problems were substantial. Figure 4 shows two examples of measurements from EPCs and the mean results from all 11 experiments. Again, it appears that facilitation reached a maximum after 20–30 msec. Facilitation was observed at 7

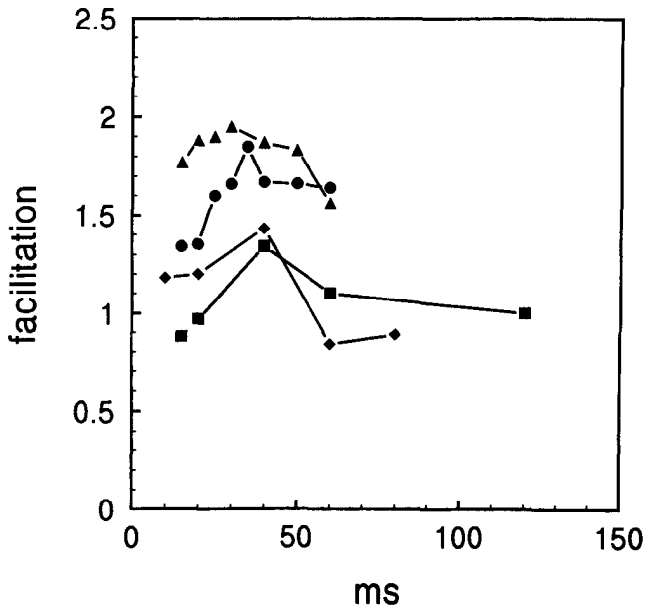

Figure 3. Facilitation of EPPs at 0°C. Three examples are shown, which are typical of the nine experiments.

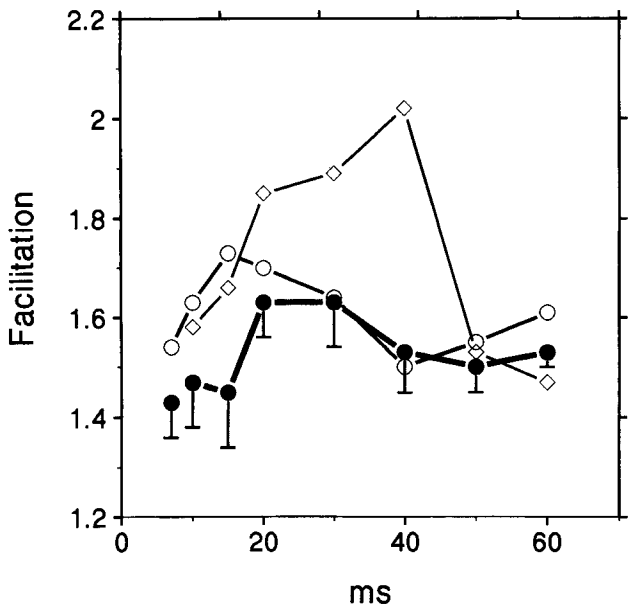

Figure 4. Facilitation of EPCs at 0°C. The *solid circles* show the average from 11 experiments (there were not 11 examples at each point). The error bars show SEM. *Open circles* and *diamonds* show two examples of single experiments: □, 80 mV holding potential; ◇, 88 mV holding potential.

msec in some cases, which was the shortest interval at which the nerve could be stimulated a second time in these preparations.

Discussion

Two methods were used to measure facilitation at relatively short intervals. The method of failures has the potential problem that occasionally the nerve may be refractory to the second stimulus. The integral method has the problem of the stimulus artifact, so there was concern that the methods used to correct for the artifact might produce systematic errors in measuring facilitation. In fact, both approaches gave the same answer: facilitation rose as the interval was lengthened, reaching a maximum at intervals between 20–40 msec.

Two possible explanations for the observations come to mind. (1) At the shorter intervals the second action potential is altered so it does not admit the usual amount of Ca^{2+}. Even though there is facilitation, it is masked because the second response is diminished by reduced Ca^{2+} influx. If Ca^{2+} entry was normal, then facilitation would be largest at the shortest intervals. Hypotheses that at the neuromuscular junction facilitation follows changes in the action potential have not fared well (Martin, 1977; Baldo et al., 1983; Zucker, 1989). (2) Facilitation does not arise full blown following the first action potential, but develops over a time course of more that 10 msec at 0°C.

The results of Dudel (1988), showing that there is no facilitation at 0°C in response to direct nerve depolarization, do not completely decide the issue, because there is no certainty that a second depolarization produces the same sequence of conductance changes. My results do not agree completely with Dudel (1988). He observed no facilitation at intervals less than 10 msec, while I saw facilitation at 7 msec. However, the conditions for the experiments were quite different: his preparations were in tetrodotoxin and the Ca^{2+} was extremely low. It would be rash to conclude that there is a significant discrepancy. Both suggest that at 0°C facilitation requires time to develop. There are a number of possible mechanisms for a delay in the appearance of facilitation, including the possibility that it depends on the presence of a Ca^{2+} elicited second messenger or Ca^{2+} acting on a site distinct from that causing exocytosis (Yamada and Zucker, 1992).

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