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MECHANISM OF FACILITATION AND DEPRESSION OF THE EXCITATORY SYNAPTIC POTENTIAL IN SPINAL MOTONEURONES

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Under certain conditions the size of the excitatory post-synaptic potential (EPSP) produced in spinal motoneurones by constant afferent stimuli may be modified independently of changes in the post-synaptic membrane potential. For example, when monosynaptic EPSPs are set up by two successive afferent volleys in the same muscle nerve, the second EPSP is larger or smaller than the first, depending upon the interval between the two stimuli (Curtis & Eccles, 1960; Fadiga & Brookhart, 1962). Monosynaptic EPSPs may also show a considerable enhancement in size for some minutes after repetitive synaptic activation (Eccles, 1953; Eccles, Krnjević & Miledi, 1959; Curtis & Eccles, 1960). This enhancement is similar in temporal course to that for post-tetanic potentiation of monosynaptic reflexes (Lloyd, 1949; see review by Hughes, 1958). Further, it has recently been found that a muscle afferent volley may depress the size of monosynaptic EPSPs in a motoneurone produced by another muscle afferent volley (Frank & Fuortes, 1957; Eccles, Eccles & Magni, 1961). This was designated 'presynaptic inhibition', since the EPSP depression was not associated with any detectable changes in the post-synaptic membrane.

The changes in synaptic efficacy causing a variation of the EPSP size under these conditions might have arisen from two sources: (1) changes in the sensitivity of the post-synaptic site and/or (2) changes in the output at the presynaptic terminals. The preceding paper (Kuno, 1964) has shown that the monosynaptic EPSP evoked by an impulse in an individual afferent fibre is composed of discrete units each with a certain probability of response. This observation provides a favourable approach for analysing the site of facilitation and depression of EPSPs. The probability of response of units to afferent impulses depends entirely upon the conditions

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of the presynaptic membrane, while the amplitude of the unit EPSPs depends upon the properties of the post-synaptic element (Katz, 1962). Based on this criterion, the present study was undertaken to test the possible source of alteration in the EPSP size. It will be shown that facilitation and depression of monosynaptic EPSPs, under the conditions described above, result from changes in the probability of generation of the unit EPSP components.

METHODS

Adult cats were made decapitate by spinal section at the first cervical segment under ether or pentobarbital sodium (Nembutal, Abbott Laboratories) anaesthesia. The dissection and experimental arrangements were essentially similar to those described in the preceding paper (Kuno, 1964). Monosynaptic EPSPs in triceps surae (TS) motoneurones were recorded with intracellular electrodes following stimulation of fine filaments dissected from the TS nerve. The size of EPSP response evoked by such an input was so small that accurate measurement was difficult at slow sweep speeds. Consequently, the experiments on successive homosynaptic EPSPs by double shocks at long intervals were replaced by observations of the responses at fast sweep speeds during repetitive stimulation. In a later stage of the present study, this difficulty associated with slow sweep speeds was removed by using the second beam of a Tektronix dual beam oscilloscope, Type RM 565, to display the slow trace on the first beam at an expanded sweep speed. The experiments on 'EPSP depression by heteronymous afferent volleys' were performed with this aid. Additional technical details are given in the appropriate sections in the Results.

RESULTS

Changes of EPSPs following homosynaptic activation

Effects of successive stimulation. Changes in monosynaptic EPSPs during successive afferent stimulation may be investigated either by recording the responses to two afferent stimuli applied at various intervals or by recording the EPSPs to repetitive afferent volleys at various frequencies. Because of technical convenience (see Methods), changes in successive EPSPs at short intervals (or high frequencies) were investigated by two afferent volleys, and those at long intervals (or low frequencies) were studied during repetitive stimulation. A previous study (Curtis & Eccles, 1960) showed that there was a good correlation in changes of amplitude of EPSPs tested by double afferent volleys and during repetitive stimulation. It is therefore likely that changes in the EPSP produced by these two procedures depend upon the same mechanism. Figure 1*A* illustrates a series of monosynaptic EPSPs in a triceps surae (TS) motoneurone produced by two successive stimuli (at an interval of about 6 msec) applied to a fine filament of the TS nerve at 0·5/sec. The simultaneous recordings of afferent impulses (lower traces) indicate that the size of the two inputs is identical and constant throughout the observations. The evoked EPSPs, on the other hand, fluctuated in size, including some failures of response (Kuno, 1964). Such fluctuations in the sizes of the

first and second EPSPs appear to occur independently, and the range of fluctuation is large compared with the expected changes in amplitude of the second responses. Consequently, facilitation or depression of the second EPSPs was not obvious from these records. In Fig. 1*B*, the amplitude of the second EPSP is plotted against the amplitude of the first

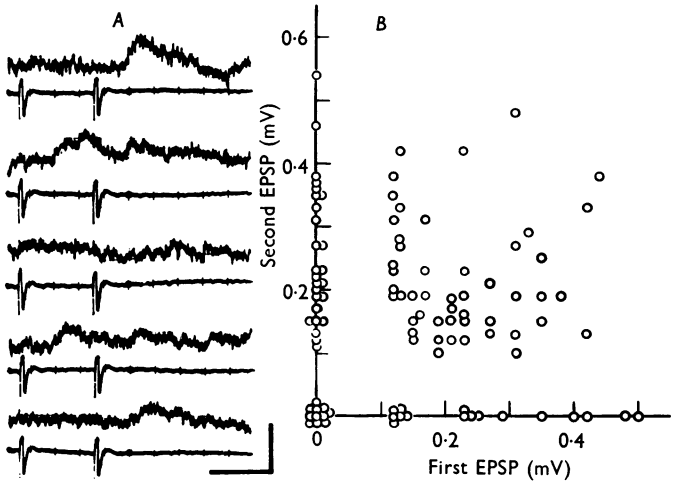

Fig. 1. *A*. Upper traces, monosynaptic EPSPs in a TS motoneurone produced by double afferent volleys (0·5/sec) in a filament dissected from the TS nerve. The filament was stimulated at intensities of twice the threshold. The number of afferent fibres measured in this filament was more than five. Lower traces, simultaneous records of the afferent impulses from the TS nerve. Time, 5 msec. Voltage, 1 mV for EPSPs. *B*. Relation between the amplitudes of the first EPSP and the second EPSP of each paired response.

EPSP for about 100 paired responses. The range of fluctuation of the second EPSPs was approximately the same whether the first EPSP was large, small, or even a failure. Obviously, the size of the second EPSP in each paired response is not related to the amplitude of the first EPSP preceding it (Fig. 1*B*). In Fig. 1*B*, it should also be noted that the number of failures of the first responses is greater than that of the second EPSP responses. This suggests that there is, on the average, a facilitation of the second EPSP responses. The results are more clearly shown in Fig. 2*A*, in which the size distributions of observed first (stippled columns) and second (open columns) EPSPs are presented. There are fewer response failures following the second stimuli than the first, and large EPSPs are evoked more often by the second stimuli than by the first.

Figure 2*B* shows a similar experiment in which the sizes of monosynaptic EPSPs evoked at two different stimulus frequencies are compared. When the stimulus frequency was increased from 0·5/sec (stippled

columns) to 5/sec (open columns), there was an increase in the number of failures as well as a decrease in the number of occurrences of large EPSPs (Fig. 2*B*).

As demonstrated in the preceding paper (Kuno, 1964), the amplitude of monosynaptic EPSPs evoked by impulses in one or a few Group Ia afferent fibres fluctuated in a manner described by Poisson's law. Under

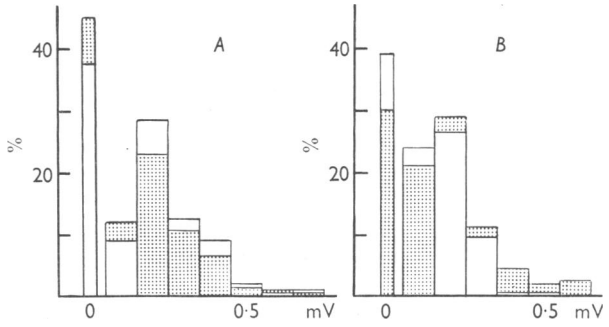

Fig. 2. *A*. Histograms showing distribution of amplitudes of the first EPSPs (stippled columns) and the second EPSPs (open columns) constructed from 200 paired responses. Ordinate, percentage of the number of occurrences relative to the total number of afferent stimuli (200) applied. Abscissa, amplitude of EPSPs in mV. Both stippled and open columns should be read from the bottom. Thus, at 0, for example, the stippled column is greater than the open column. *B*. Same as in *A*, but for EPSPs evoked by repetitive afferent stimulation at 0·5/sec (stippled columns) and 5/sec (open columns) in another experiment.

this condition, the mean number of units responding to one afferent impulse, m, may be given by two independent relations:

$$m = \frac{\text{mean size of EPSP responses } (\bar{v})}{\text{mean size of unit EPSPs } (v_1)}, \tag{1}$$

$$m = \log_e \frac{\text{number of impulses } (N)}{\text{number of failures } (n_0)}, \tag{2}$$

hence,

$$\bar{v} = v_1 \log_e \frac{N}{n_0} = v_1 m. \tag{3}$$

The relation (3) implies that if facilitation and depression of monosynaptic EPSPs occurs without associated changes in the mean size of unit EPSPs (v_1), the relative change in the average EPSP size ($\bar{v}$) is identical with the relative alteration of m calculated from equation (2). Figure 3 shows the results of fifteen experiments in which the relative change of m by two successive stimuli or during repetitive afferent stimulation was observed. Along the horizontal axis the intervals between two shocks are plotted,

but at intervals longer than 50 msec the experiments are replaced by observations during repetitive stimulation at the corresponding frequencies (see Methods). The ordinate gives the changes of m relative to that of the control responses (m_0) evoked at a rate of 0·5/sec. Since the experiments were performed only at selected intervals or frequencies, the changes of m cannot be plotted as a continuous function of stimulus frequencies. Nevertheless, the general tendency suggests that the relative change in the EPSP size at various stimulus frequencies (interrupted curve in Fig. 3, reproduced from Fig. 2*B* in Curtis & Eccles, 1960) may approximately be paralleled by the relative alteration of m.

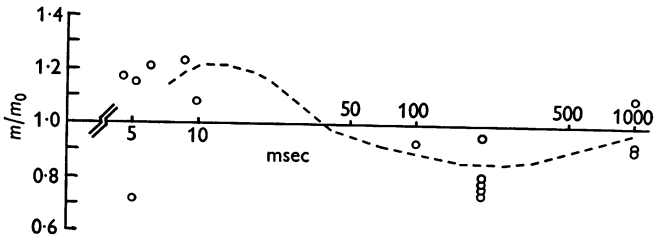

Fig. 3. Relative changes of m in EPSP responses evoked by double afferent volleys at various intervals. The results at intervals longer than 50 msec were obtained during repetitive afferent stimulation at the corresponding frequencies. All points were obtained from fifteen different experiments. m was calculated from the number of failures in about 200 responses. The value of m_0 in these experiments was 0·78–4·2, but the number of afferent fibres stimulated was not measured except in a few experiments. Abscissa, time on a logarithmic scale showing the interval between two stimuli or inverse of the stimulus frequencies. Interrupted curve, reproduced from Fig. 2*B* in Curtis & Eccles (1960), shows relative changes in the size of monosynaptic EPSPs evoked by repetitive stimulation of the whole muscle nerve at various frequencies.

Figure 3 shows that with the exception of one experiment, when the two afferent stimuli are applied at intervals between 4 and 10 msec, the second EPSPs are, on the average, larger than the first EPSP responses. This contrasts with a previous report (Curtis & Eccles, 1960) that in most cases the EPSP generated by a second afferent volley at the corresponding intevals is depressed although there is still a relative potentiation compared with the second EPSP evoked at long intervals or at very short intervals. In the one experiment in which a depression was observed in the second EPSPs evoked at a short interval, there was again no significant correlation in size between the first and second EPSPs in each paired response (cf. Fig. 1*B*). This suggests that such depression is not due to depletion of available transmitter (see Discussion).

Because of the small size of unit EPSPs and of the small change in monosynaptic EPSPs during successive stimulation, a slight change in the former could appreciably influence the latter. Figure 4 provides a test of whether the alteration in the average size of monosynaptic EPSPs during successive stimulation may be accounted for entirely by changes

in m, or some changes in the unit EPSP size also contribute to the alteration. In each experiment about 200 responses were recorded, and the mean size of EPSP responses, $\bar{v}$, was obtained. From the number of failures, m was calculated, and the unit size of EPSPs, v_1, was derived from equations (1) and (2). These parameters, $\bar{v}$, m, v_1, were expressed relative to those of the control responses ($\bar{v}_0$, m_0, v_{10}) evoked by afferent stimulation at a rate of 0·5/sec. Figure 4 shows that there is a good correlation in the relative changes between m and $\bar{v}$ (Fig. 4*A*), while the changes in the unit EPSP size (v_1) are not correlated with the facilitation

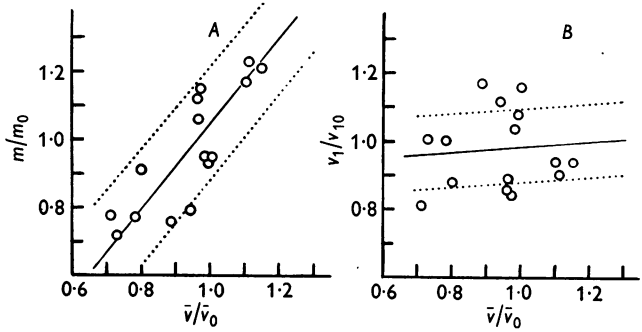

Fig. 4. *A*. Relation between relative changes of m and relative changes of the mean amplitudes of EPSP responses evoked by successive afferent volleys. Dotted lines show S.E. of the correlation. *B*. Same as in *A*, but relation between relative changes of the unit size of EPSPs and relative changes of the mean amplitudes of responses.

and depression in the mean size of EPSP responses (Fig. 4*B*). It is concluded that changes in the size of monosynaptic EPSPs by successive afferent volleys are due to changes in the probability of occurrence of unit EPSP components.

After repetitive stimulation. In the experiment illustrated in Fig. 5*A*, a fine filament dissected from the triceps surae (TS) nerve was stimulated at 1/sec, and the monosynaptic EPSPs were recorded from a TS motoneurone. Amplitudes of successively evoked EPSPs are plotted on the ordinate. At zero time, tetanic stimulation was applied to the filament for 20 sec at a frequency of 500/sec. In spite of the fluctuation in the size of EPSP responses, post-tetanic potentiation is obvious in Fig. 5*A*. The value of m in this experiment was obtained from the number of failures in about 200 EPSP responses evoked at a rate of 0·5/sec. Judging from the value of m (3·10), it may be assumed that three Group Ia fibres were used for afferent stimulation in this experiment (cf. Kuno, 1964). Figure 5*B* shows a similar experiment, but the value of m (1·21) in this experiment suggests that only one Group Ia afferent fibre was stimulated. In

contrast to Fig. 5*A*, there is no apparent increase in the size of individual EPSP responses after tetanization (Fig. 5*B*). When EPSP responses with m of less than 1·5 were tested (in four experiments), the size of individual EPSPs after tetanization usually did not markedly exceed the range of fluctuation of the responses. However, the presence of a post-tetanic potentiation of EPSPs is suggested by a decrease in the number of failures of the responses after tetanization (Fig. 5*B*). Consequently, when every fifty responses after tetanization were successively tested, there was a

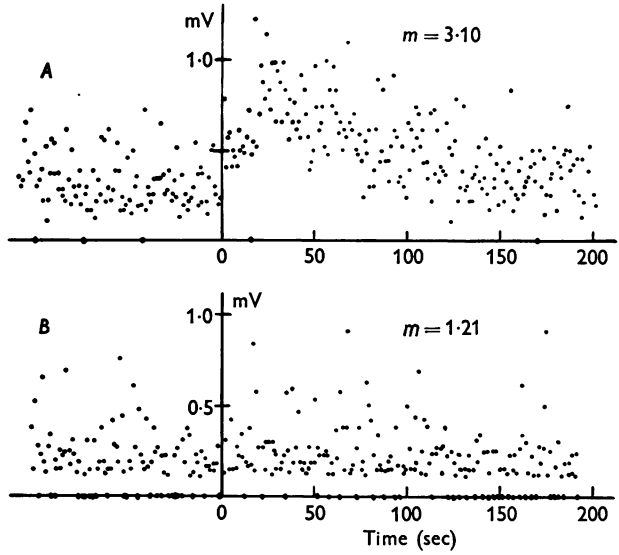

Fig. 5. *A*. Monosynaptic EPSP responses in a TS motoneurone (identified by antidromic spike) evoked by stimulation of a fine filament dissected from the TS nerve (at 1/sec). This filament contained more than five Group I fibres. Ordinate, amplitudes of the responses. Points lying on the abscissa show failures of the responses. Points to the left of the ordinate were obtained before tetanization at 500/sec for 20 sec. Points to the right of the ordinate show successive EPSP responses evoked every one sec after the tetanization. Time after tetanization in sec. m was calculated from the number of failures of responses evoked at 0·5/sec. *B*. Same as in *A*, but for another TS motoneurone. The number of Group I fibres in this filament was two.

definite increase in the mean size of EPSP responses compared with the mean sizes of each fifty responses before tetanization. This potentiation in the mean EPSP size gradually decayed to the control level with a time course comparable to that in Fig. 5*A*. Similarly, the value of m calculated from the number of failures in each fifty responses increased after tetanization and decayed to the original level in some minutes. These changes are shown in Fig. 6. Points in Fig. 6*A* are reproduced from the results in

Fig. 5*B*. The m calculated from the first fifty responses after tetanization (filled circles) is about twice as large as those in the responses before tetanization (two filled circles on the left). The value of m gradually decayed thereafter, reaching the control value within 150–200 sec after tetanization. The time course of post-tetanic potentiation in the mean amplitudes (open circles) was similar to that in m (Fig. 6*A*). Figure 6*B* shows the results of another experiment. The value of m in this experiment was 1·34 when calculated from about 200 responses evoked at a stimulus frequency of 0·5/sec. However, the post-tetanic potentiation in this experiment was unusually strong, and an obvious increase in the size of

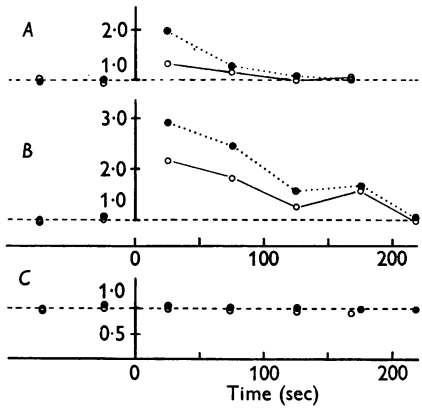

Fig. 6. *A*. m (filled circles) and the mean amplitudes of EPSP responses (open circles) calculated from every fifty responses after tetanization at 500/sec for 20 sec (to the right of the ordinate), relative to those before tetanization (to the left of the ordinate). Time after tetanization in sec. *B*. Same as in *A*, but for another experiment. *C*. The mean size of unit EPSPs in every fifty responses after tetanization (to the right of the ordinate) relative to those before tetanization (to the left of the ordinate) in two experiments. Further explanation in text.

some individual EPSPs was recognized among the first fifty responses after tetanization. Correspondingly, the potentiation both in m (filled circles) and in the mean size of EPSPs (open circles) was more striking in Fig. 6*B* than in Fig. 6*A*.

Although there was a good agreement in the total time course of potentiation between the value of m and the mean size of EPSP responses, the amount of potentiation relative to the control was always greater in m than in the mean EPSP responses (Fig. 6*A*, *B*). Such a difference was particularly marked at the beginning of potentiation, becoming gradually less until it finally became insignificant (Fig. 6*A*, *B*). As shown by equation (3), the present statistical assumptions require that the relative change in m should be identical with the relative change in the mean size

of EPSP responses ($\bar{v}$), if the mean size of unit EPSPs (v_1) remains constant. The discrepancy indicated in Fig. 6*A*, *B*, therefore, suggests that there is a change in the unit size of EPSPs after tetanization. It is possible that the repetitive synaptic activation produces a 'receptor desensitization' of the post-synaptic membrane (Thesleff, 1959; Curtis & Eccles, 1960; Eccles, 1961). If the 'receptor desensitization' takes place, the unit size of EPSPs would be reduced immediately after tetanization. Accordingly, an increase in the mean amplitude of EPSP responses would be less than that expected from an increase in the probability of unit responsiveness, until the post-synaptic 'receptor sensitivity' is restored. This surmise is tested in Fig. 6*C*, in which the results shown in Fig. 6*A* (open circles) and Fig. 6*B* (filled circles) are used. In each experiment, about 200 EPSP responses were obtained by stimulation of the dissected filament at a rate of 0·5/sec. The mean size of unit EPSPs (v_1) was calculated from equations (1) and (2). On the assumption that most unit EPSPs are larger than zero but smaller than 1·5 times the calculated mean unit size (v_1), only those EPSP responses whose size was in this range were selected from every fifty responses after tetanization. Their average values relative to the control (before tetanization) are plotted in Fig. 6*C*. Obviously, there is no significant decrease in the mean size of unit EPSPs after tetanization. The probability of response of units may be no longer constant during post-tetanic potentiation, changing with time after tetanization. Consequently, some deviation from the statistical assumptions, on which the Poisson analysis is based, may be unavoidable in this case. The difference in potentiation between m and the mean amplitude of EPSP responses (Fig. 6*A*, *B*) is probably due to the unsteady probability of unit responsiveness after tetanization.

EPSP depression by heteronymous afferent volleys

In agreement with Frank & Fuortes (1957) and Eccles, Eccles & Magni (1961), Group I afferent volleys in the biceps–semitendinosus (BST) nerve usually produced a reduction in the size of monosynaptic EPSPs in the triceps surae (TS) motoneurone. The maximum effect was obtained when the BST volleys preceded the TS stimulation by 10–20 msec. Since the BST volleys might evoke a dorsal root reflex in the TS nerve (Brooks & Koizumi, 1956; Eccles, Kozak & Magni, 1961), the EPSP depression at short intervals could be due in part to diminution of the test afferent volleys by collision with antidromic sensory impulses (Eccles, Eccles & Magni, 1961). In order to exclude this possibility, the interval between the BST conditioning and TS test stimuli was set at more than 30 msec. In all experiments, the effect of EPSP depression was intensified by applying three conditioning shocks to the BST nerve at a frequency of about 200/sec.

In two out of seven cats, however, there was no significant EPSP depression in TS motoneurones by BST afferent volleys. Accordingly, the present analysis was performed only in selected motoneurones in which the monosynaptic EPSP evoked by stimulation of the whole TS nerve was obviously depressed by the BST conditioning volleys. In spite of this selection, EPSPs in some motoneurones produced by afferent volleys in fine TS filaments were not appreciably depressed by the same conditioning volleys (cf. Fig. 8). Thus, effects of the EPSP depression by BST volleys

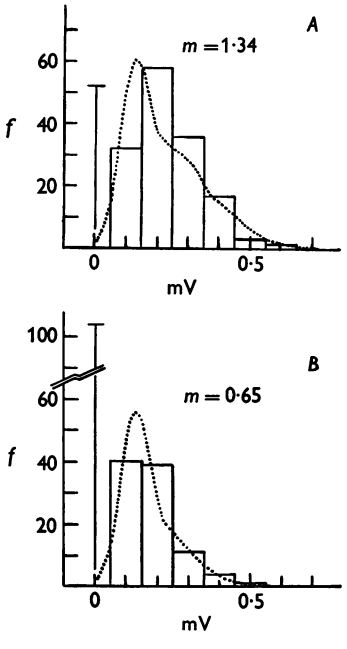

Fig. 7

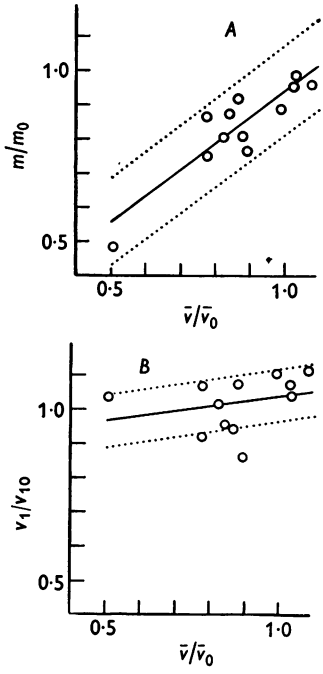

Fig. 8

Fig. 7. *A*. Histogram showing distribution of amplitudes of monosynaptic EPSPs in a TS motoneurone evoked by stimulation of a fine TS nerve filament every 2 sec. Failures of the responses shown by a vertical line at 0. The theoretical distribution drawn by the dotted curve. *B*. Same as in *A*, but three conditioning volleys in the BST nerve preceded each TS filament stimulation.

Fig. 8. *A*. Relation between relative changes of m and relative changes of the mean amplitudes of EPSP responses in TS motoneurones by the BST conditioning volleys in twelve experiments. Dotted lines show S.E. of the correlation. *B*. Same as in *A*, but relation between relative changes of the unit size of EPSPs and relative changes of the mean amplitudes of responses.

were not uniform for different TS Group Ia afferent fibres which converged on the same TS motoneurone. This suggests that the site of the EPSP depression is presynaptic.

In the experiment illustrated in Fig. 7, the effect of EPSP depression

obtained was the largest in the present study, reduction in the mean size of EPSP responses being about 50 %. Figure 7 shows the size distributions of EPSP responses evoked by stimulation of a fine filament every 2 sec with (Fig. 7*B*) and without (Fig. 7*A*) the BST conditioning volleys. By application of BST conditioning volleys, the number of failures of EPSP response was increased from 52 (Fig. 7*A*) to 104 (Fig. 7*B*). Consequently, there was a decrease in the value of m from 1·34 to 0·65. The appropriate Poisson curve was determined for the size distribution of the control responses (Fig. 7*A*) by the procedure described in the preceding paper (Kuno, 1964). The predicted distribution curve was then constructed for Fig. 7*B*, based on the assumptions that the mean size and variance of the unit EPSPs are not changed by the BST conditioning volleys. Although the number of occurrences of EPSPs in the first group is less than that predicted from the assumptions, the range of fluctuations in the observed EPSP responses approximately agrees with the theoretical curve (Fig. 7*B*). It is likely that the monosynaptic EPSP depression is not associated with any significant changes in the unit size of EPSPs. This is further supported by the analysis shown in Fig. 8, which is made by the same procedure as described in Fig. 4. In twelve experiments, the reduction in the mean size of TS monosynaptic responses ($\bar{v}$) by the BST volleys is obviously correlated with a decrease in the value of m (Fig. 8*A*), while changes in the unit size (v_1) have no significant correlation with alteration in the mean size of monosynaptic responses (Fig. 8*B*). It may be concluded that the monosynaptic EPSP depression in TS motoneurones by BST afferent volleys involved only the presynaptic mechanism.

DISCUSSION

The present study agrees with previous observations that, when monosynaptic EPSPs are set up by paired afferent volleys, the second response may be facilitated or depressed, depending upon the interval between the two stimuli (Curtis & Eccles, 1960; Fadiga & Brookhart, 1962). From the analysis presented here, it seems clear that such facilitation and depression are not associated with changes in the unit size of EPSPs but are entirely accounted for by changes in the probability of occurrence of unit EPSPs. Hence, the alteration in synaptic efficacy produced by successive afferent volleys involves the presynaptic level only. Curtis & Eccles (1960) have postulated that facilitation and depression of the second EPSP is determined by a balance between two opposed processes taking place at the presynaptic terminals by the first afferent volley: (1) mobilization of the available transmitter, and (2) depletion of the transmitter. These assumptions imply that the size of the second EPSP in each paired response would

be influenced by the number of units released by the first afferent impulse, since more depletion of the transmitter would be expected as more release of the transmitter occurs by the first impulse. However, as shown in Fig. 1*B*, the size of the second EPSPs after failures of the first responses was in the same range as when no failure occurred in the first responses. This behaviour bears similarity to that at the neuromuscular junctions of the frog (del Castillo & Katz, 1954), the crayfish (Dudel & Kuffler, 1961*a*) and the rat (Hubbard, 1963). It seems likely that the mechanisms of both potentiation and depression reside in the presynaptic terminals but are independent of the amount of transmitter released by the first impulse.

The present study has also shown that post-tetanic potentiation of monosynaptic EPSPs is restricted to changes in the presynaptic properties. It has been reported that potentiation is often followed by a late depression (Eccles *et al.* 1959; Curtis & Eccles, 1960). Such a post-tetanic depression was not clear in the present study although occasionally a relative increase in the number of failures of EPSP responses followed a period of potentiation (for example, Fig. 5*B*). It was suggested that this late depression (Curtis & Eccles, 1960), and probably the delayed onset of post-tetanic potentiation as well (Eccles, 1961), may be due to 'receptor desensitization' on the post-synaptic membrane. A similar mechanism was also considered to occur at the neuromuscular junction after repetitive stimulation of the motor nerve (Thesleff, 1959). However, this explanation seems untenable, since the unit size of EPSPs is not changed by an afferent conditioning tetanus (Fig. 6*C*). A recent study has also failed to confirm the 'receptor desensitization' at the neuromuscular junction by repetitive nerve stimuli (Otsuka, Endo & Nonomura, 1962).

The present study has provided direct evidence that the EPSP depression in a TS motoneurone by BST muscle afferent volleys is presynaptic in origin. The depression was associated with a decrease in the number of occurrences of unit EPSPs without appreciable changes in the mean size of units. This behaviour is similar to that described in the analysis of 'presynaptic inhibition' at the crayfish neuromuscular junction (Dudel & Kuffler, 1961*b*). Thus, the results presented here confirmed the previous conclusion that the EPSP depression in TS motoneurones by BST afferent volleys is a manifestation of a presynaptic mechanism of inhibition (Frank & Fuortes, 1957; Eccles, Eccles & Magni, 1961).

SUMMARY

1. Facilitation and depression of monosynaptic excitatory post-synaptic potentials (EPSPs) were studied in spinal motoneurones of the cat.

2. The test monosynaptic EPSPs were evoked by stimulation of a strictly limited number of Group Ia afferent fibres.

3. Alteration in the monosynaptic EPSPs was studied under three conditions: (1) during successive afferent stimulation, (2) after repetitive afferent stimulation (post-tetanic potentiation) and (3) following heteronymous Group I afferent volleys ('presynaptic inhibition').

4. Under all these conditions, changes in the test monosynaptic EPSPs were associated with alteration in the probability of generation of the unit EPSP components without appreciable changes in the unit size of EPSPs.

5. It was concluded that changes in the monosynaptic EPSPs under these conditions involve only presynaptic mechanisms.

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