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IN VITRO STUDIES OF PROLONGED SYNAPTIC DEPRESSION IN THE NEONATAL RAT SPINAL CORD

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SUMMARY

1. Synaptic transmission between dorsal root afferents and α-motoneurones was studied in the *in vitro* hemisected spinal cord preparation isolated from neonatal rats.

2. Repetitive stimulation of the dorsal roots depressed the monosynaptic reflex recorded from the homologous ventral roots. The depression developed within the first five to six pulses in a stimulus train and stabilized at a plateau-like level for many seconds of stimulation.

3. The magnitude of the reflex depression depended on the stimulation interval and was capable of reducing the reflex to 17% of its undepressed control during 5 Hz stimulus trains. Complete recovery from depression was obtained at stimulation intervals $\geqslant 30$ s.

4. Monosynaptic excitatory postsynaptic potentials (EPSPs) were recorded intracellularly after reduction of the activity in polysynaptic pathways by addition of mephenesin to the bathing media. These EPSPs exhibited a prolonged, frequency-dependent synaptic depression. The depression reduced the amplitude of the EPSP to 25% of the undepressed control during 5 Hz stimulus trains, and was alleviated completely at stimulus interval $\geqslant 60$ s.

5. The prolonged EPSP depression was not altered by blockade of glycinergic and type-A γ-aminobutyric acid ($GABA_A$-ergic) receptors underlying postsynaptic inhibition in the spinal cord. Injection of current steps to motoneurones before and during the prolonged depression revealed similar values of the membrane time constant and input resistance. These excluded changes in the passive properties of the motoneurone membrane as an explanation for the observed synaptic depression.

6. Extracellular recordings of terminal potentials and their accompanying synaptic fields from motor nuclei in the ventrolateral cord revealed that the frequency-dependent depression in the synaptic fields was not preceded by any detectable changes in the amplitude or the shape of the terminal potential, suggesting that the depression cannot be attributed to impairment of action potential invasion to the afferent terminals.

7. Reduction of the basic level of transmitter release in the spinal cord by increasing the Mg^{2+}/Ca^{2+} ratio of the bathing solution or by application of 2 μM of L(−)baclofen markedly diminished the synaptic potential depression at all the stimulation intervals tested in this study. Recovery from depression was evident for

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stimulation intervals $\geqslant 5$ s. Under these conditions, short tetanic trains (5 pulses at 25 Hz) revealed a substantial facilitation and potentiation of the EPSPs.

8. We suggest that prolonged depression of synaptic potentials in the neonatal rat reflects decreased transmitter output from the activated afferent terminals. The decrease in transmitter release seems best explained by postulating immature properties of the transmitter release machinery in developing synapses, which prevents sustained transmitter release during moderate stimulation rates.

INTRODUCTION

The efficacy of synaptic transmission in the central nervous system is not constant and can be modulated by the rate of activity in presynaptic pathways (Redman, 1979, 1990; Mendell, 1984; Burke, 1987). High-frequency activation of primary afferents in the adult cat *in situ* induces simultaneous facilitation, potentiation and depression of the excitatory postsynaptic potentials (EPSPs) evoked in α-motoneurones (Curtis & Eccles, 1960; Kuno, 1964; Redman, 1979; Hirst, Redman & Wong, 1981; Lev-Tov, Pinter & Burke, 1983; Collins, Honig & Mendell, 1984; Mendell, 1984; Lev-Tov, Meyers & Burke, 1988). Facilitation and potentiation are predominant at the beginning of stimulus trains, but are then masked by synaptic depression (Curtis & Eccles, 1960; Kuno, 1964; Lev-Tov *et al.* 1983, 1988). Understanding of these frequency modulation phenomena in the mammalian spinal cord is impeded, in part, by the inability to control the ionic environment of spinal neurones *in situ*. On the other hand, *in vitro* studies of synaptic transmission in neonatal mammalian spinal cord are complicated by EPSP depression of unknown origin during low-frequency stimulation (Kudo & Yamada, 1985; Fulton & Walton, 1986) without detectable facilitation or potentiation.

The present work was aimed at studying the synaptic transmission and its modulation in spinal cords isolated from neonatal rats. Our major findings were that the low-frequency EPSP depression, had a more prolonged time course than previously described (Kudo & Yamada, 1985) and that the prolonged depression is of presynaptic nature and is unaltered by reducing the activity in polysynaptic pathways to motoneurones or by blockade of glycinergic and type-A γ-aminobutyric acid ($GABA_A$-ergic) receptors. The depression did not involve changes in the passive properties of the motoneurone membrane or the degree of invasion of presynaptic action potentials to the terminal arbor. The prolonged depression was markedly reduced by lowering the basic level of transmitter release and could be then transformed into facilitation and potentiation by high-frequency stimulation. A preliminary report of some of our data has appeared in abstract form (Lev-Tov, Pinco & Lavy, 1990).

METHODS

Preparation

Experiments were performed on thirty-one spinal cords isolated from 6- to 8-day-old neonatal rats (Otsuka & Konishi, 1974) anaesthetized by injection of pentobarbitone (35 mg/kg, I.P.). The spinal column was quickly removed and transferred into a dissection chamber superfused at 15 ml/min with cooled (5–10 °C) and oxygenated (95% O_2/5% CO_2) low-Ca^{2+} (0·2 mM), high-Mg^{2+} (5 mM) Krebs solution (composition of the normal Krebs solution in mM: NaCl, 120; KCl, 4; $CaCl_2$, 2; $MgSO_4$, 1; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11) pH = 7·3. The spinal cord was hemisected

surgically, after removal of the dura mater, and transferred with the dorsal and ventral root of the lumbar segments L1–L6 to an experimental chamber continuously superfused at 8 ml/min with oxygenated normal Krebs solution (see above) at room temperature (24–26 °C) for 45–60 min of pre-incubation, prior to electrophysiological recordings. In the most recent experiments sucrose was substituting for NaCl in the dissection solution (at 240 mM, to maintain a constant osmolarity) to reduce the hypoxic damage to the preparation (Aghajanian & Rasmussen, 1989).

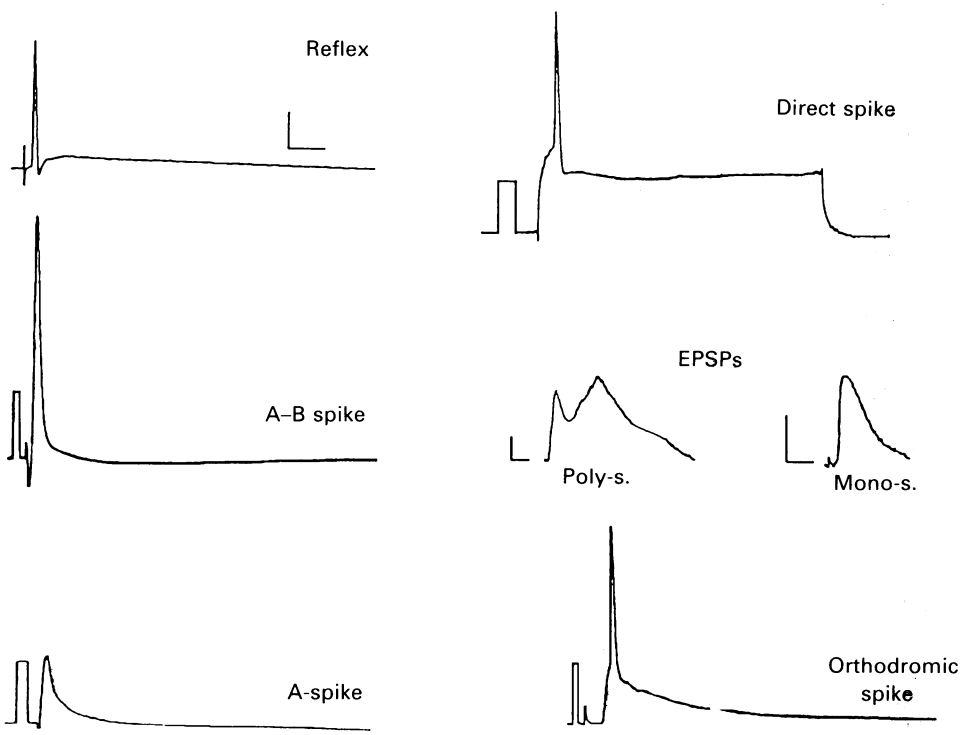

Fig. 1. Electrophysiological recordings from the neonatal rat spinal cord. Reflex: the monosynaptic reflex and root potential evoked in L5 ventral root by stimulation of the L5 dorsal root. (10-sweep average, calibration: 1 mV, 20 ms). A–B spike: the antidromic spike evoked by cathodic stimulation of the ventral root of L5. Note a prolonged after-hyperpolarization. (8-sweep average, calibration: 20 mV, 2 ms). A-spike: an initial segment spike elicited in a motoneurone by antidromic stimulation of L5 (8-sweep average, calibration: 25 mV, 2 ms). Direct spike: four-sweep average of a spike elicited in an α-motoneurone by injection of a current step (2·3 nA, 80 ms) via the bridge circuit of the intracellular amplifier. Calibration: 20 mV, 10 ms. EPSPs: EPSPs elicited in α-motoneurones by graded stimulation of the L5 dorsal root. The polysynaptic EPSPs (Poly-s, 8-sweep average) was recorded from a preparation bathed in normal Krebs solution; calibration: 1 mV, 4 ms. The monosynaptic EPSP (Mono-s, 8-sweep average) was recorded from a different preparation bathed in a mephenesin (1 mM)-containing Krebs solution. Calibration: 5 mV, 5 ms. Orthodromic spike: an orthodromic spike (4-sweep average) elicited by suprathreshold stimulation of the L5 dorsal root. Calibration: 25 mV, 2 ms.

Stimulation and recordings

The homologous dorsal and ventral roots (usually of L5 or L4) were placed in suction electrodes for either stimulation or recording. Intracellular recordings were performed using 40–100 MΩ micropipettes filled with 3 M-potassium acetate. Motoneurones were impaled from the lateral

aspect of the hemisected cord following removal of the pia mater, and identified by the presence of antidromic (A–B) or initial segment (A) spikes (Fig. 1). Motoneurones with membrane potential more positive than −60 mV were discarded. The input resistance of the motoneurones was measured by the spike height method (Frank & Fuortes, 1956) or by injection of a series of 80 ms current steps at varying intensities through the balanced bridge of the amplifier (Axoclamp 2A, Axon Instruments). Synaptic activation of motoneurones was done by graded stimulation of the dorsal roots (see Fig. 1, EPSPs and Orthodromic spike). In many of the cases, recordings of monosynaptic EPSPs were obtained after reducing the activity in polysynaptic pathways to motoneurones by addition of mephenesin (1 mM; Sigma Chemical Co.) to the bathing media, and stimulation of split macrofilaments of the dorsal root (Fig. 1, EPSPs, Mono-s.).

The delay between the stimulus artifact and onset of the EPSPs generated by this method was 2–3 ms at room temperature in most of the cases. This delay corresponded to synaptic delays of 1 ms or less (measured from the positive peak of the extracellularly recorded terminal potentials to the onset of the accompanying synaptic fields, see Fig. 6). The monosynaptic nature of the EPSPs is also supported by the similarity in the shape of the EPSPs at different stimulation frequencies (see Fig. 5).

The viability of the preparation was tested by monitoring the monosynaptic reflex (Fig. 1, Reflex), the antidromic or the directly elicited (Fig. 1, Direct spike) spikes during the experiments. Deterioration of the preparation occurred usually 12–18 h after its isolation. Extracellular recordings of antidromic and synaptic fields were obtained using 5–7 MΩ glass micropipettes, filled with 2 M-NaCl (see Fig. 6).

Data acquisition

Data were continuously recorded on a PCM recorder (Neurodata), digitized using a fast A/D converter (Computerscope, R-C Electronics) and saved on the hard disc of the computer (Olivetti M-280) for further off-line analysis.

Statistical analysis

The among-group differences of tested parameters were examined using one- or two- (see below) way analysis of variance (ANOVA). When significant differences were indicated in the F ratio test ($P < 0{\cdot}001$), the significance of differences between means of any two of these groups was determined using the modified Tukey method for multiple comparisons with an α of 0·05 (Zar, 1984).

RESULTS

Depression of the monosynaptic reflex

Low-frequency stimulation of a dorsal root depressed the monosynaptic reflex and the root potentials recorded from the homologous ventral root. The upper panel in Fig. 2*A* shows the monosynaptic reflexes and root potentials recorded from the ventral root of L5 during graded stimulation of the dorsal root of L5 at 2 s intervals. After an initial run-down (the first five to six responses in a train, Fig. 2*A* upper and lower panels) the reflex amplitude reached a plateau-like level which could be maintained with minor fluctuations for many seconds of stimulation (Fig. 2*A*, lower panel). The amplitude run-down and the plateau depression depended on the stimulation frequency, showing an increase upon shortening of the stimulus intervals (Fig. 2*A*, lower panel). This is also shown in the upper panel of Fig. 2*B* in which computer averaged records of the reflex sampled during the plateau phase of repetitive trains at 2, 5, 10 and 20 s intervals (left to right) are superimposed. The largest reflex amplitude in each one of the experiments was obtained upon a 60 s interval of dorsal root stimulation. The average amplitude at 60 s intervals was therefore used to normalize the averaged responses elicited at each of the shorter interstimulus intervals. The mean values of averaged normalized reflex amplitudes

in six different experiments are shown (means ± S.D.) in the left part of the lower panel of Fig. 2*B*. These data were used to calculate the percentage depression ($D_p i$) of the reflex at any tested interpulse interval, using the general equation:

$$D_p i = (1 - R_i/R_{max}) \times 100, \quad (1)$$

where R_i is the averaged response at a given stimulation interval, and R_{max} is the averaged response elicited at a 60 s stimulation interval, in each of the experiments.

The dependence of D_p on the interstimulus interval is shown in the right part of the lower panel of Fig. 2*B*. As expected, D_p was inversely related to interpulse interval of dorsal root stimulation, and recovered only at an interpulse interval of $\geqslant 30$ s ($D_p 30 < D_p 10$; $D_p 30 = D_p 60$, one-way ANOVA, followed by the Tukey method for multiple comparisons $\alpha = 0{\cdot}05$).

Depression of the composite EPSPs

In order to evaluate the underlying mechanism of the prolonged depression described above, we studied EPSPs recorded intracellularly from α-motoneurones in the neonatal rat spinal cord. Following identification of a motoneurone and its characterization (see Methods), we applied a series of stimulus trains at nine different intervals in a random order with intertrain intervals of up to 5 min (according to the frequency of the preceding train) and assessed the frequency dependence of the EPSP amplitude. Collection of the complete set of data from a given motoneurone required more than 60 min of intracellular recordings. Such recordings (1–8 h) were obtained from forty-nine motoneurones in twenty-two different preparations. Motoneurones at this postnatal age (6–8 days) had antidromic spikes with a mean amplitude of 69·9 ± 8·4 mV ($n = 34$, 13 out of the 49 motoneurones analysed in this study, and 21 additional motoneurones) and a mean input resistance of 12·6 ± 6·0 MΩ ($n = 39$, 17 out of the 49 motoneurones analysed in this study and 22 additional motoneurones). The spike was followed by prolonged after-hyperpolarization (Fig. 1, A–B spike) and quite often by an after-depolarizing hump (see Fulton & Walton, 1986).

The EPSPs studied in this work were elicited by graded stimulation of the dorsal roots, which activate monosynaptic and polysynaptic pathways (Fig. 1, EPSPs, Poly-s, see Baldissera, Hultborn & Illert, 1981). It was possible to record monosynaptic EPSPs in isolation in many of the motoneurones following addition of 1 mM-mephenesin to the bathing media (Fig. 1, EPSPs, Mono-s.), which has been shown to suppress activity in polysynaptic pathways in the adult cat spinal cord (Wright, 1954; Longo, 1961) and in the isolated spinal cord of the embryonic (Ziskind-Conhaim, 1990) and neonatal rat (Fulton & Walton, 1986). Figure 3*A* shows the development of the monosynaptic EPSP depression during repetitive stimulation of the dorsal root at 2 s intervals (upper panel) in the presence of 1 mM-mephenesin. The EPSP run-down was rather rapid (5–6 pulses) and followed by stabilization of the amplitude at a plateau-like level (Fig. 3*A*, upper and lower panels). The initial run-down and the plateau of the EPSP depression depended, as in the case of the monosynaptic reflex (see above), on the frequency of synaptic activation (Fig. 3*A*, lower panel). Computer-averaged records sampled at the 'plateau' phase during repetitive stimulations at 1, 2, 5 and 10 s intervals of dorsal root stimulation are

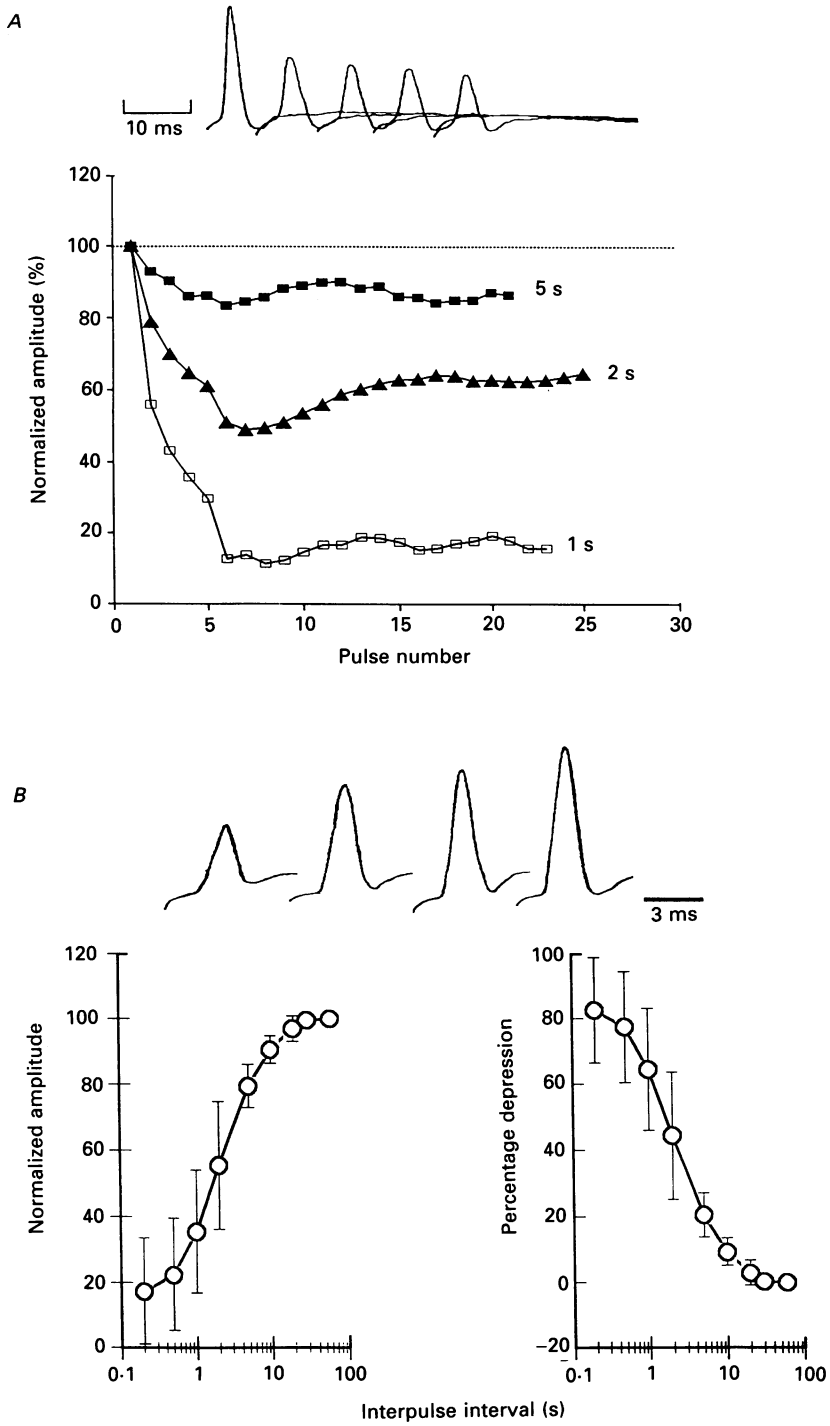

Fig. 2. Prolonged depression of the monosynaptic reflex. *A*, run-down in the amplitude of the monosynaptic reflex during repetitive stimulation. Upper panel: five consecutive reflexes recorded from the L5 ventral root during repetitive stimulation of the homologous dorsal root at 2 s intervals. The traces were superimposed with arbitrary time lags. Lower

shown (left to right, respectively) in the upper panel of Fig. 3*B*. The percentage depression of the EPSP at a given stimulation interval ($D_p i$) was calculated, using the relation:

$$D_p i = (1 - V_i/V_{max}) \times 100, \quad (2)$$

where V_i is the averaged EPSP amplitude elicited upon a given stimulation interval in a given motoneurone and V_{max} is the largest EPSP amplitude averaged in the nine-interval stimulation series in that motoneurone. Similar to the reflex data, the EPSPs recorded from preparations bathed in normal Krebs solution reached V_{max} in each of the recorded motoneurones at stimulus intervals of 60 s.

The average dependence of D_p on the stimulation interval in twelve motoneurones recorded from seven different preparations in the presence of mephenesin is shown in the lower panel of Fig. 3*B*. Unlike the monosynaptic reflex data, which are insensitive to subthreshold synaptic events, EPSPs were easily detected at even short stimulation intervals at which the monosynaptic reflex was virtually abolished. Nevertheless, the EPSP depression was again alleviated only at interpulse intervals $\geqslant 60$ s ($D_p 30 < D_p 10$; $D_p 60 < D_p 30$; one-way ANOVA, followed by Tukey method, $\alpha = 0{\cdot}05$).

Postsynaptic factors and the prolonged depression

Blockade of postsynaptic inhibition

In order to rule out possible involvement of polysynaptic inhibitory pathways in the mechanisms accounting for the prolonged EPSP depression we blocked the $GABA_A$-ergic (for review see Bormann, 1988) and the glycinergic (Werman, Davidoff & Aprison, 1968; for review see Krnjevic, 1974) receptors in the spinal cord by bicuculline and strychnine, respectively (for reviews see: Krnjevic, 1974 and Bormann, 1988), and recorded EPSPs at different stimulation intervals. Figure 4*A* shows three pairs of superimposed intracellular records of EPSPs (upper record in each pair) and inhibitory postsynaptic potentials (IPSPs; lower record in each pair) generated in an L5 motoneurone (with resting potential more negative than the IPSP reversal potential; see legend for Fig. 4*A*) by stimulation of the homologous and the L3 dorsal roots respectively, before (Normal) and after (Str; Bicuc) addition of 1 μM-strychnine and 10 μM-bicuculline to the normal Krebs bathing medium. The reversed IPSP virtually disappeared 10 min after addition of the drugs. At the same

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panel: run-down in the amplitude of the monosynaptic reflex recorded from same preparation during repetitive stimulation at 1, 2 and 5 s intervals. Data were normalized by the first response in each series, and smoothed by the modified moving-bin method (Lev-Tov, 1987), bin = 5, dbin = 1 responses. *B*, dependence of the reflex depression on the stimulation interval. Upper panel: computer-averaged records (8 sweeps each) of the reflexes recorded from L5 ventral root during repetitive stimulation of the homologous dorsal root at 2, 5, 10 and 20 s intervals (left to right). Averages were taken immediately following the initial amplitude run-down in each series, and are shown superimposed with constant time lags for convenience. Lower panel: the means $\pm$ S.D. of the averaged reflex amplitudes obtained upon various stimulation intervals in six different experiments. Computer averages in each experiment were taken at each stimulation interval and their peak amplitudes were normalized by the amplitude obtained upon stimulation at 60 s intervals in the respective experiment (left). These data were also used to calculate the percentage depression (D_p) of the monosynaptic reflex at the same stimulation intervals (right) according to eqn (1) (see text).

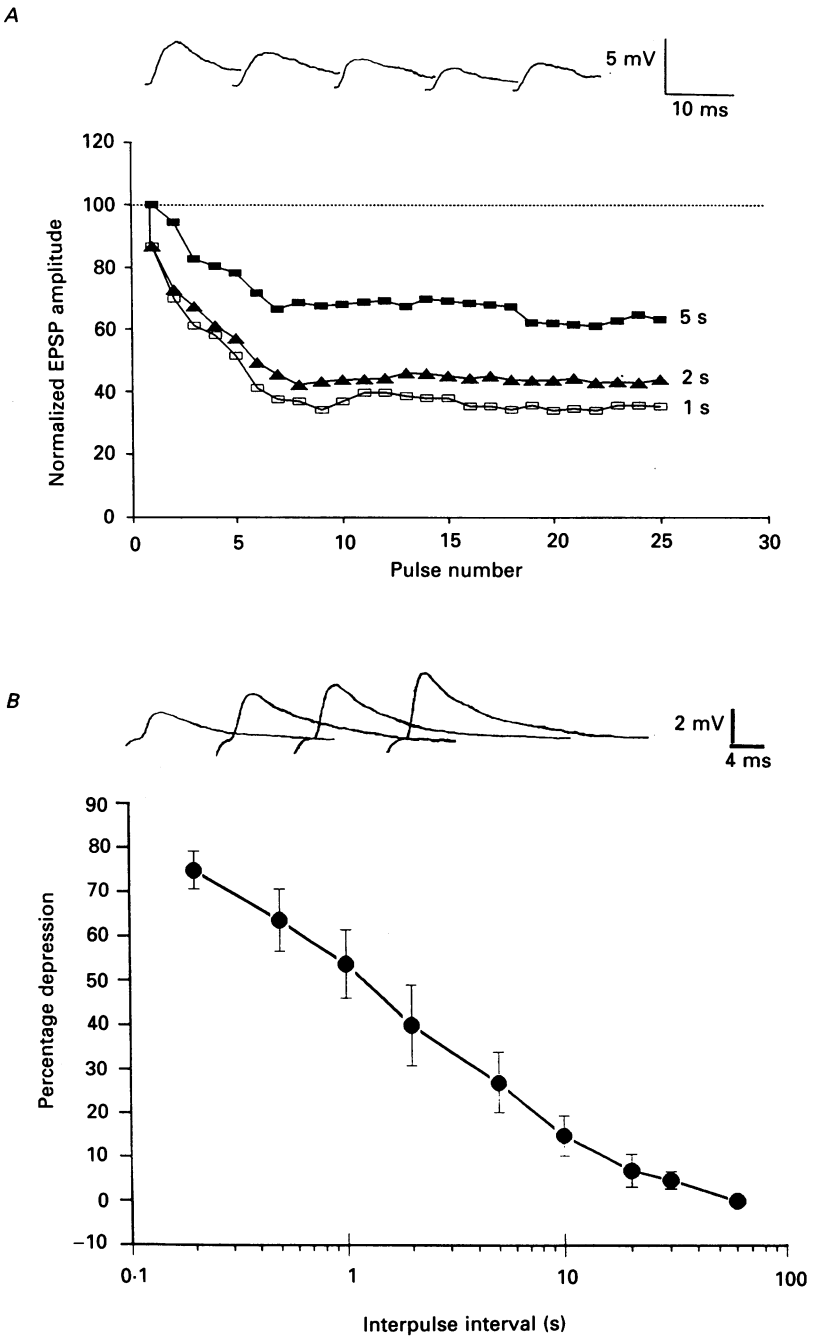

Fig. 3. Prolonged depression of the monosynaptic EPSP. *A*, run-down in the amplitude of the monosynaptic EPSP during repetitive stimulation. Upper panel: intracellular recordings of five consecutive EPSPs (single sweeps) recorded from a motoneurone during repetitive stimulation of the L5 dorsal root at 2 s intervals in the presence of 1 mM-mephenesin. Records were superimposed for convenience. Lower panel: run-down in the amplitude of the EPSPs elicited in the same motoneurone shown in *A* during repetitive stimulation at 1, 2 and 5 s intervals. Data were normalized by the mean EPSP amplitude ($n = 16$) obtained upon stimulation at 60 s intervals in the same motoneurone. Modified

time, both the mono- and the polysynaptic components of the 'homologous' EPSPs were markedly enhanced. These changes can be attributed to blockade of the inhibitory pathways in the spinal cord. Figure 4*B*, shows four computer-averaged records of EPSPs elicited in a different motoneurone by graded stimulation of the dorsal root at 1, 2, 5 and 10 s intervals (left to right) after addition of 1 μM-strychnine and 10 μM-bicuculline to the mephenesin-containing (1 mM) normal Krebs solution. The dependence of these EPSPs on the stimulation intervals was no different from that of the normal control EPSPs elicited at the same stimulation intervals in a mephenesin-containing medium (see Fig. 3). These findings were confirmed by recordings made from nine motoneurones in four different preparations.

The motoneurone input resistance and membrane time constant

Further assessment of a possible involvement of postsynaptic mechanisms in the neonatal synaptic depression was accomplished in two different ways: (1) comparison of the waveforms of the EPSPs recorded upon different stimulation intervals, and (2) comparisons of the membrane time constant extracted from the voltage transients generated by injection of current steps to motoneurones prior to and during synaptic depression.

Figure 5*A* shows superimposed computer-averaged records of EPSPs elicited in a motoneurone by graded stimulation of the dorsal root at 1, 2, 5 and 10 s intervals (left to right, left panel). Down-scaling of these EPSPs to the peak amplitude of the EPSP elicited upon a 1 s stimulation interval enabled comparison of their waveforms (Fig. 5*A*, right panel). The waveforms of the EPSPs elicited at these stimulation intervals showed close similarities, indicating that the amplitude differences cannot be ascribed to changes in postsynaptic membrane properties. Figure 5*B* shows computer-averaged records of the membrane response to 0·2 nA, 80 ms hyperpolarizing current steps injected to a motoneurone via the bridge circuit of the amplifier, 1 s before (left) and 1 s after (right) a five-pulse stimulus train applied to the dorsal root at 1 Hz (middle), in the presence of 1 mM-mephenesin, Despite the severe EPSP depression induced by the train, the steady-state voltage and the rate of its development were closely similar to those recorded before the stimulus train. The membrane time constant was analysed by fitting regression lines to the late component of the natural logarithmic transforms of the voltage transients generated before and after synaptic activation (Fig. 5*C*; for review see Rall, 1977). The membrane time constants calculated for this motoneurone were 10·9 and 11·2 ms before and after the conditioning EPSPs, respectively. Thus the input resistance of the motoneurone (9·5 MΩ before and 10·2 MΩ after the train; calculated from the

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moving bin, bin = 5, dbin = 1 responses. *B*, dependence of the EPSP depression on the stimulation interval. Upper panel: computer-averaged records (16-sweeps each) of EPSPs sampled from a motoneurone upon dorsal root stimulation at 1, 2, 5 and 10 s intervals (left to right). Averages were taken following the initial amplitude run-down in each series. Records were superimposed for convenience. Lower panel: the means ± S.D. of the percentage depression of the EPSPs (D_p, calculated by eqn (2)) recorded at different stimulation intervals from ten different motoneurones, seven different experiments. The average EPSP amplitudes at each stimulation interval were normalized by the maximal averaged amplitude ($n = 16$) obtained for each stimulation series in each of the recorded motoneurones.

steady-state voltage developed by each current pulse) and the membrane time constants were unaltered during the prolonged synaptic depression. Similar results were found in three other motoneurones tested in the same way, in two different experiments.

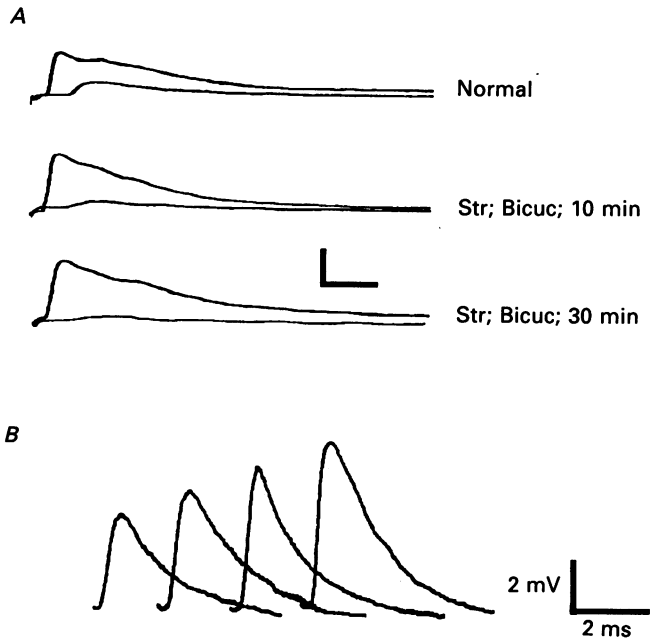

Fig. 4. The effects of strychnine and bicuculline on the prolonged EPSP depression. *A*, computer-averaged records (8 sweeps each) of EPSPs (upper trace in each pair) and reversed IPSPs (lower trace in each pair) recorded from a motoneurone in the L5 segment before (Normal), and 10 and 30 min after (Str; Bicuc) addition of 1 μM-strychnine and 10 μM-bicuculline to the normal Krebs solution. The IPSPs generated by stimulation of a caudal filament of the L3 dorsal root reversed polarity as the membrane potential changed spontaneously from -65 to -73 mV. Stimulation interval 10 s. Calibration = 5 mV, 12 ms. *B*, computer-averaged records (8 sweeps each) of monosynaptic EPSPs evoked in a motoneurone at 1, 2, 5 and 10 s intervals after addition of 1 μM-strychnine and 10 μM-bicuculline to a mephenesin-containing (1 mM) normal Krebs solution.

Presynaptic factors and the prolonged depression

Invasion of presynaptic action potentials

Extracellular recordings of synaptic fields and their preceding terminal potentials were performed in three different experiments using 5–7 MΩ micropipettes filled with 2 M-NaCl. The extracellular pipette was advanced in the spinal cord while recording antidromic fields upon ventral root stimulation. As the electrode reached a motor nucleus (indicated by an increase in the amplitude of the antidromic field and by shortening of its rise time, Fig. 6, arrow-head), the antidromic stimulation was switched to orthodromic synaptic stimulation. Averaged records of the resultant terminal potentials and synaptic fields elicited at 1, 2 and 5 s intervals are shown in Fig. 6 (left to right). The substantial decrease in the synaptic field at the shorter stimulation intervals (compare 5 to 1 s interval) was not preceded by any detectable

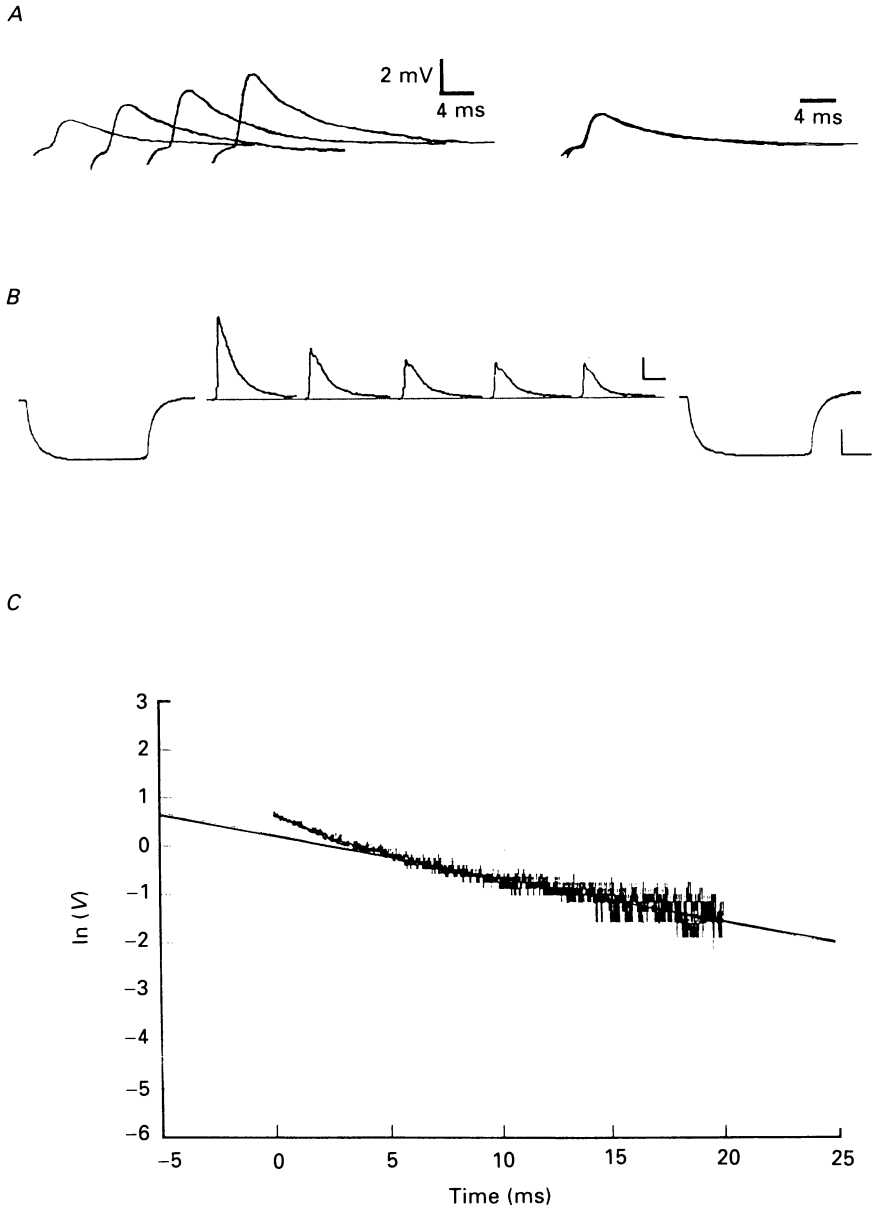

Fig. 5. Passive properties of the motoneurone membrane during prolonged depression. *A*, the waveform of monosynaptic EPSPs elicited at different stimulation intervals. Left panel: four computer-averaged records of monosynaptic EPSPs (16 sweeps each) elicited in a motoneurone by graded stimulation of the dorsal root in the presence of 1 mM-mephenesin at 1, 2, 5 and 10 s intervals (left to right). Right panel: the EPSPs shown in the left panel were scaled down to the peak amplitude of the EPSP elicited at 1 s stimulation intervals and superimposed. *B*, the voltage responses (32-sweep averages) to hyperpolarizing current steps (0·2 nA, 80 ms) injected to a motoneurone before (left) and after (right) application of five consecutive stimuli to the dorsal root at 1 s intervals. Computer-averaged records of the EPSPs elicited by these stimuli (8 sweeps each) are

changes in the amplitude of the terminal potentials (TPs) and their shape (expanded scale, upper traces).

The baseline of transmitter release

The results described in the previous sections indicated that the prolonged low-frequency EPSP depression might be attributed to a prolonged decrease in release of the excitatory transmitter upon moderate rates of synaptic activation. Since the synaptic depression during repetitive stimulation reported in the adult cat spinal cord depends on the level of transmitter release (Lev-Tov *et al.* 1988), we tried to test the effect of decreasing the baseline of transmitter release on the frequency dependence of the monosynaptic EPSPs. This was done by two alternative ways: (1) decreasing the calcium and increasing the magnesium concentration of the bathing medium, and (2) addition of the $GABA_B$ agonist L-baclofen which has been shown to decrease transmitter release in the spinal cord (Lev-Tov *et al.* 1988; Edwards, Harrison, Jack & Kullmann, 1989; Peng & Frank, 1989; Konnerth, Keller & Lev-Tov, 1990) by decreasing presynaptic calcium influx (see Dunlap & Fishbach, 1981; Dolphin and Scott, 1986; Robertson & Taylor, 1986; Bormann, 1988; Lev-Tov *et al.* 1988; Bowery, 1989; Wang & Dun, 1990).

(1) *Effects of low* Ca^{2+}, *high* Mg^{2+}. Reduction of the Ca^{2+} concentration to 0·85 mM and elevation of the Mg^{2+} concentration to 6–7 mM blocked the monosynaptic reflex very rapidly. Intracellular recordings from α-motoneurones under these conditions, revealed that the monosynaptic EPSPs were reduced from a control level of $7{\cdot}9 \pm 3{\cdot}5$ mV ($n = 10$) to $2{\cdot}7 \pm 1{\cdot}0$ mV ($n = 12$; see also Fig. 7*B*). Repetitive activation of the dorsal root under these conditions resulted in significant changes in the behaviour of the EPSPs: the initial run-down in the EPSP amplitude observed in the control conditions (Normal Krebs, lower panel of Fig. 7*A*) was less severe (upper panel of Fig. 7*A* and Low calcium in the lower panel of Fig. 7*A*), and the severe EPSP depression observed normally following the initial run-down (Normal Krebs in the lower panel of Fig. 7*A*) was partially alleviated (Low calcium in the lower panel of Fig. 7*A*). Moreover, the maximal EPSP amplitude obtained for a series of stimulus trains was found at stimulation intervals shorter than 60 s in four out of the twelve cells analysed in this section. This could be attributed to fluctuations in the 16-sweep averages of the low-amplitude EPSPs recorded under these conditions. Figure 7*B* (lower panel) shows the amplitude of averaged EPSPs recorded from the same motoneurone at different intervals of dorsal root stimulation, first, in low Ca^{2+}, high Mg^{2+} (Low calcium) and then in normal Krebs (Normal Krebs) solutions. Note that the average EPSP depression observed at most of the stimulation intervals was lower in low-Ca^{2+}, high-Mg^{2+} solution than in normal Krebs solution. Four computer-

shown in the middle. Records were superimposed for convenience. The input resistance calculated from the steady-state voltage was 9·5 and 10·2 MΩ before and after the train, respectively. The input resistance calculated by the spike height method in this cell was 9·3 MΩ. Calibration: EPSPs, 5 mV, 30 ms; voltage transient, 2 mV, 20 ms. *C*, superimposed semilogarithmic transforms of the onset of the voltage transients shown in *B*. The slopes of the linear regression lines (continuous lines) fitted to the late component of the voltage onset were −0·0917 and −0·0893 before and after the dorsal root stimulation, corresponding to membrane time constants of 10·9 and 11·2 ms respectively. All the experiments were performed in normal Krebs solution with 1 mM-mephenesin.

averaged records sampled following the initial EPSP run-down at 1, 2, 5 and 10 s intervals of dorsal root stimulation in the low-Ca^{2+}, high-Mg^{2+} solution are shown in the upper panel of Fig. 7*B* (left to right respectively). The differences between these EPSPs were rather small. Figure 7*C* compares the average D_p observed at various interstimulus intervals in preparations bathed in normal Krebs (●, 10 motoneurones

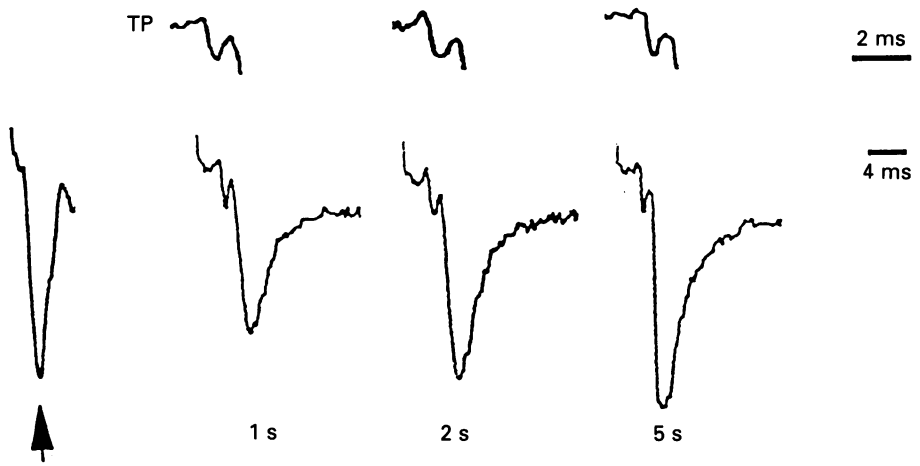

Fig. 6. Terminal potentials and synaptic fields during the prolonged depression. Averaged records of an antidromic field (32 sweeps, arrow-head) elicited at 2 s intervals, and three orthodromic fields (64 sweeps each) elicited at 1, 2 and 5 s intervals. The terminal potentials preceding each of the synaptic fields (synaptic delays of 0·95–1·05 ms) are shown in an expanded scale (TP) above each of the corresponding synaptic fields. Note that the marked changes in the synaptic fields are not preceded by changes in TP.

in 7 different experiments) and in low-Ca^{2+}, high-Mg^{2+} solutions (○, 12 motoneurones in 4 different experiments). As expected the mean D_p induced by repetitive stimulation in the low-Ca^{2+}, high-Mg^{2+} solutions was lower than that induced in normal Krebs solutions in most of the tested intervals (for example, $D_p 0{\cdot}5$ was reduced from $63{\cdot}5 \pm 7{\cdot}0$ to $36{\cdot}5 \pm 8{\cdot}8\%$; $D_p 2$ from 40 ± 9 to $18{\cdot}7 \pm 7{\cdot}7\%$, etc.). The differences between the D_p values observed in preparations bathed in low-Ca^{2+}, high-Mg^{2+} and normal Krebs solutions were statistically significant (two-way ANOVA, followed by the modified Tukey method, $\alpha = 0{\cdot}05$). The recovery from the depression in preparations bathed in low-Ca^{2+}, high-Mg^{2+} solutions was evident at intervals $\geqslant 5$ s of dorsal root stimulation ($D_p 5 < D_p 1$; $D_p 10 < D_p 2$; $D_p 5 = D_p 10 = D_p 20 = D_p 30 = D_p 60$), whilst the depression under normal conditions was alleviated only at intervals $\geqslant 60$ s ($D_p 30$ s $< D_p 10$ s, $D_p 60 < D_p 30$; two-way ANOVA, and the modified Tukey method, $\alpha = 0{\cdot}05$).

In four out of twelve motoneurones, V_{max} was obtained at intervals shorter than 60 s. The statistical analysis of the D_p values was therefore performed using the actual V_{max}, and also using V_{60} as V_{max}. Both analyses gave similar results. The D_p values in low Ca^{2+}, high Mg^{2+} shown in Fig. 7*C* were calculated using V_{60} as V_{max} for convenience.

Recordings of EPSPs from nine additional motoneurones in three different preparations bathed in low-Ca^{2+}, high-Mg^{2+} solution were performed in the presence of 1 mM-mephenesin, in order to rule out the effects of polysynaptic pathways. The

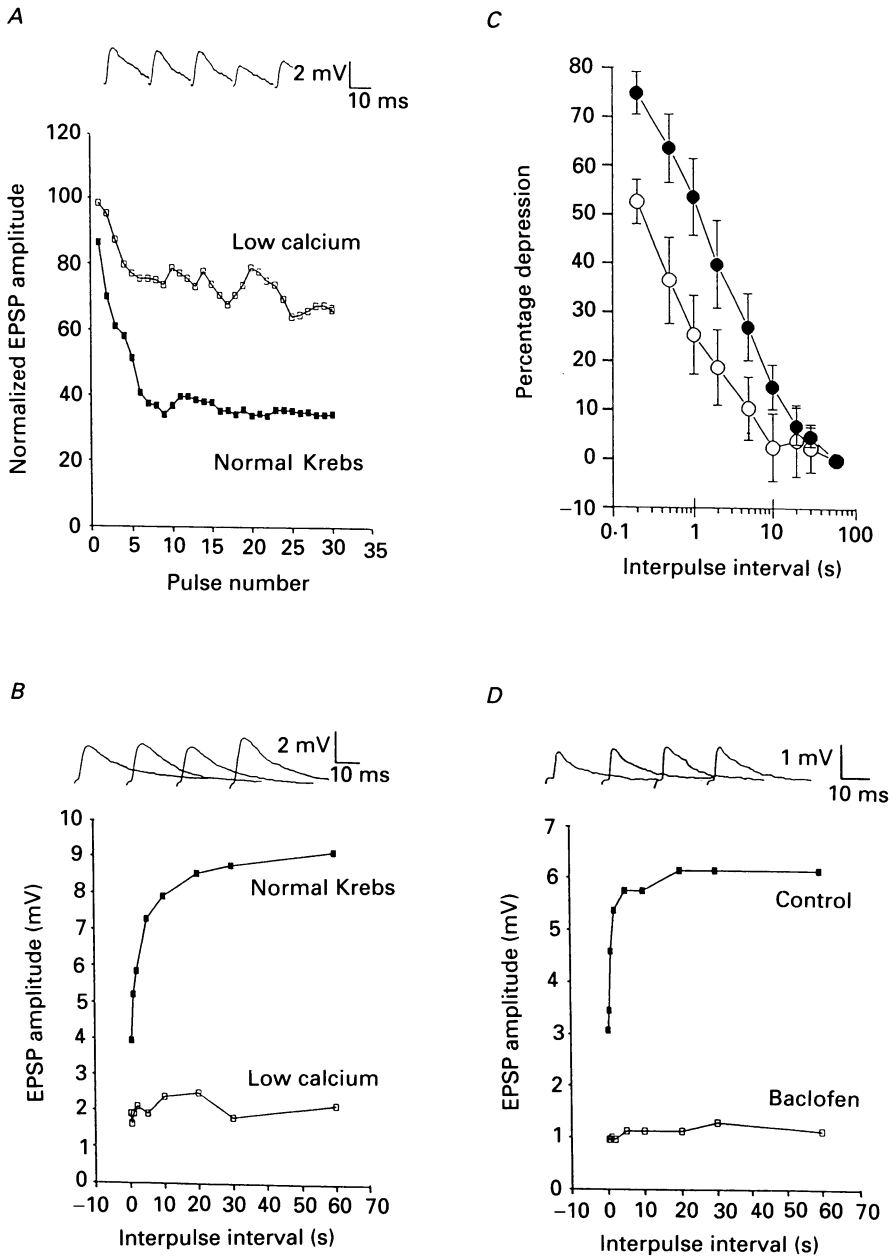

Fig. 7. The dependence of the prolonged EPSP depression on the baseline of transmitter release. *A*, run-down in the EPSP amplitude in low-Ca^{2+}, high-Mg^{2+} solutions. Upper panel: five consecutive EPSPs recorded from a motoneurone during repetitive stimulation at 1 s intervals in a 0·75 mM-Ca^{2+}, 7 mM-Mg^{2+} Krebs solution. Lower panel: run-down in the EPSP amplitude during 30 pulse trains applied at 1 Hz, in normal (Normal Krebs) and 0·75 mM-Ca^{2+}, 7 mM-Mg^{2+} (Low calcium) Krebs solution. EPSPs were recorded from two different motoneurones, the amplitudes were normalized by the mean value ($n = 16$) of the maximal EPSP amplitude obtained for each series of stimulus trains in the two different data sets respectively. Data were smoothed by the modified moving bin method, bin = 5, dbin = 1 responses. *B*, dependence of the EPSP amplitude on the stimulation

results were closely similar to those obtained in the absence of mephenesin (D_p 0·2 = 29·4 ± 8·3; D_p 0·5 = 38·4 ± 14·6; D_p 1 = 24·3 ± 9·0; D_p 2 = 27·1 ± 13·2; D_p 5 = 21·1 ± 8·1; D_p 10 = 6·3 ± 11·7; D_p 20 = 10·7 ± 7·0; D_p 30 = 16·5 ± 11·2 and D_p 60 = 3·7 ± 5·0).

(2) *Effects of L(−)baclofen.* Addition of low concentrations of the $GABA_B$ agonist L(−)baclofen (1–3 μM) to preparations perfused with normal Krebs solution caused a substantial decrease in the amplitude of the EPSPs (from 7·9 ± 3·5 mV, n = 10 to 2·2 ± 1·13 mV, n = 5) evoked by graded stimulation of the dorsal root. Figure 7*D* (upper panel) shows four computer-averaged traces (16 sweeps each) sampled following the initial EPSP run-down during repetitive stimulus trains at 1, 2, 5 and 10 s intervals after addition of 2 μM-L(−)baclofen (left to right). As in preparations bathed in low-Ca^{2+}, high-Mg^{2+} media, there were only small differences between the amplitudes of these EPSPs. Figure 7*D* (lower panel) shows the averaged EPSP amplitudes recorded from the same motoneurone, during repetitive trains applied at 0·5, 1, 2, 5, 10, 20, 30 and 60 s intervals, before (Control) and after (Baclofen) addition of 2 μM-L(−)baclofen to a mephenesin-containing normal Krebs solution. The severe depression induced at the higher stimulation frequencies in the control series was virtually abolished after addition of baclofen. Similar results were obtained in four more motoneurones sampled from three different preparations.

(3) *Development of facilitation and potentiation.* As the basic level of transmitter release was lowered either by changing the Mg^{2+}/Ca^{2+} ratio of the bathing media or by addition of L(−)baclofen, it became possible to induce double-pulse facilitation of the composite EPSPs (see Jahr & Yoshioka, 1986, for low Ca^{2+}, high Mg^{2+}) or facilitation and potentiation by short repetitive trains. Figure 8*A* shows five consecutive traces of EPSPs recorded from a preparation bathed in normal Krebs solution. These EPSPs were elicited by dorsal root stimulation at 40 ms intervals. A severe synaptic depression developed towards the end of the train. Recordings of EPSPs from a different preparation bathed in low-Ca^{2+}, high-Mg^{2+} solution showed that the same tetanic train induced marked facilitation and potentiation of the EPSPs (Fig. 8*B*). Similar conversion of depression to frequency facilitation was

interval. Upper panel: average records (16 sweeps each) of EPSPs generated in a motoneurone following the initial EPSP run-down during repetitive stimulation at 1, 2, 5 and 10 s intervals, in 0·75 mM-Ca^{2+}, 6 mM-Mg^{2+} Krebs solution. Lower panel: the average EPSP amplitudes generated in the same motoneurone at various stimulation intervals in low-Ca^{2+}, high-Mg^{2+} (Low calcium) solution, and after 45 min of superfusion with normal Krebs solution (Normal Krebs). *C*, frequency dependence of D_p in low-Ca^{2+}, high-Mg^{2+} solution. The dependence of D_p (means + S.D.) on the stimulation interval in low-Ca^{2+} (0·6–0·75 mM), high-Mg^{2+} (6–7 mM; ○) and in normal (●) Krebs solution. Data are based on means of computer-averaged records at each stimulation interval from twelve motoneurones in four different preparations for the low-Ca^{2+}, high-Mg^{2+} solution, and from ten motoneurones in seven different preparations for the normal Krebs solution. *D*, effects of baclofen on the EPSP amplitudes. Upper panel, average EPSPs (16 sweeps each) recorded from a motoneurone following the initial EPSP run-down during repetitive stimulation at 1, 2, 5 and 10 s stimulation intervals after addition of 2 μM-L(−)baclofen to a mephenesin-containing normal Krebs solution. Lower panel: the average EPSP amplitudes (16 sweeps each) recorded from the same motoneurone following the initial EPSP run-down during repetitive stimulation at different stimulation intervals before (Control) and 30 min after (Baclofen) addition of 2 μM-L(−)baclofen to the normal Krebs bathing medium.

found in each one of eight additional motoneurones recorded from five different preparations.

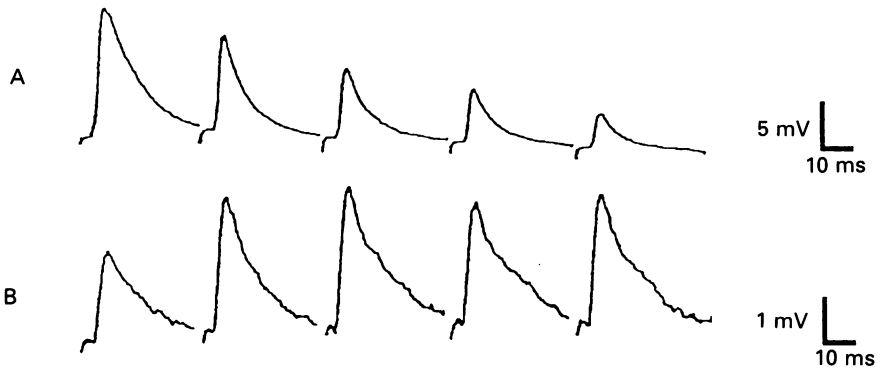

Fig. 8. Tetanic depression and potentiation of the monosynaptic EPSP in the neonatal rat spinal cord. Computer-averaged records (4 sweeps each) of monosynaptic EPSPs evoked by short stimulation trains (5 pulses, 40 ms interpulse intervals) in two different motoneurones (*A* and *B*). The preparations were bathed in a mephenesin-containing normal (*A*) and 0·75 mM-Ca^{2+}, 6 mM-Mg^{2+} (*B*) Krebs solutions, respectively. Stimulus artifacts were clipped for convenience.

DISCUSSION

Prolonged depression in the neonatal rat spinal cord

The present study has characterized the frequency dependence of excitatory synaptic transmission between dorsal root afferents and α-motoneurones in the isolated spinal cord of neonatal rats. The excitatory transmission in this system is mediated by release of an excitatory amino acid, most probably glutamate (Jahr & Voshioka, 1986; Konnerth *et al.* 1990; Jiang, Shen & Dun, 1990; Ziskind-Conhaim, 1990). The transmitter activates the NMDA (*N*-methyl-D-aspartic acid; for review see Mayer & Westbrook, 1987) and non-NMDA receptor subtypes (Konnerth *et al.* 1990; Jiang *et al.* 1990; Ziskind-Conhaim, 1990), which are suggested to be co-localized on the postsynaptic membrane (Konnerth *et al.* 1990, see also Bekkers & Stevens, 1989 for hippocampal cells). As has been reported for embryonic chick (Lee & O'Donovan, 1991), the rat (Ziskind-Conhaim, 1990), the neonatal rat (Kudo & Yamada, 1985; Fulton & Walton, 1986), and the kitten (Eccles & Willis, 1965) spinal cord, stimulation of afferent pathways using short-interval double-pulses (Jahr & Yoshioka, 1986) or moderate rates of repetitive stimulation (down to 0·1 Hz; Kudo & Yamada, 1985) induced substantial depression of the EPSPs. We have found that the depression developed within five to six stimuli and had a much longer time course than that reported before (Kudo & Yamada, 1985). Complete recovery of the reflex and EPSP amplitudes was obtained only as the interval between successive stimuli in a repetitive train reached 30 and 60 s respectively. The depression intensified with stimulation frequency and could reduce the EPSP amplitude down to 25% of its maximal value during 5 Hz stimulus trains. The facilitation and potentiation which are normally observed at the first stage of high-frequency activation in the adult

spinal cord preparations (Curtis & Eccles, 1960; Kuno, 1964; Collins *et al.* 1984; Mendell, 1984; Lev-Tov *et al.* 1988) could not be detected at any of the tested stimulation frequencies in the neonatal rat spinal cord bathed in normal Krebs solution.

Pre- or postsynaptic mechanisms?

In principle, the depression might have been induced either by pre- or postsynaptic mechanisms. The involvement of changes in the passive properties of the motoneurones due to the activation of postsynaptic inhibitory pathways by dorsal root stimulations, has been excluded by the findings that the average D_p was not altered by blockade of the $GABA_A$ and glycine postsynaptic receptors in the spinal cord, and by the finding that the input resistance and time constant of motoneurones were not altered during the time course of the depression. Another postsynaptic mechanism which might contribute to the prolonged depression is desensitization of the glutamate receptors on the postsynaptic membrane. Recent studies have shown rapid desensitization of the non-NMDA-mediated glutamate response in cultured chick spinal neurones (Trussell & Fishbach, 1989) and rapid desensitization of the glutamate response in crayfish muscles (Dudel, Franke & Hatt, 1990). Such desensitization may directly affect the duration of synaptic currents in these systems and may also depress the responses to closely spaced repetitive stimuli. However, a complete resensitization to glutamate has been demonstrated in chick motoneurones to occur 40 ms following 1 ms ionophoretic pulses of glutamate (these 1 ms pulses generated currents which closely matched the synaptic currents in this preparation; Trussell & Fishbach, 1989) and 3 ms following 10 ms pulses of glutamate in the crayfish muscle (Dudel *et al.* 1990). Therefore, it seems unlikely that the substantial and prolonged depression detected in our raw data even for the second stimulation pulses in repetitive trains at intervals as low as 5, 10 or 20 s, could be accounted for by desensitization. To summarize, it seems that the contribution of desensitization to the prolonged synaptic depression is an unlikely possibility for most of the interstimulus intervals used in our study. We therefore suggest that the prolonged EPSP depression in the neonatal rat spinal cord reflects a decreased transmitter output.

Possible mechanisms for the decrease in transmitter release

There are several presynaptic mechanisms which might decrease the release of excitatory transmitter from the activated afferents: (1) activation of presynaptic inhibitory pathways, (2) partial conduction blockade of presynaptic action potentials, and (3) reduced efficacy of the transmitter release machinery.

(1) *Presynaptic inhibition.* Conditioning of group Ia afferents to extensor motoneurones by short stimulus volleys in Ia afferents of non-antagonist muscles reduces the amplitude of Ia EPSPs in the adult cat spinal cord, without affecting postsynaptic properties (for reviews see: Burke & Rudomin, 1977; Nicoll & Alger, 1979; Redman, 1979). This phenomenon is known as presynaptic inhibition and has been shown to be mediated by polysynaptic (Burke & Rudomin, 1977; Baldissera *et al.* 1981) GABAergic pathways involving $GABA_A$ (Nicoll & Alger, 1979; Bormann, 1988) and probably $GABA_B$ presynaptic receptors (Borman, 1988; Lev-Tov *et al.*

1988; Bowery, 1989; Edwards *et al.* 1989; Peng & Frank, 1989; Wang & Dun, 1990). Activation of these presynaptic inhibitory pathways by dorsal root stimulation might, in principle, contribute to the prolonged depression described in our study. The involvement of $GABA_A$-mediated presynaptic inhibition in the prolonged synaptic depression is ruled out by the findings that blockade of the $GABA_A$ receptors by bicuculline did not affect the level and time course of the prolonged synaptic depression. An involvement of presynaptic $GABA_B$ receptors in the prolonged depression cannot be completely excluded but is rather unlikely, since most of the experiments in this study were performed in the presence of mephenesin, which appeared to reduce the activity in polysynaptic pathways.

(2) *Branch point blockade.* High-frequency activation brings about long-term changes in axonal excitability (Raymond, 1979), which may interfere with information flow particularly in branching axons (branch-point blockade; Hatt & Smith, 1976; Spira, Yarom & Parnas, 1976; Grossman, Parnas & Spira, 1979; Lüscher, Ruenzel & Henneman, 1983). Development of such branch-point blockade in the terminal arborization of presynaptic afferents during repetitive stimulation may decrease the release of neurotransmitter. Our data provide two lines of evidence indicating that the mechanism of the prolonged synaptic depression does not involve enhancement of branch-point blockade of the presynaptic action potentials. The terminal potential preceding the extracellularly recorded synaptic field reflects the summated activity in the terminal arbor adjacent to the recording electrode (Munson & Sypert, 1979; Lev-Tov *et al.* 1983, 1988). The terminal potential has been successfully used to monitor conduction blockade of presynaptic action potentials to Ia afferent terminals during presynaptic inhibition in the cat spinal cord (Sypert, Munson & Fleshman, 1980). In the present results, these terminal potentials (TPs) were unaltered during repetitive stimulation at frequencies that were sufficient to cause substantial frequency-dependent reduction in their accompanying synaptic fields (Fig. 6; see also Fulton & Walton, 1986; Jahr & Yoshioka, 1986). Furthermore, as the basic level of transmitter release was decreased either by replacing the normal Krebs with low-Ca^{2+}, high-Mg^{2+} bathing solution, or by addition of L(−)baclofen (neither treatment is thought to interfere with the invasion of spikes to the terminal arbor, see Jahr & Yoshioka, 1986), the depression was virtually abolished, revealing high-frequency facilitation and potentiation of the monosynaptic EPSPs.

We therefore suggest that, at least for most of the activation frequencies tested in the present work, there was no significant contribution of branch-point blockade to the prolonged EPSP depression, and that, as in the case of the short-term synaptic depression in the adult spinal cord (Lev-Tov *et al.* 1983; 1988), the prolonged depression in the neonatal spinal cord depends on the basic level of transmitter release (see Betz, 1970; Barrett & Magleby, 1976; for the neuromuscular junction).

(3) *Reduced efficacy of the transmitter release machinery.* In the two preceding subsections we discussed the evidence indicating that the prolonged decrease in transmitter release does not involve presynaptic inhibition or branch-point blockade of presynaptic action potential. The most likely remaining explanation for the prolonged EPSP depression has, therefore, to do with a frequency-dependent reduction in the efficacy of the transmitter release machinery of the synapse. One of the possible underlying mechanisms of the prolonged depression is the one suggested

to account for the short-term synaptic depression observed in the adult spinal cord (Curtis & Eccles, 1960; Kuno, 1964; Lev-Tov *et al.* 1983, 1988) and the neuromuscular junction (Betz, 1970; Barrett & Magleby, 1976). This mechanism involves either partial depletion of synaptic vesicles and/or inactivation of presynaptic release sites following an excessive release of transmitter (Betz, 1970). The differences in the time courses of these adult and neonatal types of depressions may be ascribed to a smaller population of synaptic vesicles (Ziskind-Conhaim, 1990) or the releasable pool of synaptic vesicles in the neonatal synapse, to slower recycling rates of synaptic vesicles following release, or partial filling of synaptic vesicles, or perhaps other immature properties of the developing synapses in the neonatal rats. The fact that reduction of the release level alleviated the depression and revealed facilitation and potentiation, which are known to co-exist with synaptic depression in the adult spinal cord (Curtis & Eccles, 1960; Kuno, 1964; Lev-Tov *et al.* 1983; Mendell, 1984; Lev-Tov *et al.* 1988) further support these assumptions. These findings also show that the mechanisms that account for facilitation and potentiation are present in neonatal rats at these post-embryonic ages, but are masked by the predominant long-term depression.

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