Importance of stimulation paradigm in determining facilitation and effects of neuromodulation

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Abstract

Evoked synaptic activity within the CNS and at the neuromuscular junction in most in vivo preparations studied occurs not with single isolated stimuli, but with trains, or bursts, of stimuli. Although for ease in studying the mechanisms of vesicular synaptic transmission one often uses single discrete stimuli, the true mechanisms in the animal may be far more complex. When repetitive stimuli are present at a nerve terminal, often a heightened (i.e., facilitated) postsynaptic potential can be as a result. Facilitation is commonly used as an index of synaptic function and plasticity induced by chronic stimulation or by neuromodulation. The mechanisms that give rise to facilitation are thought to be the same that may underlie short-term learning and memory [C.H. Bailey, E.R. Kandel, Structural changes accompanying memory storage. Annu. Rev. Physiol. 55 (1993) 397–426]. Differences in short term facilitation (STF) are seen depending on the conventional stimulation paradigm (twin pulse, train, or continuous) used to induce facilitation. Thus, a battery of paradigms should be used to characterize synaptic function to obtain a closer understanding of the possible in vivo conditions. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Communication by neurons within the CNS and in the periphery of an actively moving animal, for the most part, occurs through bursts of neural activity. Tonic muscles are induced to contract by neurons that fire in bursts or trains rather than in continuous low frequencies. This type of neurotransmission can result in a facilitated post-synaptic response. Historically, crayfish neuromuscular preparations have provided substantial insights in the basic mechanisms of synaptic transmission [1–7,11,35,47–49,52–54,59–62] which has been shown to be applicable not only to other crustacean neuromuscular junctions (i.e., lobsters and crabs), but also vertebrate chemical synapses in general [13,38,39,55]. Among the vast body of research characterizing synaptic efficacy, different forms of facilitation have been used to draw hypotheses to explain the underlying the basis of chemical transmission. Facilitation of synaptic transmission occurs when there is an increase in a response due to previous activity at the synapse. Depending on the experimental conditions, quite different interpretations of synaptic plasticity arise (see the following references: [9,10,44,49,57]). Two types of facilitation, long term facilitation (LTF) and short term facilitation (STF), have been well described at the crayfish NMJ [8,50,56,57]. LTF is the result of stimulation at a high frequency for a given amount of time. This prolonged stimulation leads to an increased post-synaptic response when an additional single stimulus is given after a period of minutes to hours [45,50]. LTF is achieved through the activation of the cAMP second messenger system [31]. STF, on the other hand, is a transient increase in the amplitude of subsequent excitatory post-synaptic potentials (EPSPs) due to two or more stimuli given within a short period of time. The mechanism of STF is due, in part, to a buildup of residual Ca$^{2+}$ within the pre-synaptic cell. This buildup leads to an increase in neurotransmitter release and thus an increase in the EPSP amplitude [8,21,27,28,33,63,64]. The time course of STF may be affected by removal of free Ca$^{2+}$ in the pre-synaptic cell [46,47]. However, other possibilities and
modifications of past concepts for facilitation have been suggested [12,21,54]. Most animal muscles are not activated by a single neural impulse, but instead by a barrage of motor nerve activity, to overcome the inertia for movement. Such activity is readily observed when measuring EMG activity, so that when investigating the synaptic properties of neuromuscular preparations using facilitation protocols, it is advantageous to use multiple stimuli because they better mimic physiological conditions of motor neurons when compared to measuring events from isolated stimuli.

The neuromuscular junctions of the crayfish are amenable for study of the mechanisms underlying synaptic transmission and facilitation because of the few and identifiable neurons that innervate a given muscle, and the large axons allow microelectrodes to be used to inject compounds, such as pharmacological agents, directly into pre-terminal regions [32,41]. Also, the morphology and ultrastructure of nerve terminals is directly comparable to physiological function [6,17,18,20,23].

In particular, the opener muscle preparation of the crayfish is ideal for investigating different facilitation paradigms since the entire muscle is innervated by a single excitatory tonic motor neuron. The opener muscle is divided into three anatomical regions: proximal, central, and distal. Studies are underway to determine if regional variation exists in facilitation induced by several different stimulation approaches. Linder [44] has shown that the distal region facilitates more than the central region during twin pulse facilitation. As the delay between stimuli was increased, the central muscle fibers begin to show more facilitation involved giving continuous stimulation at a constant frequency of 1 Hz and allowing the facilitation to plateau to a new baseline.

The induction of STF was obtained using three methods. The first was to apply two stimulus pulses relatively close together. This procedure is referred to as twin pulse facilitation. The time between stimulus pulses was varied from 5 to 60 ms, and the frequency of stimulation was varied for three conditions: 0.1, 0.5 and 1.0 Hz. The second procedure consisted of giving a train of 10 pulses at various intervals (every 1, 2 or 10 s). The frequency of stimulation within the train was 30 or 50 Hz. In order to determine the decay of facilitation, a train of 10 pulses was followed by an eleventh pulse at given intervals, and the amplitudes were measured. A third method for inducing facilitation involved giving continuous stimulation at a constant frequency (1, 10 or 30 Hz) and allowing the membrane potentials to plateau to a new baseline.

2. Materials and methods

2.1. General

All experiments were performed using the first or second walking legs of crayfish, Procambarus clarkii, measuring 6–10 cm in body length (Atchafalaya Biological Supply, Raceland, LA). Animals were housed in an aquatic facility and fed dried fish food and carrots. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld’s solution (in mM: 205 NaCl; 5.3 KCl; 13.5 CaCl₂ · 2H₂O; 2.45 MgCl₂ · 6H₂O; 0.5 HEPES adjusted to pH 7.4). Crayfish were induced to autotomize the first or second walking leg by forcefully pinching at the merus segment. During the dissections of the opener muscle, the bathing medium was exchanged every 20 min with chilled saline at 14°C until the dissections were completed.

2.2. Physiology

The excitatory axon was selectively stimulated by placing a branch of the leg nerve (from the merus segment) into a suction electrode connected to a Grass S88 Stimulator [34]. Only muscle fibers from the ventral side of the opener muscle were used [17]. The intracellular muscle potentials were amplified by a microelectrode preamplifier (Almost Perfect Electronics, Basel, Switzerland) and were observed using a Hameg analog/digital oscilloscope. Signals were simultaneously recorded using a Vetter PCM VHS tape recorder and digitized using MacLab/s v3.5.

Preliminary reports of this data have previously been presented in abstract form [24–26].

subtracted from the result. The subtraction of the numerical value one ensures that if no facilitation is occurring (the amplitudes of the first and last responses are the same), the facilitation index \( (F_e) \) will be zero. To analyze continuous stimulation experiments, initial and final baseline measurements were compared to determine the amount of increase to the new plateau. In addition, the amplitudes of the initial EPSPs were taken and compared with the amplitudes of EPSPs in the plateau region.

The amplitudes of the EPSPs were measured using the MacLab® v3.5 programs Scope or Chart. The signals recorded on the computer were calibrated to the raw traces observed on the oscilloscope.

3. Results

There are several ways to index the degree of facilitation. One method is by giving two identical stimulus pulses (twin pulse, Fig. 1A) within a short period of time (less than 100 ms). A second method is to give a train of pulses at various frequencies given within a short temporal range (100 ms, Fig. 1B). Comparing the ratio derived from the last and first EPSP amplitudes commonly used to determine the facilitation index \([44]\). In addition, continuous stimulation at a given frequency may be applied (Fig. 1C) so that the steady state of the EPSP amplitudes may be compared with the initial responses or to responses of continuous stimulation at a different frequency.

3.1. Twin pulse

Twin pulse facilitation occurs when two identical stimulus pulses are given within a short amount of time, resulting in a larger EPSP amplitude following the second stimulus pulse (Fig. 1A). There are two stimulation parameters that one needs to be concerned with when providing
Fig. 3. EMG recordings of the intact crayfish opener muscle. (A) Excitatory postsynaptic potentials recorded when the claw is opened quickly and then maintained in the open position. (B) Instantaneous frequency of motor neuron firing during the opening of the chelae.

twin pulses of stimuli. One is the pulse frequency (interval between twin or train pulse onset) and pulse delay (interval within a twin pulse set). Fig. 2A shows mean facilitation indexes (Fe) when measured under a stimulation paradigm of 0.1, 0.5 and 1 Hz in producing the onset of the twin pulses with a constant 20 ms delay between the two pulses for five preparations. Within all preparations, decreasing frequency of paired pulses resulted in a decrease in Fe ($p < 0.05$, rank sum test). This indicates that with less time between the induction of twin pulses the nerve terminal may not be re-setting to resting conditions, potentially be due to residual released calcium from temporary bound stores within the nerve terminal [21].

In addition to variation in stimulation frequency, the delay between pulses in a set of twin pulses may be altered. The delay between stimulus pulses was varied to 10, 20, 40, and 60 ms, as illustrated in Fig. 2B. As the delay between identical stimulus pulses increased, the facilitation index decreased within a preparation ($n = 5$, means ± S.E.M.).

3.2. Trains

Train facilitation is a less utilized method for measuring the facilitation index of neuromuscular preparations. However, electromyogram (EMG) recordings of the opener muscle indicate that short bursts of activity are present in the intact leg opener muscle (Fig. 3). The activity profile in the instantaneous frequency histogram indicates that when the chela is opening, a neuron fires bursts or trains of action potentials which then taper off. Therefore, the range of 20 to 85 Hz in short bursts is a feasible approach to

Fig. 4. Method of analysis for train facilitation. (A) When a train of 10 stimulus pulses is given in a short period of time, the rise of the 10th EPSP often begins on the decay of the 9th. (B) To determine the correct 10th EPSP amplitude, the time at the peak of the 10th pulse was determined. On a train of nine pulses, the amplitude was taken at a time point on the decaying 9th pulse that is equivalent to the time of 10th peak. This amplitude was subtracted from its baseline to yield the correction factor. This correction factor was then subtracted from the peak of the 10th EPSP less its baseline, yielding the correct 10th EPSP amplitude.
experimentally induce facilitation for physiologically relevant assessment.

Measuring train facilitation is a more complex task than that of measuring twin pulse facilitation. The train facilitation paradigm used gives a series of 10 identical stimulus pulses within a short period of time (e.g., 300 ms). Because these stimulus pulses are given so close together, often an EPSP will rise on the decay of the previous EPSP, as illustrated in Fig. 4A. This leads to difficulty in accurately determining the amplitude of the 10th EPSP. Measuring from the baseline to the peak will result in too large an EPSP amplitude measure. Likewise, measuring from trough to peak represents an EPSP that is too small because of the differences in the timing of the events and decay of the 9th potential. To correct these measurement errors, the amplitude of the 10th EPSP was measured from baseline to peak to get the absolute difference. The time at the peak of the 10th EPSP was also determined. In a train of nine stimulus pulses, the time to the peak of the 10th EPSP was measured on the decay of the 9th EPSP. The amplitude at this time was subtracted from the baseline to give the correction factor. The correction factor was then subtracted from the absolute difference baseline to peak of the 10th EPSP , yielding the corrected 10th EPSP amplitude. This method is outlined in Fig. 4B.

Another problem in measuring the facilitation index using a train paradigm occurs when no EPSP above the noise level can be measured after the 1st stimulus pulse. As the 2nd and 3rd stimulus pulses are given, however, the EPSPs facilitate and a response may be measured. As an added means of comparison, the amount of facilitation was also found by altering the equation for Fe to compare the 10th EPSP to the 3rd EPSP, rather than the 1st EPSP. The results of (10th EPSP/1st EPSP) - 1 and (10th EPSP/3rd EPSP) - 1 from the same recording were 10.43 and 4.86, respectively, using a 0.1 Hz train rate with a 30 Hz stimulation frequency to obtain 10 responses. These results demonstrate two factors: first, Fe values are directly variable with EPSP amplitudes used to measure them, and secondly, there is a greater facilitation from the 1st EPSP to the 10th than the 3rd to the 10th EPSP. Since by the 3rd EPSP there was already a substantial facilitation, the overall difference to the 10th EPSP is not that large. In very low output terminals the first EPSP can be so close to baseline, essentially zero, that extraordinarily large facilitation indexes (> 100) may result. Very small changes in the first EPSP amplitude can drastically alter the Fe value. This indicates that this type of measure is not useful for comparative purposes among low-output terminals.

As seen with twin pulses, differences in Fe were also observed when the frequency interval between train sets was varied. Fig. 5A illustrates how Fe is altered over time when given a shorter interval frequency of 0.5 Hz (closed circles) vs. 0.1 Hz (open circles) while maintaining a 30 Hz, 10 pulse train for a single preparation. The Fe index is larger for the shorter interval between trains in all five preparations tested for the central muscle fibers (p < 0.05, rank sum test), although the absolute values among preparations varied.

In addition, higher train frequency at the same 0.1 Hz interval between trains gives rise to a larger Fe in the same preparation. When the train frequency was increased from 30 to 50 Hz a substantial Fe increase was observed (Fig. 5B). The general trend shown is the same for all five preparations obtained from central muscle fibers (p < 0.05, rank sum test), although absolute values varied.

3.3. Continuous stimulation

STF is obtained quickly when continuous stimulation paradigms are used (Fig. 1C). Three stimulation frequencies were used in this experiment: 1, 10 and 30 Hz (Fig. 6). Similarly, two different methods of measuring STF were utilized. For the first method, the mean amplitude...
was measured once the EPSPs had reached a plateau. By dividing the mean amplitude at 10 Hz by the mean amplitude at 1 Hz and subtracting the value 1, a facilitation index was established. Analogously, the mean of the plateau at 30 Hz over that at 10 Hz or 1 Hz can be used. The second method compares the EPSP amplitude at the plateau with the EPSP amplitude produced by single stimuli at the beginning of stimulation. As calculated from the values given in Fig. 6, these two methods will result in varied Fe values. The Fe value for the 10 to 1 Hz ratio was 4.58, 0.65 for the 30 to 10 Hz, and 8.26 for the 30 to 1 Hz ratio for the first method. The second method resulted in an Fe of 4.29 for 1 Hz, 27.37 for 10 Hz, and 36.29 for 30 Hz.

3.4. Effects of serotonin (5-HT) on facilitation

5-HT enhances synaptic transmission at crayfish opener neuromuscular junctions [29,30,37]. We are not aware of any study that has systematically compared various methods of inducing facilitation with the effects of the well established neuromodulator 5-HT. Here we report that interpretation of neuromodulator effects on facilitation are dependent upon the stimulation paradigm used. In a maintained recordings, both twin pulse and train stimulation were given before and after the bath was exchanged with saline containing 100 nM 5-HT. The preparations were given 15 min to incubate before 10 twin pulses were given at 0.1 Hz frequency at a 20 ms delay followed by a 5 min rest. After the rest, trains were given at a 30 Hz frequency with a 0.1 Hz train rate. These same stimulation conditions were also used before the application of 5-HT. Table 1 shows that the twin pulses gave rise to a much larger percent change in the Fe than for the 30 Hz train induced Fe, thus suggesting the importance of using various stimulation paradigms in determining neuromodulator roles in the mechanisms underlying facilitation.

4. Discussion

The performance of synaptic transmission has been quantified in several ways and by several methods. At times, choice of the best paradigm to use when investigating the effects of pharmacological agents, synaptic plasticity, or other conditions is confounded. In order to understand basic mechanisms of transmission, the simplest of procedures are warranted [43], although in a behaving animal more complex patterns of activity exist at synaptic sites, as indicated in the EMG recording for the opener muscle (Fig. 3A). In an earlier report [18], various analysis procedures of synaptic current quantal responses were shown to have significant impact [13] in quantifying synaptic efficacy in invertebrate NMJ and vertebrate CNS systems. In this paper, the purpose is to raise the awareness, that depending upon the stimulation conditions different interpretations can be reached. In illustrating this point, a particular recording session was subjected to twin pulses (0.1 Hz interval with a 40 ms delay), trains (0.1 Hz interval with 10 stimuli at 30 Hz), and continuous stimulation (30 Hz), with 10-min rest periods between the changing conditions, to compare the Fe values for each paradigm. The twin pulse gave a Fe value of 1.73, and the trains ([10th EPSP/1st EPSP] – 1) resulted in an Fe value of 10.43, and the continuous stimulation resulted ([plateau EPSP/initial EPSP] – 1) in an Fe value of 2.07.

The effects of 5-HT and other neuromodulators that are found in the hemolymph of crustaceans are known to have pronounced effects on synaptic transmission at crayfish neuromuscular junctions [29,30,32,36,37] and may also be responsible for alterations in behavior of the entire animal [14,15,42]. Since the effects of 5-HT on twin pulse facilitation results in a larger increase in Fe value, one may be lead to conclude that 5-HT increases facilitation substantially. But, when a train of 10 stimuli are given at 30 Hz (0.1 Hz train interval) the EPSPs may start to plateau before the 10th EPSP as shown in the data presented in Table 1. A lower facilitation in the presence of 5-HT would give an impression that 5-HT reduced facilitation, although for twin pulses or lower frequencies of train stimuli, the degree of facilitation would actually be larger because the initial EPSPs would start at a lower level. This
illustrates the point that different postulations on the mechanisms of facilitation can be proposed that may be in error unless various stimulation conditions are examined.

Despite nearly four decades of investigating the opener muscle in the crayfish as a model preparation to investigate synaptic properties, few comparative studies have been undertaken to examine the effects of various stimulation conditions on synaptic transmission [34,44]. No reports have appeared, of which we are aware, that examine the combined effects of stimulation paradigms and neuromodulators on this preparation. In addition, most of the literature in this specialty area contains data obtained by different stimulation conditions making it difficult to objectively compare results and their meaningfulness among reports. Investigations in this area are needed to have a more complete understanding of synaptic properties under varying activity profiles. This will allow correlation of the effects of locomotive commands and motor output in a more meaningful manner [23] and will provide better information for modeling of synaptic transmission [21,59] and locomotion. Since there is a growing interest in neuromodulator effects on synaptic circuitry and synaptic function [14,16,22,51,58], wide ranges of stimulation procedures that mimic normal activity may also provide unique insights related to in vivo conditions. Although there are numerous paradigms that can be tested that we have not described, the common stimulation and analysis procedures mentioned in this report can be utilized, to provide a more complete understanding of the processes involved in synaptic differentiation and plasticity. Likewise, use of a combined range of conditions in vertebrate and invertebrate CNS and peripheral systems will be beneficial in understanding the mechanisms underlying transmission.

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