THE ROLE OF THE INTERSTIMULUS INTERVAL IN HETEROSYNAPTIC FACILITATION IN *APLYSIA CALIFORNICA*

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INTRODUCTION

Sensitivity changes of *Aplysia* neurons following afferent stimulation at different time intervals were observed first by Segundo and co-workers[14,15]. A related phenomenon, 'heterosynaptic facilitation' as described by Kandel and Tauc in *Aplysia*[7-9] represents the application of a classical conditioning paradigm to electrophysiological stimulation and recording at the level of the single neuron and — in the cases of unitary test responses — perhaps of the single synapse. A unitary synaptic potential (EPSP, test response) caused by test stimulation of one afferent nerve, is facilitated for many minutes after a strong electrical stimulus (priming shock) to another nerve. Kandel and Tauc[8] showed that after a priming shock to one of the nerves or connectives was delivered, most recorded nerve cells responded unspecifically with sensitization (pseudoconditioning) of the test responses to test shocks applied to any of the non-priming nerves. At 3 occasions in their study a nerve cell was found which needed a temporal pairing between test and priming stimulation to achieve heterosynaptic facilitation. The interstimulus interval (ISI) in this case was 350 msec and selected on the basis of behavioral observations. With this fragmentary data only and without quantitative measures the question is still open whether and to what degree the interval between test shock and priming shock is a determining factor of the degree of heterosynaptic facilitation (HSF). It appears of importance to gather data about such time relations in order to distinguish HSF more clearly from other known changes of synaptic efficacy. Jahan-Parwar and von Baumgarten[5,20] have observed that 'partial specificity' of HSF exists in many of the seemingly unspecific cells, since pairing of the test and priming stimuli was more effective than unpaired stimulation.

The present investigation aimed at the determination of the ISI between test shock and priming shock which would result in maximal amplitude and duration

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of HSF. In this study, for each preparation, HSF was studied using different ISIs. The same cell and when possible the same synapse was tested for HSF, using ISI of 150 msec, 250 msec, 350 msec and so on up to 2 sec. It will be suggested from the data that an ISI of 350 msec was optimal for the evocation of a HSF of maximal amplitude and maximal duration.

METHODS

Preparation

Specimens of *Aplysia californica* were obtained by air express from Los Angeles and kept not longer than one week in artificial seawater (Instant Ocean, Aquarium System Inc., Wickliff, Ohio). A refrigerated, biologically filtered 'instant ocean system' of the same firm was used to maintain the animals. The *Aplysia* were exposed to constant fluorescent light and were all in good physical condition. The main ganglia of the nervous system were dissected out quickly and pinned down in the usual way on a silastic base and covered with artificial seawater. The different nerves and connectives were laid over bipolar platinum stimulation electrodes which were connected through a switchboard with electronic stimulators (for details see ref. 21). The maintenance of constant temperature in the recording chamber by a thermoelectric device (13 $\pm$ 0.5°C) was of high importance in order to obtain comparable test responses over the experimental period of over an hour.

Recording technique

Ultrafine micropipettes filled with 2.5 *M* KCl and a tip resistance of between 3 and 10 MΩ were used for intracellular recording. A Bak-unity gain high impedance preamplifier and a Tektronix 502, DC coupled oscilloscope were used for amplification and display of the recorded signals. The sweep of the oscilloscope was synchronized with the test stimulator and the swept records were photographed by a Grass camera. The shutter and the film drive of the camera were also synchronized with the stimulus. For observation and comparison of test responses an RM-546 Tektronix storage oscilloscope was used.

Stimulation technique

A Grass double stimulator (S8) with two stimulus isolation units was used for the test and the priming stimulation. The bipolar stimulation electrodes were platinum wires submerged in the seawater. The distance between the stimulation electrodes and the recorded cell varied in different experiments between 5 and 15 mm. An electronic unit automatically inversed the polarity of the test and priming stimulus every time in an alternating sequence in order to avoid the polarization of the stimulus electrode. Tests comparing stimulation with alternating polarity and with constant polarity under otherwise constant conditions revealed much better stability of the test response

when the polarity of the stimulus was alternating. Stability of the test response was especially important in unitary EPSPs. The test stimulus was adjusted so that it was barely suprathreshold for a unitary or small compound EPSP when delivered every 10 sec throughout the duration of the experiment. When paired stimulation was performed, the test stimulus was followed by a stronger priming shock to another nerve or connective. The intensity of the priming shock was between 7 and 15 V and suprathreshold for a single action potential. Repetitive action potentials were avoided. Only cases which showed a clear HSF or heterosynaptic inhibition (HSI) were selected for the rest of the experiment. The priming shock was delivered in typical cases only once and in a few cases a second time 10 sec later with inversed polarity. After each pairing trial a pause of at least 10 to 15 min was allowed before the next pairing was performed. The ISI between test shock and priming shock in the pairings was changed in subsequent pairing trials from shorter intervals to longer intervals in a randomized fashion.

There was one deviation from this procedure of random use of intervals between test and priming stimuli. When what turned out to be the optimal interval, producing the largest *EPSP* occurred early in the series of trials, it was naturally followed by trials with EPSPs of lesser amplitude. In these instances we tested for fatigue of the test response by using the optimal interval again on a last trial. If HSF of a size and duration comparable to the first use of this interval was obtained, then we concluded that the lesser HSF observed with other intervals was *not* due to fatigue or accommodation.

Statistical analysis

Because of the possibility of spontaneous changes in responsiveness of recorded neurons, a statistical evaluation of all recorded potentials was carried out even in the cases which appeared quite obvious by pure observation. The mean amplitude of the test EPSP was calculated for the period of the last 4 min before pairing. The standard deviation of the amplitudes of compound EPSPs differed considerably from cell to cell and nerve to nerve, but only cases were evaluated in which the standard deviation did not exceed 20% of the mean value. For unitary EPSPs the standard deviation of the amplitude did not exceed 10% of the mean value. The effect of the paired stimulation on the amplitude of the test response was determined by application of Snedecor's testing procedure[17] to the difference of the control response and the test response after pairing. Because the effect of pairing had a declining time course, the mean value was calculated for fractions of a minute (which corresponds to 3 samples of the same stimulus polarity) immediately after pairing and for 2 min periods (6 samples of the same stimulus polarity) up to 15 min after the priming shock. During this 15 min period, the test response is said to be facilitated by the pairing procedure, as long as the mean value of the test response was elevated significantly over the mean value of the control response. In the beginning of HSF, this evaluation was usually significant at the 0.1% level and towards the very end of the HSF decreased to the 5% level. Even lower levels of statistical significance were taken to indicate the decay

of HSF. The statistical significance of the HSF amplitude was calculated by comparing the mean value of the test responses before and after pairing using suitable statistical procedures such as published by Snedecor[17]. Similar statistical principles were applied also in the procedure of pooling several cases as used for the average curves in Figs. 2 and 3.

Criteria for unitary EPSPs

EPSP had to meet the following criteria to be considered unitary[8,18]: (1) All or nothing behavior when the strength of the test stimulation of the nerve or connectives was changed. (2) Constancy of a relatively short latency period within 5%, when stimulation frequencies higher than 5/sec were used. (3) No steps in the raising or falling phase of the potential.

Recorded cell types

Two compound EPSPs were recorded in the giant cell (R2) of the abdominal ganglion. One unitary EPSP was recorded in the giant cell of the left pleural ganglion. Two compound EPSPs and one unitary EPSP were recorded in unidentified cells of the abdominal ganglion in an area between the right upper quadrant giant cell and the midline, on the dorsal surface of the ganglion. Six unitary and 5 compound EPSPs were recorded from unidentified cells surrounding the right side of the giant cell of the left pleural ganglion on the dorsal surface. No significant differences concerning the optimal ISI were found so far, recording from different cells. However, concerning the relatively small number of recorded neurons and the difficulty and sometimes impossibility to relocate unidentified cells in different preparations, no conclusions can be made yet concerning the question of whether different neurons might have different optimal ISIs.

RESULTS

Selection of evaluated cases

Our studies of ISI were made in the course of a long-lasting study of drug influence on HSF (Hukuhara and von Baumgarten[4]). Since very few recordings are stable enough to allow comparison of test and priming responses over a prolonged period, the data published here represent only a small fraction of the total of recorded cases. More than 50 *Aplysia californica* were used and more than 100 cells penetrated during this study for the results reported in this paper. Only 7 unitary and 9 compound EPSPs were selected for evaluation. The criterion for selection was solely the ability of the recorded neuron to yield a stable resting membrane potential without significant shifts and of a statistically comparable control value of the amplitude of the test EPSPs over the whole experimental period of 1–2 h. Cases which showed fatigue or adaptation either of the test response at the repetition rate of one every 10 sec or of the priming response were discarded.

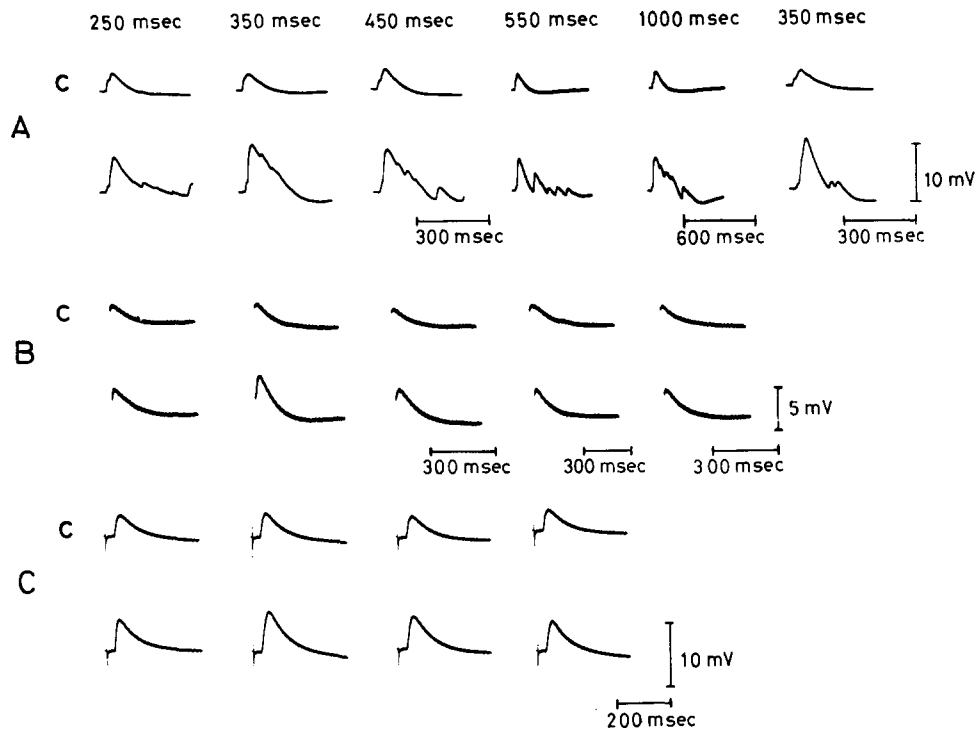

Fig. 1. Comparison of HSF of three different cells at various ISIs. The vertical columns represent HSF at various ISIs as indicated at the top. The upper trace of each pair is the control EPSP (c) taken immediately before the pairing. A is taken from a compound EPSP. B and C are taken from unitary EPSPs. In all three cases HSF was larger at the 350 msec ISI than at any other interval. A, right upper quadrant giant cell. Test nerve: left connective; priming nerve: branchial nerve. B, unidentified cell in the left pleural ganglion. Test nerve: left connective; priming nerve: branchial nerve. C, unidentified cell in the right lower quadrant of the abdominal ganglion. Test nerve: left connective; priming nerve: branchial nerve.

Amplitude of heterosynaptic facilitation

Fig. 1 shows the effect of various ISIs on the amplitude of HSF in one compound (A) and two unitary (B and C) EPSPs. In all three cells, a 350 msec ISI gave a stronger HSF than other ISIs. Fig. 2 shows the maximal amplitude of the test response after priming expressed in percent of the control level of the test response plotted for different ISIs in 7 different neurons. Three of the 7 investigated units showed a clear peak of the HSF amplitude at an interval of 250 msec, one compound EPSP was facilitated best when an interval of 350 msec was used. The 3 cells with the 350 msec optimum showed a sharp decline of the HSF amplitude when shorter or longer intervals were used.

Total duration of heterosynaptic facilitation

By the total duration of HSF, we mean the time period during which the mean value of the test response after priming significantly exceeds the mean value of the

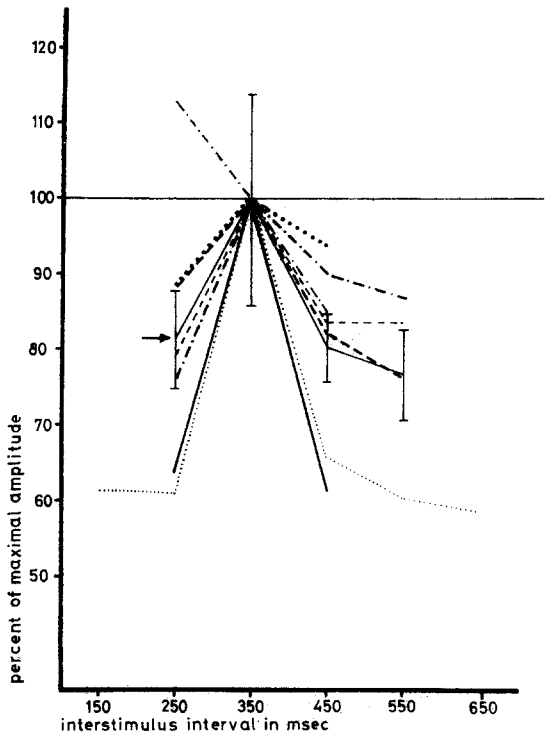

Fig. 2. Amplitude of HSF at various ISIs. The ordinate indicates the maximal amplitude of HSF expressed in percent of the mean control value. The abscissa indicates various ISIs. The different curves were obtained from 7 different cells; three of them were unitary, four were compound EPSPs. The solid line represents the mean values, the arrow ± standard deviation. The plotting indicates a clear peak of HSF at the 350 msec ISI.

control test response (at least 5% significance level). After the relatively weak priming stimuli which we applied, this period was found to vary in different cells from 2–4 min. Stronger stimuli gave much longer HSF but were arousing reverberating activity in the ganglion and had to be avoided. The total duration of HSF was found to depend even more on the ISI than the amplitude of HSF. In all 8 tested cases the maximum duration of HSF was obtained when the interval was 350 msec (Fig. 3). Two cases of the unitary EPSPs were partial exceptions in so far as the duration of HSF was the same for the 250 and 350, or in another cell for the 350 and 450 msec interval. Even in these 2 cases, the duration of HSF decreased when longer or shorter intervals were tried. It should be noted that the duration effect was also best at 350 msec in the case which made the exception in Fig. 2.

Fig. 4 shows the time course of HSF for various intervals. The figure reflects again how the amplitude of HSF depends on the ISI with a clear optimum at 350 msec. The initial decline of HSF is steepest within the first minute and levels exponentially thereafter.

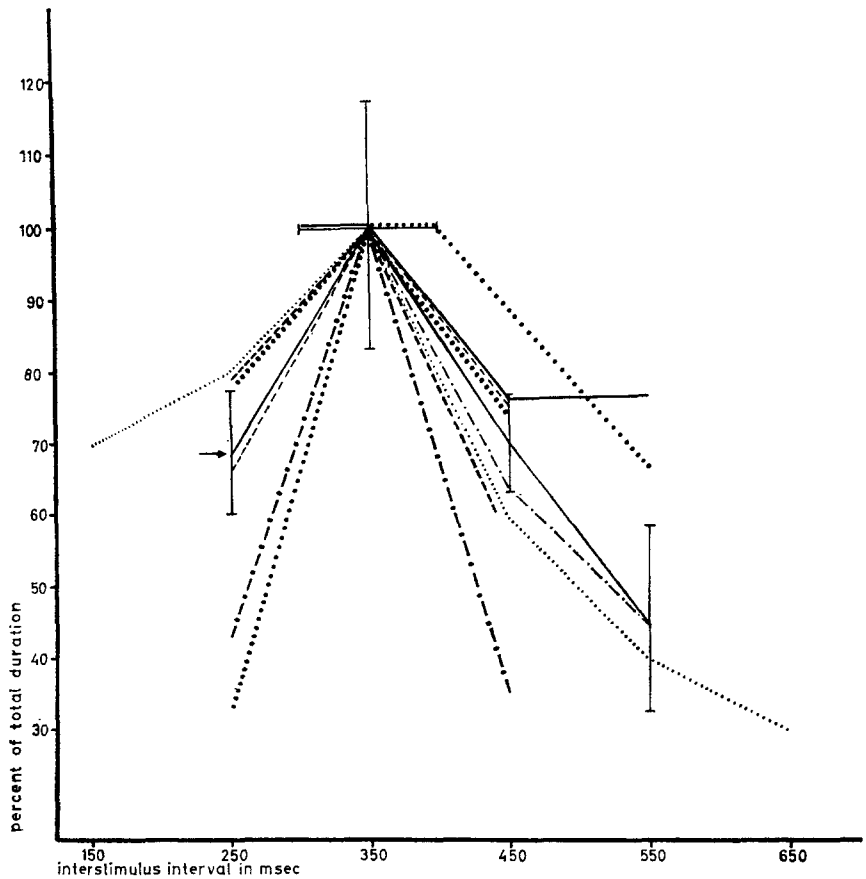

Fig. 3. Duration of HSF at various ISIs. The different curves were obtained from 9 different cells; 5 were unitary, and 4 compound EPSPs. The solid line represents the mean values, the arrow ± standard deviation (absolute value: 135.3 ± 19.8 sec). The plotting indicates that the longest HSF was obtained when the ISI was 350 msec.

Unitary test responses

Test responses, which fulfilled the criteria for unitary EPSPs (see above) presumably arise from a single synaptic terminal. Eight cases met the standards for evaluation. In 6 of these cases the amplitude increased after paired stimulation was performed (Fig. 1B and C). Two other unitary cases did not show changes of amplitude, but were silent in one of the stimulation polarities during the control test but could be triggered by the test stimuli which followed paired stimulation (Fig. 5). Amplitude measurements of HSF could consequently not be performed in these units, but the duration could be determined, taking the appearance of the unitary EPSP in the weaker polarity as criterium. The optimum providing the longest HSF for both of these unitary EPSPs was 350 msec.

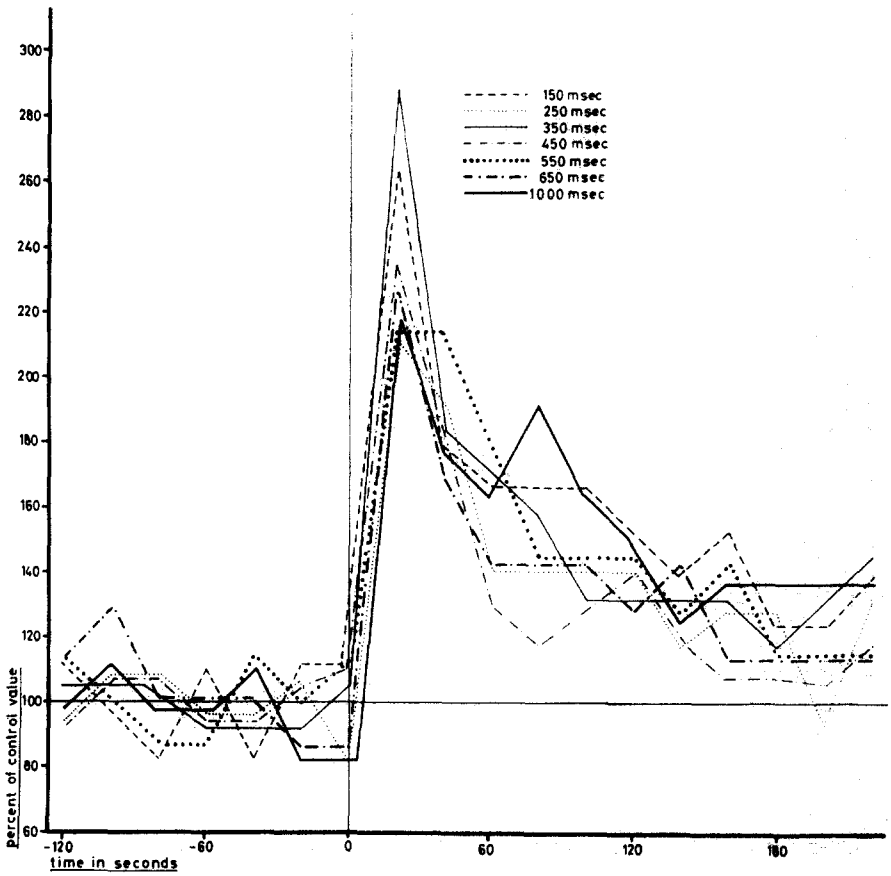

Fig. 4. Time course of HSF at various ISIs. The different curves were taken from the same compound EPSPs, but different ISIs were used. The highest amplitude was reached as can be seen from the curve at a 350 msec ISI. This figure does not indicate the total duration of HSF.

Heterosynaptic inhibition[18]

Only 2 neurons were found which showed heterosynaptic inhibition and had a test response which was stable enough to perform the complete experiment. Both cases were unitary EPSPs. One was of the amplitude-changing type (Figs. 6 and 7), and showed a maximum depression of its amplitude at an ISI of 350 msec. The other unitary EPSP was of the all or nothing HSF type (see above) and was inhibited only when an ISI of 350 msec was used.

DISCUSSION

Our studies about the optimal interval for HSF are based on experiments with 16 EPSPs. This number is relatively small, but the following evidence supports our statement that 350 msec (250–450 msec, middle 350 msec) is an optimal ISI for hetero-

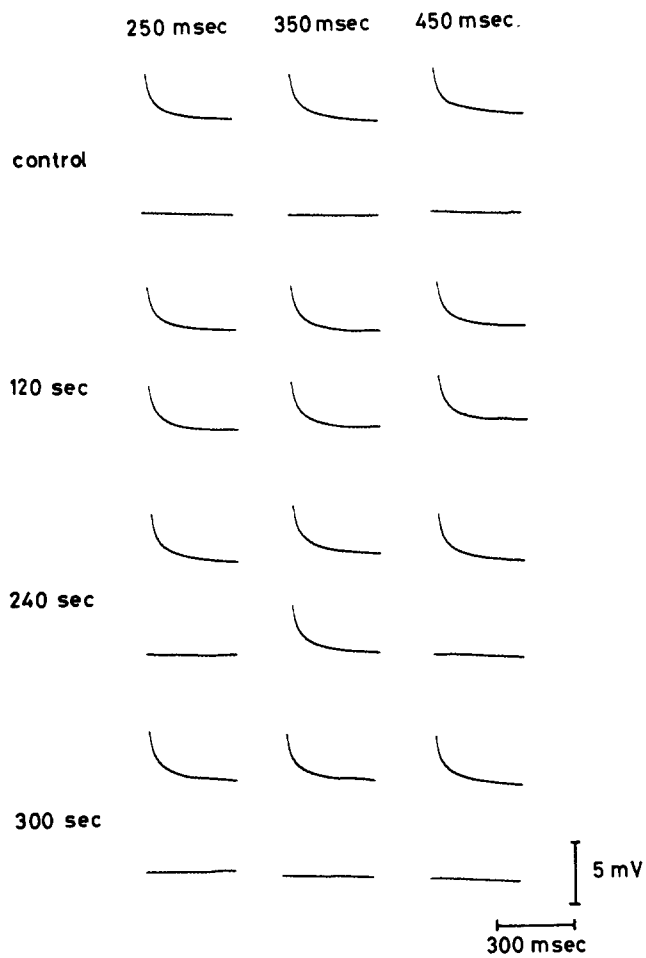

Fig. 5. HSF of the all or nothing type in a unitary EPSP. The upper trace represents always one polarity, the lower trace the other polarity of the test shock. The horizontal rows show each the control EPSP and a sample of the EPSP 120, 240, and 300 sec after the paired stimulation. The vertical columns are recordings taken at 3 different ISIs. The unitary EPSPs appear only in one polarity of the test shocks during the controls. After HSF they appear regularly at both polarities. The time period during which the test response appears at both stimulus polarities depends on the ISI and was longest at the 350 msec interval. Left pleural ganglion giant cell. Test nerve: left connective; priming nerve: tentacular nerve.

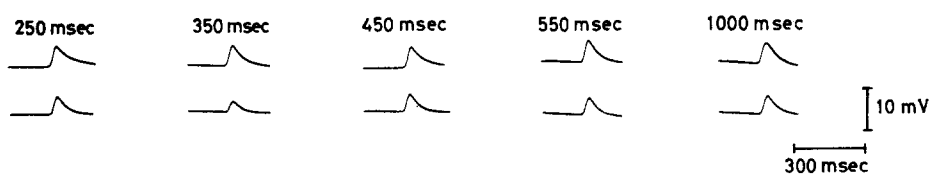

Fig. 6. HSI in a unitary EPSP at different ISIs. The different ISIs are indicated at the top of the figure. The upper trace in the horizontal row is the controlled EPSP immediately before paired stimulation. The lower row represents the heterosynaptic effect 20 sec after priming. It can be seen that the strongest HSI appeared when ISI was 350 msec. Unidentified cell in the left pleural ganglion. Test nerve: left tentacular nerve; priming nerve: right tentacular nerve.

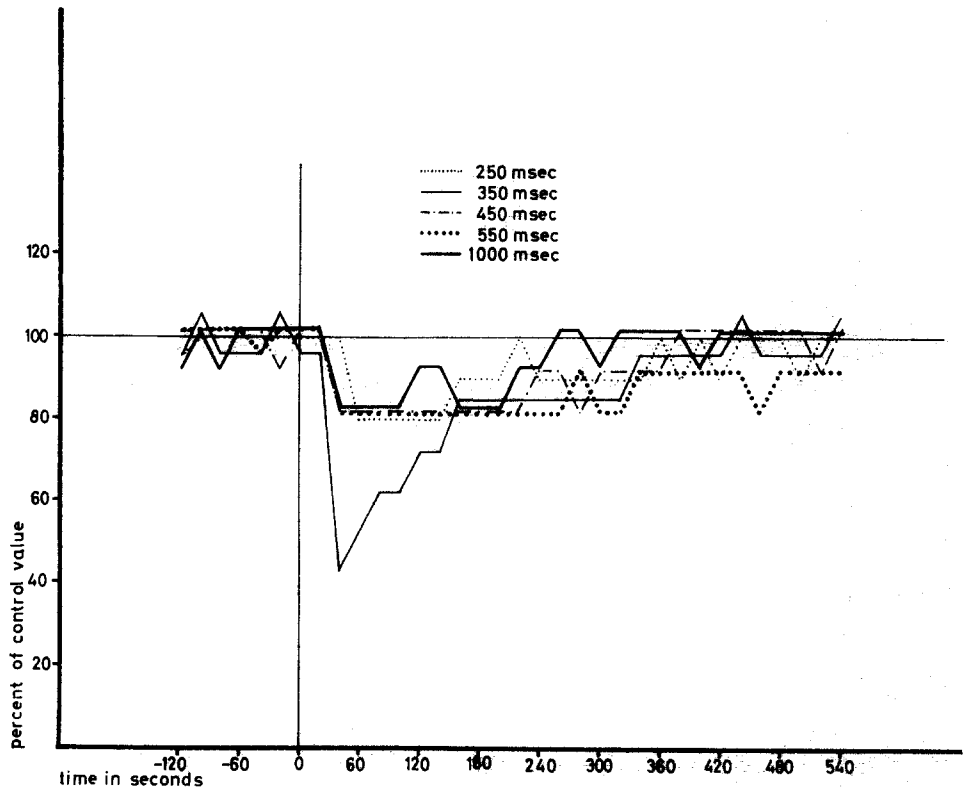

Fig. 7. Time course of HSI at various ISIs. The different curves were taken from the same unitary EPSP at various ISIs. The largest depression appears in the curve which represents an ISI of 350 msec.

synaptic facilitation: (1) In 14 out of 16 cases we obtained a stronger or longer-lasting HSF with 350 msec ISI than with any other tested interval. The two other cases showed brief plateaus which included the 350 msec interval and do not contradict our general finding. (2) The statistical methods applied to each of the 14 cases affirmed that the 350 msec ISI produced significantly greater HSF than the other tested intervals. (3) The 16 evaluated EPSPs represented the most stable and reliable cases from over 100 cases. All cases with unstable test responses were not included in the formal data analysis even though many showed the same results as the evaluated cases with stable test responses. The dependence of HSF on the ISI was observed in compound as well as in unitary EPSPs. The all or nothing character of unitary EPSPs let it appear possible that they are mediated to the recorded neuron by a single synapse. This does not mean, of course, that the test pathway was necessarily monosynaptic.

The shortness and constancy of the latency period, however, favor the view that such EPSPs are presumably monosynaptic[8]. The measured latencies of the test and priming response were always shorter than 10% of the applied ISIs and the observed variations of these latencies were shorter than 2% of the ISIs. For this reason the measurement of the most effective ISI was essentially not contaminated by any

changing polysynaptic delays or varying conduction time from the site of stimulation to the recorded cells.

The present study reconfirmed and extended our earlier observations (on compound EPSPs) that pairing for most cells showing HSF was more effective than priming alone[5]. The precise determination of which ISI between priming and test shock can be regarded as unpaired, was difficult in this paper. The priming shock is always related in time to the foregoing or following test shock. The present study adds more quantitative measurements to this question. So far we can judge by the latency and wave form the test response before and after pairing was triggered by synaptic transmission from the same axonal terminal. A close temporal relation between the depolarization of the synaptic terminal of the test neuron and the depolarization of the priming neuron must be a prerequisite of at least a fraction of the observed facilitation.

Since there are at present no conclusive explanations about the physiological mechanisms of HSF in general, it is not possible to explain what relevant physiological processes or changes at the membrane level go on during the short, but important, time period of the ISI interval. Kandel and Tauc[9], on the basis of the unchanged properties of the postsynaptic membrane during HSF, and of the ineffectiveness of pairing intracellular stimulation with a test shock, favor the hypothesis of presynaptic facilitation. Theoretically such a gating influence of a synapse could be effected in many different ways — hyperpolarization of the test terminal (presynaptic facilitation) being only one. Before we have more data on the minimal delay of the HSF and no direct recording of the test neuron, it appears to be difficult to speculate on the possible mechanisms.

In any case, it is probable from the results that the test synapse or synapses undergo changes of their susceptibility to the gating influence of an impulse in another afferent channel (priming impulse). This cycle starts with the arrival of the test impulse and reaches an optimum after 350 msec.

While posttetanic potentiation certainly can contribute to the total effect of HSF[21], it can be excluded in certain experimental conditions[16,19]. In the present experiment, with unitary EPSPs, no reverberating 'tetanizing' activity of the test neuron was noted during the continuous observation of the membrane potential of the recorded cell after paired stimulation. Also another possibility has to be taken into consideration that paired stimulation activates more interneurons which receive convergent impulses from both the priming nerve and the test nerve. These interneurons could in turn exert somehow a controlling influence on the test synapse. In favor of this hypothesis are the observations of Segundo *et al.*[14,15], who found in *Aplysia* that stimulation with certain frequencies (and therefore certain time intervals) is more effective than using other intervals. Our optimal ISI of 350 msec fits well to the optimal timing stimuli in some of Segundo's experiments.

HSF has been proposed as a possible basic mechanism for information storage and memory at the cellular level[6]. HSF displays features of unspecific sensitization as well as specifically improved connections between simultaneously excited nervous structures[5,8]. Only the fraction of the HSF which is specific could be compared to

psychological conditioning. In a classical conditioning situation rechanneling of information from one channel to another occurs. Prerequisite for this event in psychological experimentation is a close temporal relationship between the activation of both afferent channels with an optimum of less than 1 sec[1,2,10–13,22]. This paper provides evidence for the same prerequisite in the specific fraction of HSF. The similarity in duration of the optimal ISI in psychological conditioning experiments and in HSF does not, of course, prove that the mechanisms involved are necessarily similar. On the other hand, this similarity might stimulate further experimentation along these lines.

SUMMARY

(1) From a great number of recordings, 16 cases were selected in which heterosynaptic facilitation (HSF) or inhibition (HSI) could be repeated without fatigue of the test or priming responses for a period up to 2 h.

(2) Eight of these recordings represented unitary, the rest compound EPSPs. The unitary responses could be divided into those which changed their amplitude during HSF or HSI and those which were facilitated or inhibited in an all or nothing fashion by heterosynaptic interference.

(3) Various interstimulus intervals (ISIs) for the test and the priming stimulation ranging from 250 to 2,000 msec were tested. The interval which produced the highest amplitude of HSF was between 250 and 450 msec (approximately 350 msec) in 15 out of 16 cases. Shorter or longer intervals showed less heterosynaptic facilitation. No differences of the optimal ISI were found in unitary as compared to compound synaptic potentials.

(4) The interval which was correlated to the longest total duration of HSF was also 350 msec.

(5) In 2 cells, HSI was found to be strongest and longest when ISIs of 350 msec were used.

(6) Since in psychological conditioning experiments ISIs of the same magnitude have been found to be optimal, this paper further allows indicating another similarity between HSF and behavioral conditioning.

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