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EFFECTS OF SOME DIVALENT CATIONS ON SYNAPTIC TRANSMISSION IN FROG SPINAL NEURONES

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SUMMARY

1. Synaptic transmission between dorsal root afferents and motoneurones was studied in the isolated and hemisected spinal cord of frogs, using intracellular and extracellular recording techniques, and ionic substitutions of divalent cations in the bathing fluid.

2. Delayed components of excitatory post-synaptic potentials (e.p.s.p.s) evoked in motoneurones by dorsal root supramaximal stimuli, as well as the Ca^{2+}-dependent slow after-hyperpolarization which follows antidromic spikes, were reversibly blocked by superfusing the cords with 'Ca^{2+}-free' media containing Co^{2+} (4 mM) or Mg^{2+} (6–10 mM). However, short latency e.p.s.p.s persisted in these media for more than 8 hr.

3. The minimum synaptic delay of the Co^{2+} and Mg^{2+}, resistant e.p.s.p.s, measured from the peak negativity of the extracellularly recorded presynaptic spike to the onset of the e.p.s.p., was 0·3 msec at 10 ± 1 °C.

4. The Co^{2+}, Mg^{2+}-resistant e.p.s.p.s were graded, and could be elicited by stimulation of segmental or adjacent roots. Those evoked by each of two adjacent roots showed linear summation when the roots were stimulated simultaneously.

5. The Co^{2+}, Mg^{2+}-resistant e.p.s.p.s decreased in amplitude at stimulating frequencies between 10 and 100 Hz, and with paired stimuli at intervals shorter than 20–40 msec. These reductions in amplitude were paralleled by decreases in amplitude of the presynaptic population spike.

6. Solutions free of divalent ions, containing EGTA (2 mM) abolished the Co^{2+}, Mg^{2+}-resistant e.p.s.p.s. They remained blocked for a variable time after returning to Ca^{2+}-free Ringer containing Mg^{2+} (8 mM). Their continued abolition at this stage is probably not due to changes in electrical properties of motoneuronal membranes. Eventually, the Mg^{2+}-resistant e.p.s.p.s started recovering in the Ca^{2+}-free Ringer containing Mg^{2+}. The time of onset of this recovery depended on the duration of exposure to EGTA.

7. Sr^{2+} (2–11 mM), although less effective than Ca^{2+}, restored the composite e.p.s.p.s evoked by dorsal root supramaximal stimuli, as well as the Ca^{2+}-dependent slow after-hyperpolarization of the motoneurone. The composite e.p.s.p.s could not be restored with Ba^{2+} (2–10 mM).

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8. The results suggest that the Co^{2+}, Mg^{2+}-resistant e.p.s.p. is generated by electrical coupling between some afferent fibres (probably primary afferents) and motoneurones. The after-effects of EGTA treatments probably reflect uncoupling of electrotonic junctions. In contrast, the delayed components of the composite e.p.s.p.s are generated through chemical synapses whose divalent cation requirement is similar to that of the neuromuscular junction.

INTRODUCTION

The most studied synapse in the vertebrate central nervous system is probably that between primary afferent fibres and motoneurones and yet its mechanism of transmission is not fully understood. One issue still in question is whether transmission at this synapse is entirely chemical or involves an electrical component (Rall, Burke, Smith, Nelson & Frank, 1967). In the cat spinal cord the uncertainty arises mainly from the fact that excitatory post-synaptic potentials (e.p.s.p.s), evoked by stimulation of *I*a fibres, do not always behave as expected from the chemical model (Coombs, Eccles & Fatt, 1955) when polarizing currents are applied in the soma (Werman & Carlen, 1976; Edwards, Redman & Walmsley, 1976).

It is well known that evoked neurotransmitter release is a calcium dependent process, which can be inhibited by divalent cations such as Mg^{2+}, Mn^{2+} and Co^{2+} (Del Castillo & Engbaek, 1954; Jenkinson, 1957; Miledi, 1966; Katz & Miledi, 1969*a*, *b*; Meiri & Rahamimoff, 1972; Weakly, 1973). Studies of the effects of these cations on the excitatory monosynaptic potentials evoked in frog motoneurones by dorsal root stimulation in the isolated cord preparation have also yielded equivocal results. For instance, addition of Mg^{2+} (12–20 mM) to the bathing fluid reduced the amplitude, but did not completely abolish the evoked synaptic potentials (Katz & Miledi, 1963). Similar results were obtained if Ca^{2+} was removed from the external fluid (Dambach & Erulkar, 1973); although it was later reported (Erulkar, Dambach & Mender, 1974) that transmission was blocked, when bath [Ca^{2+}] was decreased to 0·1 mM and [Mg^{2+}] increased to 5–11 mM.

Recent investigations have shown that either Mn^{2+} (1–2 mM) or Co^{2+} (4–5 mM) completely blocked the monosynaptic e.p.s.p.s evoked by dorsal root stimulation (Barrett & Barrett, 1976; Sonnhof, Richter & Taugner, 1977). In contrast, Shapovalov, Shiriaev & Velumian (1978) have shown that some monosynaptic e.p.s.p.s persist for several hours, when the cord is exposed to Ca^{2+}-deficient, high Mg^{2+} (5–20 mM) media or when either Mn^{2+} (2 mM) or Co^{2+} (5 mM) are added to the bathing fluid.

In the present study we compared the 'chemical' *vs.* the 'electrical' models of transmission between dorsal root afferent fibres and motoneurones of the isolated and hemisected spinal cord of the frog, using ionic substitution of several divalent cations in the bathing medium. Our results suggest that transmission in this pathway involves a monosynaptic component which is electrically mediated. This conclusion is in agreement with the recent work of Shapovalov *et al.* (1978) and Shapovalov & Shiriaev (1978) which appeared as our work was being completed.

Preliminary accounts of our findings have been presented (Alvarez-Leefmans, De Santis & Miledi, 1978*a*, *b*).

METHODS

Preparation. Experiments were performed on the isolated and hemisected spinal cord of the frog, *Rana temporaria*. The method of isolating the cord and recording the root potentials was essentially that used in previous work (Katz & Miledi, 1963; Miledi & Szczepaniak, 1975). After sagittal hemisection, the hemi-cord was transferred to a chamber (3·5 ml. volume), whose bottom was covered by a Sylgard (Dow Corning Corp., Mich., U.S.A.) plate on which the hemi-cord was fixed with pins. For recording and stimulation of spinal roots, the central stumps of ventral and dorsal roots of segments 7–9 (Katz & Miledi, 1963) were drawn into close fitting suction electrodes. The roots were stimulated with square-wave pulses of 0·05 –0·1 msec duration and the stimulus strength was adjusted to be supramaximal for root potentials.

Intracellular recording. Micropipettes filled with 4 M-K acetate or 3 M-KCl, having 10–60 MΩ resistance, were used for intracellular recording. Conventional recording equipment was used. The present study is based on more than 150 motoneurones satisfactorily impaled, having stable membrane potentials (50–80 mV). Cells with membrane potential or antidromic spikes of less than 50 mV were rejected.

To record extracellular field potentials from the base of the dorsal horn, the tip of a micro-electrode (filled with Ringer or 2M-NaCl) was placed in the region where the amplitude of the triphasic presynaptic action potential generated by the dorsal root volley was maximal. This region corresponds to the Substantia gelatinosa, where according to anatomical (Liu & Chambers, 1957; Szekely, 1976) and electrophysiological (Brookhart & Fadiga, 1960) evidence, the bulk of the dorsal root afferent fibres end or pass through.

Solutions. The composition of the normal Ringer solution was (mM): NaCl 114, KCl 2, $CaCl_2$ 2, Hepes (N-2-hydroxyethypiperazine-N′-2-ethanesulphonic acid) 10, and D-glucose 1 g/l. The final pH was adjusted to 7·2 after bubbling with 95 % O_2 + 5 % CO_2 during 30 min. The stability of the pH was checked during the course of the experiment. Variations in the composition of the normal Ringer solution are indicated where appropriate, and included removal of Ca^{2+} and its substitution by 2–11 mM-Mg^{2+}, Sr^{2+}, Ba^{2+} or Co^{2+}.

'Ca^{2+}-free medium' was prepared using bidistilled deionized water, and carefully cleaned plastic ware. The reagents used to prepare the 'Ca^{2+}-free medium' were: NaCl (Aristar, B.D.H.) and KCl (Aristar, B.D.H.) which are listed as containing no more than 3 and 0·1 p.p.m. Ca, respectively. In some experiments, concentrations of divalent cations in the bathing solution were buffered with 0·25–2 mM-EGTA (ethyleneglycol-bis-(β-amino-ethyl ether)N,N′-tetra-acetic acid). The concentration of free calcium in a mixture of known composition was calculated using the following equation (cf. Portzehl, Caldwell & Rüegg, 1964; Schwarzenbach, Flaschka & Irving, 1969),

$$[Ca^{2+}] = \left[\frac{\text{Ca-EGTA}}{\text{EGTA}}\right] \times \frac{1}{K'} \tag{1}$$

where K' is the combined apparent association constant. K' varies with pH, and therefore $[Ca^{2+}]$ varies with pH. K' was calculated for pH = 7·2, using the association constants given by Portzehl *et al.* and Schwarzenbach *et al.* K' calculated for EGTA and Ca^{2+} is $10^{7\cdot09}$, for strontium $10^{4\cdot59}$, for barium $10^{4\cdot49}$ and for Mg^{2+} $10^{1\cdot3}$.

In addition to Ca^{2+} due to contamination (assumed as 100 μM, and very probably an over-estimate), other divalent cations such as Mg^{2+}, Ba^{2+} or Sr^{2+} were added to the solution. EGTA binds both Ca^{2+} and the other divalent cations (Me^{2+}) and, therefore, $[Ca^{2+}]$ depends on the concentration of Me^{2+}, and vice versa. The reactions of EGTA with Ca^{2+}, Me^{2+} and H^+ are equivalent to two competing reactions such that after applying the mass action law, the following equation is obtained,

$$\left[\frac{\text{Ca-EGTA}}{\text{Me-EGTA}}\right] = \left[\frac{Ca^{2+}}{Me^{2+}}\right]\frac{K'}{K''} \tag{2}$$

where K'' is the combined apparent association constant for Me^{2+}. Equation (2) was used to estimate $[Ca^{2+}]$ and $[Me^{2+}]$ in the presence of EGTA, at pH = 7·2.

In some experiments, the cords were pre-washed with Ca^{2+}-free Ringer containing 2 mM-EGTA *without* the addition of any divalent cation. After 50–135 min, EGTA was removed or reduced to 0·25–0·5 mM, and Mg^{2+}, Ba^{2+} or Sr^{2+} were added. In experiments in which Co^{2+} was

added to the 'Ca^{2+}-free medium', EGTA could not be used since it binds more strongly to Co^{2+} than to Ca^{2+}.

The preparation was perfused with a steady flow (1 ml./min) of oxygenated bathing solutions. However, on several occasions it was necessary to stop the flow, to obtain stable intracellular recordings. Most experiments were done at 10 ± 1 °C, but a few were done at 7 ± 1 °C, as specified in the appropriate section.

RESULTS

Motoneurone responses in normal Ringer

When cords were superfused with normal Ringer, the typical motoneurone responses to dorsal root supramaximal stimuli consisted of a composite excitatory post-synaptic potential (dorsal root e.p.s.p.) with one or more spikes superimposed (Figs. 1*A* and 9*C*). Only on very few occasions was the earliest component of the dorsal root e.p.s.p. sufficient to generate an action potential in the motoneurone. This is consistent with previous observations suggesting that motoneurone action potentials in the isolated frog spinal cord are rarely triggered by monosynaptic e.p.s.p.s (Katz & Miledi, 1963; Dambach & Erulkar, 1973; Erulkar *et al.* 1974).

The synaptic delay of the first component of the dorsal root e.p.s.p. was measured from the peak negativity of the extracellularly recorded, triphasic, presynaptic spike to the onset of the e.p.s.p. (see also section on *Synaptic delay* and Fig. 2). The minimum delay at 10 °C was 0·3 msec. Data pooled from four preparations gave a mean delay of 0·7 msec ± 0·1 S.E. (range 0·3–1·4 msec; $n = 11$ motoneurones). Synaptic delays measured as described, are subject to several errors and are likely to represent overestimates of the true value (cf. Katz & Miledi, 1965*c*). In spite of these errors, which will be discussed below, the figures obtained are considerably smaller than those reported for the neuromuscular junction where the minimum synaptic delay at 10 °C is 1·4 msec (see Fig. 4 of Katz & Miledi, 1965*a*).

The measured synaptic delays are consistent with the idea that the earliest component of the dorsal root e.p.s.p. is generated through a monosynaptic pathway. In contrast, the minimum delay for the second component of the e.p.s.p. (Fig. 1*A*) was found to be 2·6 msec. The mean delay for this component was 3·0 msec ± 0·1 S.E. (range 2·6–4·0 msec; $n = 11$ motoneurones). Frequently a third and even a fourth component of the dorsal root e.p.s.p. were seen. All these components have been thought to be generated by the successive arrival of impulses along different internuncial pathways (Katz & Miledi, 1963); but in the absence of more direct evidence we shall refer to them as the delayed components of the dorsal root e.p.s.p., without implying whether they are poly- or monosynaptic. The amplitude of the earliest component of the dorsal root e.p.s.p. elicited by successive stimuli (1–2 Hz) of constant strength showed little variability for each particular cell. In contrast, the delayed components showed considerable fluctuations in amplitude (Fig. 1*A*).

Antidromic spikes recorded in normal Ringer (Fig. 1*B*) were followed by after-potentials with the features described by Magherini, Precht & Schwindt (1976), and Barrett & Barrett (1976). One of the after-potentials, namely the slow after-hyperpolarization is relevant to the present study (Figs. 1*C* and 8*C*), since it has been suggested to be a calcium mediated K^+ current (Krnjević & Lisiewicz, 1972; Barrett & Barrett, 1976).

Effects of Co^{2+}

Superfusion of the cord with Ca^{2+}-free Ringer containing 4 mM-Co^{2+}, with or without Mg^{2+} (2–5 mM), reversibly abolished the delayed components of the dorsal root e.p.s.p. as well as the Ca-dependent slow after-hyperpolarization which follows the antidromic spike (Fig. 1*F*). However, even after more than 8 hr in Co^{2+} media, dorsal root stimulation evoked monosynaptic e.p.s.p.s in motoneurones (Fig. 1*D*).

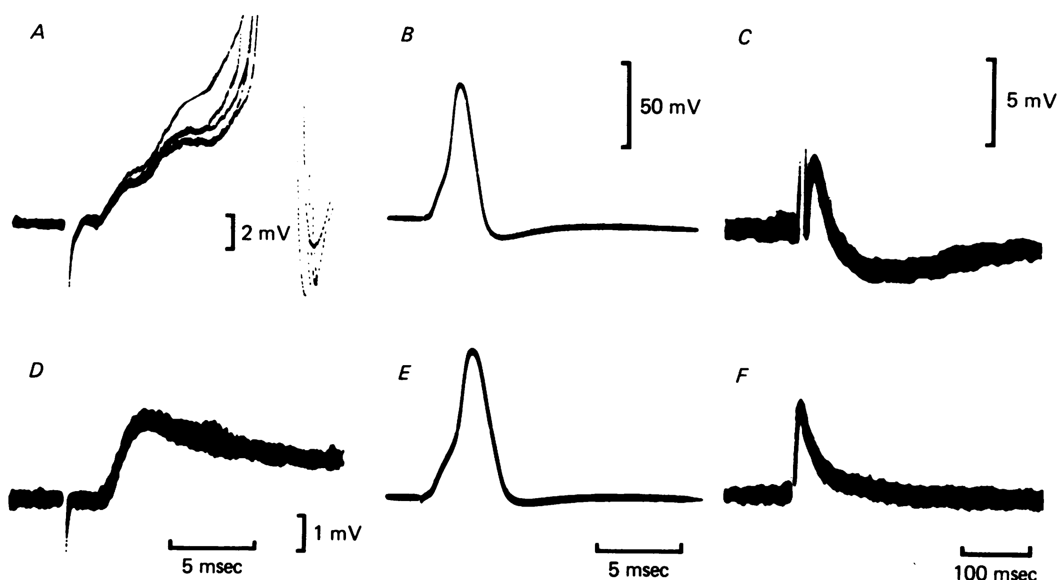

Fig. 1. Examples of intracellularly recorded motoneurone responses evoked by dorsal or ventral root stimulation in normal Ringer (*A*–*C*), and *ca.* 100 min after superfusion with Ca^{2+}-free Ringer containing 4 mM-Co^{2+} (*D*–*F*). *A*, composite e.p.s.p. evoked in normal Ringer by dorsal root supramaximal stimuli. The falling phase of spikes was retouched for clarity. *D*, Co^{2+}-resistant e.p.s.p. evoked by dorsal root supramaximal stimuli. *B*. and *E*, antidromic spikes evoked in normal Ringer (*B*) and Co^{2+} Ringer (*E*). *C* and *F*, antidromic spikes as in *B* and *E*, but at higher gain and slower sweep to show after-potentials. Note slow after-hyperpolarization in *C* and its absence in *F*. All traces consist of five or more superimposed sweeps at 1/sec. Traces *A*–*C* come from one cell, and *D*–*F* from another cell. Resting potential in *A*–*F* was – 65 mV. Temp. = 10 ± 1 °C.

The average rise time of the Co^{2+}-resistant dorsal root e.p.s.p. at 10 °C was 2·0 msec ± 0·1 S.E. (n = 13 cells; range of 1·3–2·7 msec), having average half-decay time of 7·8 msec ± 1·1 S.E. (range 2·3–13·8 msec), and average peak amplitude of 2·4 mV ± 0·2 S.E. (n = 13 cells; range of 0·6–4 mV). Similar results were obtained in a medium containing 5 mM-Co^{2+} and 1 mM-Ca^{2+}, or in Ca^{2+}-free Ringer containing 6–10 mM-Mg^{2+} (Fig. 8*G*–*I*).

Figs. 2 and 3 show that the monosynaptic e.p.s.p. which persisted in Co^{2+} (Fig. 2*B*) or Mg^{2+} (Fig. 3*A*) media, was a potential generated across the motoneurone membrane, as evident from the negligible field potential recorded just outside the cell (Fig. 2*C*). In most cases, the monosynaptic e.p.s.p. was seen to arise from a sharp negative-going potential which was preceded by a larger positive-going potential

(Fig. 3). Similar positive–negative potentials have been seen preceding post-synaptic potentials intracellularly recorded both in squid giant synapses (Miledi & Slater, 1966), and in vertebrate motoneurones (Jankowska & Roberts, 1972; Watt, Stauffer, Taylor, Reinking & Stuart, 1976; Shapovalov *et al.* 1978) and they probably represent field potentials generated at the close end of presynaptic terminals (Katz & Miledi, 1965*b*), which can be seen by the intracellular post-synaptic electrode. That these presynaptic action potentials were not generated across the motoneurone

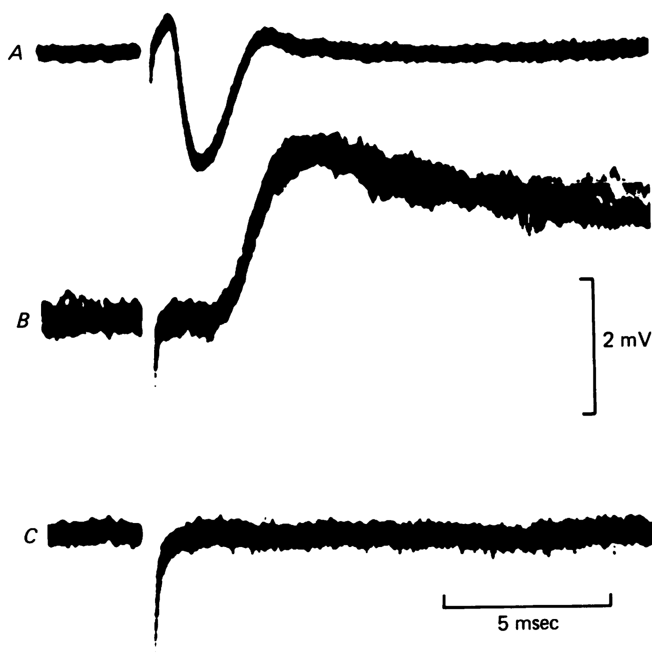

Fig. 2. Time relations between presynaptic spike extracellularly recorded at the base of the dorsal horn (*A*) and Co^{2+}-resistant e.p.s.p. intracellularly recorded (*B*) from the soma of one motoneurone, in response to dorsal root supramaximal stimuli. Records obtained after 105 min superfusion with Ca^{2+}-free Ringer containing 4 mM-Co^{2+}. *C*, potential recorded outside this motoneurone in response to the same dorsal root stimuli. Traces consist of five superimposed sweeps at 1/sec. Resting potential in *B* was −65 mV. Temp. = 10 °C.

membrane was made evident either by subtracting the extracellular from the intracellularly recorded potentials or simply by visual inspection of the recorded potential (e.g. Fig. 3). On very few occasions, the negative going phase of these presynaptic components was followed by a relatively slow and small positivity.

Some properties of the Co^{2+}-resistant e.p.s.p.

Spatial summation. The Co^{2+}-resistant e.p.s.p. was generated by low threshold dorsal root afferent fibres. It was finely graded, increasing its amplitude in proportion to the strength of the dorsal root stimulus, and with the concomitant increase in amplitude of the triphasic presynaptic spike recorded extracellularly. This suggests that the Co^{2+}-resistant e.p.s.p. results from the sum of smaller components generated by individual afferent fibres converging onto the motoneurone.

Co^{2+}-resistant e.p.s.p.s could also be evoked by stimulation of at least the adjacent dorsal root, implying convergence of heterosegmental afferent fibres onto motoneurones. Fig. 4 illustrates a typical experiment in which e.p.s.p.s evoked from two roots, were recorded in the same motoneurone. The dots represent the averaged e.p.s.p. evoked by stimulation of dorsal root IX and the asterisks that obtained by stimulation of dorsal root VIII. The open circles represent the e.p.s.p. elicited when

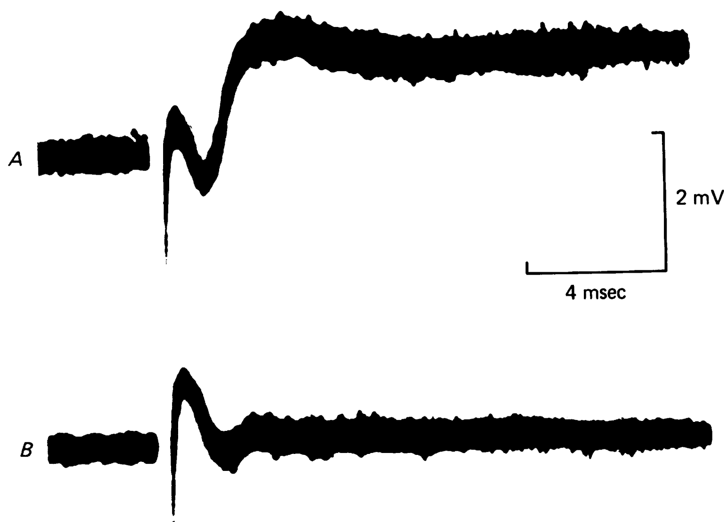

Fig. 3. Potentials evoked in a motoneurone in response to dorsal root stimuli, *ca.* 60 min after Ca^{2+}-free Ringer containing Mg^{2+} (8 mM) was admitted to the bathing fluid. *A*, Mg^{2+}-resistant e.p.s.p. recorded intracellularly, and (*B*) field potential recorded outside the motoneurone. Traces consist of ten superimposed sweeps at 2/sec. Resting potential in *A* was -76 mV. Temp. $= 10 \pm 1$ °C.

both roots were simultaneously stimulated, and the crosses are the instantaneous sum of the dorsal roots IX plus VIII responses. The results show that the e.p.s.p. evoked by stimulation of these two adjacent roots exhibited near perfect linearity of potential summation when both roots were stimulated simultaneously.

In most motoneurones examined, the depolarization brought about by simultaneous stimulation of two adjacent roots was below the motoneurone firing threshold.

Synaptic delay. A useful aid in distinguishing between chemical and electrical transmission is the measurement of the synaptic delay (Katz & Miledi, 1965*c*; Miledi & Slater, 1966; Bennett, 1977). The synaptic delay of the Co^{2+}-resistant e.p.s.p. was measured from individual or superimposed records as those shown in Fig. 2*A*–*C*, but photographed at higher sweep speed. The delay was taken as the time interval between the negative peak of the presynaptic spike recorded extracellularly in the base of the dorsal horn (see Methods and Fig. 2*A*) and the beginning of the intracellularly recorded e.p.s.p. (Fig. 2*B*). Measurements obtained in this way are subject to errors in estimating the value of the 'synaptic delay' as defined by Katz & Miledi (1965*c*). This is due to the fact that unlike at the neuromuscular junction (Katz & Miledi, 1965*b*), in the spinal cord it is not possible to place the recording electrode on the terminals of the presynaptic axons. This will introduce

time lags due to terminal conduction whose magnitude will obviously depend on how far from the terminals the tip of the presynaptic recording electrode is located. The other main error is due to uncertainty in determining the exact onset of the e.p.s.p., which is sometimes obscured by field potentials which are intracellularly recorded, and by electrotonic attenuation due to the cable properties of the dendrites (Rall, 1967). In addition, while it is possible at the neuromuscular junction to record from a single presynaptic fibre, in the spinal cord the presynaptic action potential represents the combined action currents of many fibres. The error due to asynchrony

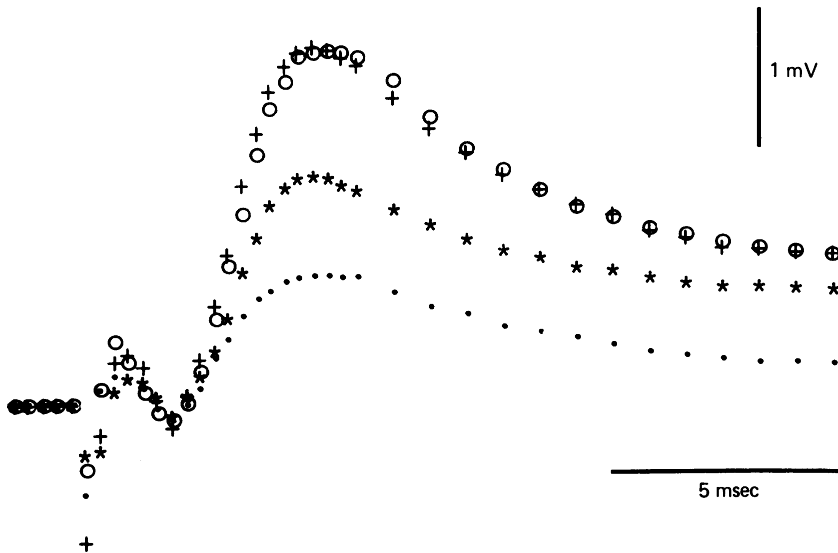

Fig. 4. Co^{2+}-resistant e.p.s.p.s evoked in a motoneurone by supramaximal stimulation of two adjacent roots. Potentials were averaged (n = 10) and digitized. ●, e.p.s.p. evoked by dorsal root IX; *, e.p.s.p. evoked by dorsal root VIII; ○, e.p.s.p. elicited when both roots (VIII and IX) were simultaneously stimulated; +, instantaneous sum of the e.p.s.p.s evoked by dorsal roots IX plus VIII. Data obtained 2 hr after Ca^{2+}-free, 4 mM-Co^{2+}-Ringer was admitted to the bath. Resting potential, −78 mV. Temp. = 9 °C.

of the volley of impulses conducted by the dorsal root fibres was minimized by placing its stimulating electrode as closely as possible to the entry of the root into the cord (see below). The error due to field potentials monitored by the intracellular electrode was practically eliminated by subtracting the extracellular from the intracellularly recorded potentials. The minimum delay for the Co^{2+}-resistant e.p.s.p. measured in this way was less than 0·3 msec (0·25–0·3 msec), but values as high as 1·3 msec were obtained. The data pooled from four preparations gave an average delay of 1·0 msec ± 0·1 S.E. (nine motoneurones, temp. = 10 ± 1 °C). For each particular motoneurone there were no latency and amplitude fluctuations of the e.p.s.p. comparable to those described at the end-plate (Katz & Miledi, 1965*b*, *c*).

In order to counteract possible errors due to temporal dispersion of individual action potentials in the presynaptic volley, the synaptic delay was also measured between the first positive peak of the triphasic presynaptic field potential and the onset of the monosynaptic e.p.s.p. This criterion was based on the supposition that

the arrival of the fastest elements of the afferent volley may have coincided with the first positive peak of the triphasic field potential. The minimum delay measured in this manner was 1 msec at 10 ± 1 °C.

In motoneurones in which a presynaptic spike could be recorded intracellularly (e.g. Fig. 3*A*), subtraction of the extracellular from the intracellularly recorded potential showed that the onset of the e.p.s.p. coincided with the negative peak of the presynaptic action potential.

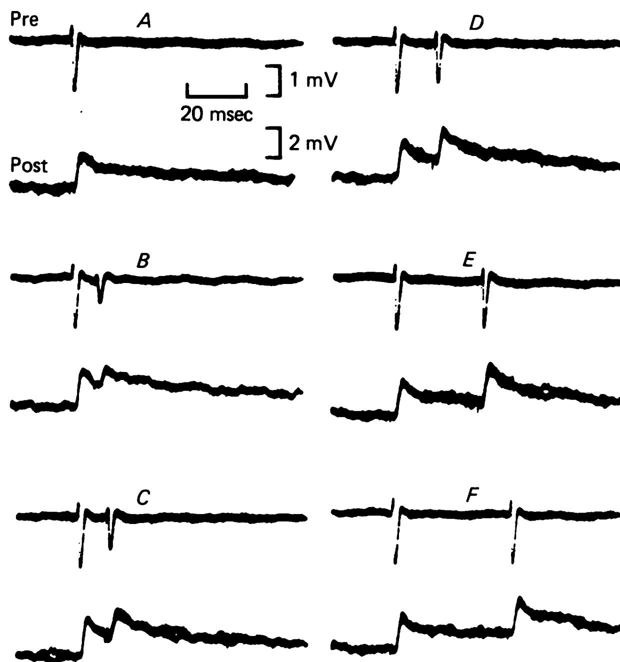

Fig. 5. Effect of dorsal root conditioning volleys on the presynaptic spike potential (Pre) recorded in the base of the dorsal horn, and the Co^{2+}-resistant e.p.s.p. (Post) set up by subsequent test volleys delivered to the same dorsal root (IX), at various interstimulus intervals. The strength of the maximal stimulus was the same for conditioning and test volleys. Resting potential = −65 mV. Sampling started 90 min after Ca^{2+}-free Ringer containing 4 mM-Co^{2+} was admitted to the bath. Each trace consists of two superimposed sweeps. Temp. = 10 °C.

Effects of repetitive or paired DR stimuli. The Co^{2+}-resistant e.p.s.p. produced by two supramaximal stimuli delivered at different intervals to the same dorsal root exhibited simple temporal summation for intervals which outlasted the relative refractory period of the presynaptic fibres (Fig. 5*F*). The peak amplitude of the second e.p.s.p. (obtained by subtraction of the control response from the summed response) was reduced as the interval between stimuli was shortened (Figs. 5 and 6*A*). The depression of the e.p.s.p. was accompanied by a concomitant decrease in amplitude of the presynaptic action potential in approximately direct proportion (Fig. 6*B*). Recovery of the test e.p.s.p. occurred at interstimulus intervals between 20 and 40 msec (Fig. 6*A*). There was no detectable facilitation of either the e.p.s.p. or the presynaptic spike at the explored intervals (1–100 msec).

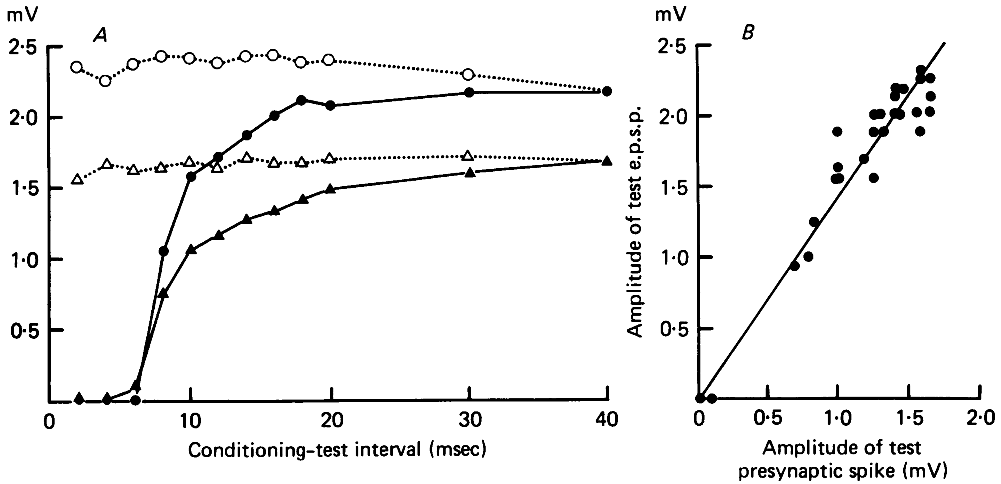

Fig. 6. Relation between amplitude of presynaptic spike and amplitude of Co^{2+}-resistant e.p.s.p.s at various interstimulus intervals. *A*, relative peak amplitude of the test e.p.s.p. (●) obtained by subtraction of the conditioning response from the summed response, the peak amplitude of conditioning e.p.s.p. (○), and amplitudes of conditioning (△) and test (▲) presynaptic spikes, plotted against volley interval, for series partly illustrated in Fig. 5. Each symbol represents the average of six responses. *B*, relation between peak amplitude of the test e.p.s.p. against the amplitude of the test presynaptic spike obtained at the interstimulus intervals shown in *A*. The regression line passes within 0·01 mV of the origin. Correlation coefficient = 0·98 (n = 36).

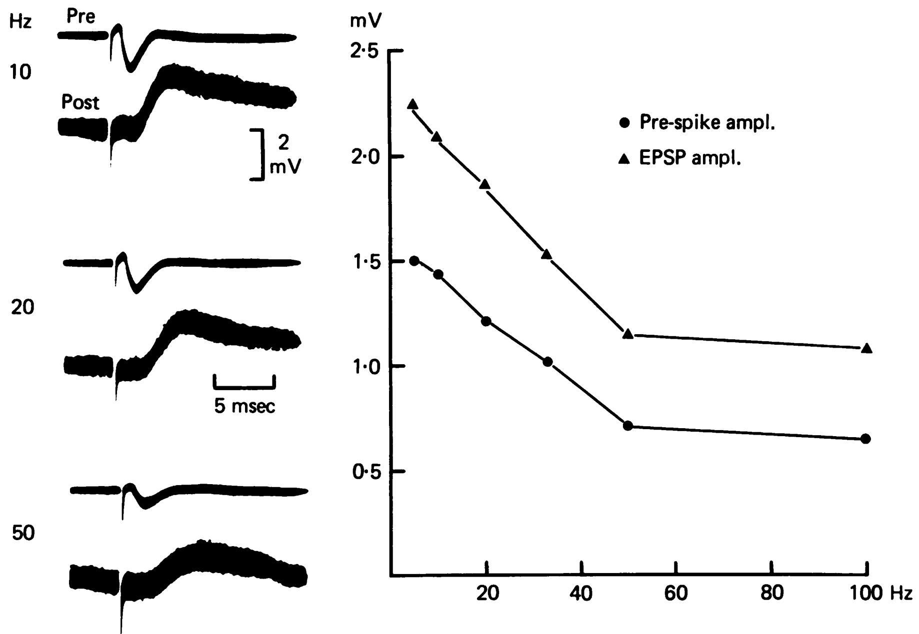

Fig. 7. Effect of tetanic stimulation of dorsal root IX on the presynaptic spike (Pre) and the Co^{2+}-resistant e.p.s.p. (Post) recorded intracellularly from a motoneurone. Sample traces on the *left* consist of several superimposed sweeps over first 3 sec of each train of stimuli at the indicated frequencies. *Right*: plot of peak amplitude of e.p.s.p. (▲), and amplitude of extracellular presynaptic spike (●) averaged over first 6 sec of each train of stimuli, against frequency. Data obtained *ca.* 2 hr after Ca^{2+}-free Ringer (4 mM-Co^{2+}) was admitted to the bath (temp. = 10 °C, resting potential, −65 mV).

The effects of tetanic stimulation of the dorsal root on the pre-synaptic spike recorded at the base of the dorsal horn and the e.p.s.p. were studied at frequencies ranging between 1 and 100 Hz. In these experiments the peak amplitude of superimposed e.p.s.p.s and the amplitude of the presynaptic spike averaged for the first 6 sec of each train of stimuli were plotted against frequency. A typical experiment is illustrated in Fig. 7. At low stimulus frequencies (1–5 Hz) the amplitude of the e.p.s.p. was not appreciably modified. However when the frequency was raised beyond 5–10 Hz the e.p.s.p. started decreasing in amplitude. There were no complete absences (failures) of e.p.s.p.s in the explored frequency range. Fig. 7 further shows, once more, that the reduction in amplitude of the e.p.s.p. produced by repetitive stimulation was paralleled by decreases in amplitude of the presynaptic action potential.

The decrease in e.p.s.p. amplitude observed during repetitive stimulation may be caused by a failure to activate some axons during their relative refractory period, but it may also be that with high frequency stimulation nerve impulses fail to be propagated along the entire terminal branching of some presynaptic nerve fibres (cf. Krnjević & Miledi, 1958, 1959).

Effects of Ca^{2+}-free Ringer and EGTA on synaptic transmission and action potentials

The effects of Ca^{2+} removal from the bathing fluid on synaptic transmission and action potentials were investigated using three experimental procedures which will be denoted I, II and III. In procedure I, the hemicords were subject to the following sequence of solution changes: *A*, normal Ringer; *B*, Ca^{2+}-free Ringer containing 8 mM-Mg^{2+} for 1–4 hr (during this period the presence of the Mg^{2+}-resistant e.p.s.p. was ascertained); *C*, Ca^{2+}-free Ringer containing 2 mM-EGTA with no other divalent ion substituting for Ca^{2+} (50–135 min); and *D*, EGTA was removed, and Mg^{2+} was added (2–8 mM). Motoneurones were sampled at different times throughout the experiment. Sometimes it was possible to follow the same cell during different changes in the bath composition.

Procedure II consisted in superfusing the hemicords with Ca^{2+}-free Ringer containing 2 mM-EGTA and 6–8 mM-Mg^{2+} for several hours; that is there was no prewashing period with solutions free of divalent ions containing EGTA. In procedure III, the hemicords were superfused with Ca^{2+}-free media containing 6–10 mM-Mg^{2+}, but EGTA was not added at any stage. Before Ca^{2+} was removed in any of the three procedures, several motoneurones were impaled in normal Ringer to ascertain the condition of the cord.

Following procedure I, 20–60 min after Ca^{2+}, Mg^{2+}-free Ringer containing 2 mM-EGTA was admitted to the bath, Mg^{2+}-resistant e.p.s.p.s were blocked. Preceding the abolition of synaptic potentials, motoneurones exhibited repetitive firing, and could be discharged by small amplitude, short latency e.p.s.p.s, until eventually both, antidromic and orthodromically elicited spikes failed. The failure of antidromic invasion reversed 10–30 min after removal of EGTA and addition of Mg^{2+} (2–8 mM). However, the Mg^{2}-resistant e.p.s.p. remained blocked for variable periods of time, depending on the duration of the pre-treatment with EGTA, until their eventual

recovery. For instance, when EGTA pre-treatments did not exceed 50–80 min, the Mg^{2+}-resistant e.p.s.p.s. recovered fully within 20–60 min, after superfusion with Ca^{2+}-free media containing Mg^{2+} started. However, when pre-treatments with EGTA lasted for more than 2 h, the synaptic potentials failed to appear for at least 2–4 hr after returning to the Ca^{2+}-free media containing Mg^{2+} (Fig. 8*D*). Under such

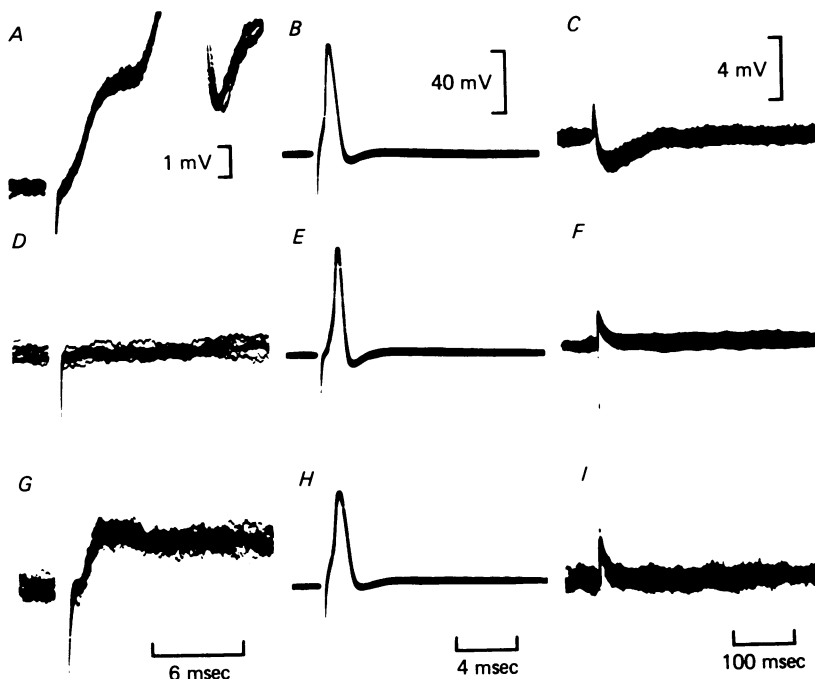

Fig. 8. After-effects of Ca^{2+}-free Ringer containing EGTA on synaptic and action potentials. The hemisected cord was superfused with Ca^{2+}-free Ringer containing 2 mM-EGTA during 133 min, after which Ca^{2+}-free Ringer containing Mg^{2+} (8 mM) and no EGTA was admitted to the bath. Intracellularly recorded motoneurones responses evoked by dorsal root (*A*, *D* and *G*), and ventral root stimulation (*B–C*, *E–F*, and *H–I*) are shown in normal Ringer (*A–C*); 70 min (*D–F*) and 165 min (*G–I*) after EGTA was removed and Mg^{2+} (8 mM) was added to the Ca^{2+}-free media. *A*, composite e.p.s.p. evoked in normal Ringer by dorsal root stimuli, *D*; note the absence of Mg^{2+}-resistant e.p.s.p. in response to same dorsal root stimuli, *G*, appearance of Mg^{2+}-resistant e.p.s.p. *B*, *E* and *H*, antidromic spikes. *C*, *F* and *I*, same antidromic spikes as in *B*, *E* and *H*, but at higher gain and slower sweep to show slow after-hyperpolarization. Note the absence of the latter in *F* and *I*. All traces consist of ten superimposed sweeps at 1/sec. Traces *A–C*, *D–F* and *G–J* are typical responses from three different cells located in same spinal segment, and having resting potentials of −70 mV (*A–C*); −58 mV (*D–F*) and −65 mV (*G–I*). Temp. = 10 ± 1 °C.

conditions, motoneurones could support action potentials (Fig. 8*E*). The Mg^{2+}-resistant e.p.s.p.s started reappearing after 2–4 h of washing with the Ca^{2+}-free media containing Mg^{2+} (Fig. 8*G*), but the Ca^{2+}-dependent slow after-hyperpolarization which follows the antidromic spike did not recover (Fig. 8*I*).

If cords were superfused with Ca^{2+}-free Ringer containing 6–8 mM-Mg^{2+} and 2 mM-EGTA (procedure II), as during the period of pretreatment with EGTA in procedure I, stable intracellular recordings were difficult to obtain. Only in eight out

of fifteen impaled motoneurones (five cords), supramaximal stimulation of dorsal roots evoked small, short latency e.p.s.p.s, having 0·4–0·5 mV peak amplitude. If two adjacent roots were stimulated simultaneously, these potentials could reach up to 1 mV peak amplitude. In the other seven cells there were no e.p.s.p.s generated by dorsal root stimuli, even when two roots were stimulated simultaneously, but the positive-negative presynaptic spike could be recorded intracellularly.

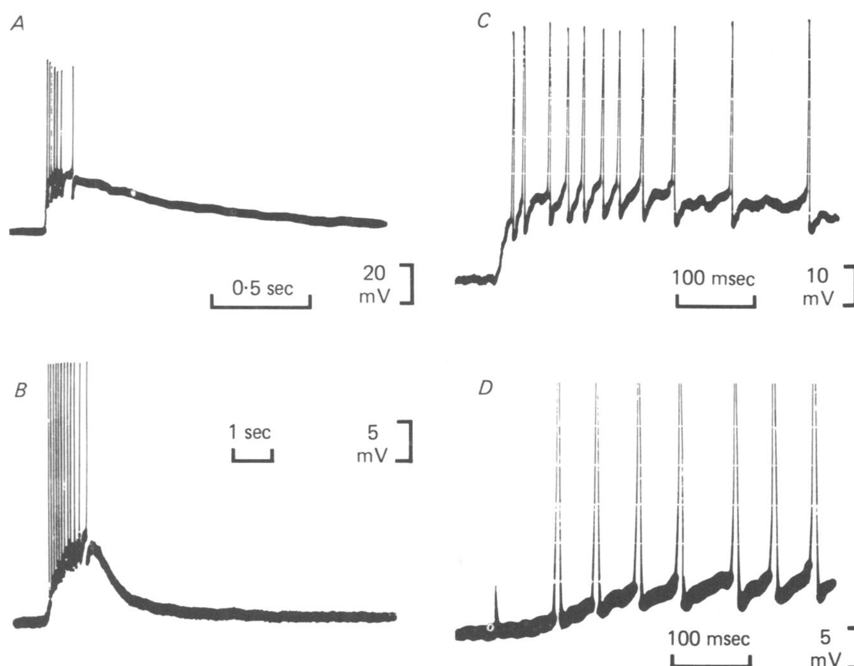

Fig. 9. Effect of replacement of Ca^{2+} by Sr^{2+} on composite e.p.s.p.s evoked in motoneurones by supramaximal dorsal root-stimulation. *A* and *C* were recorded in normal Ringer (resting potential = − 68 mV). The hemicord was superfused with Ca^{2+}-free Ringer containing 2 mM-Mg^{2+} and 0·25 mM-EGTA (during 70 min, after which superfusion with Ringer containing 2·5 mM-Sr^{2+} and 0·5 mM-EGTA was started; *B* and *D* were obtained 50 min after superfusion with Sr^{2+}-Ringer (resting potential = − 55 mV). Temp. 9·5 °C. Spikes were retouched for clarity.

In cords perfused with Ca^{2+}-free medium containing 6–10 mM-Mg^{2+}, without EGTA (procedure III), dorsal root stimuli evoked e.p.s.p.s. similar to the ones described in Co^{2+} medium, having average peak amplitude of 1·4 mV ± 0·18 S.E. (n = 9 motoneurones), time to half decay of 5·4 msec ± 1 S.E. and time to peak of 2·3 msec ± 0·35 S.E. The properties of these Mg^{2+}-resistant e.p.s.p.s were similar to those described for the Co^{2+}-resistant e.p.s.p.s. As in the Co^{2+} experiments, the slow after-hyperpolarization which follows the antidromic spike, was abolished in Ca^{2+}-free media containing 6–10 mM-Mg^{2+}.

Effect of replacing Ca^{2+} with Sr^{2+} or Ba^{2+} on synaptic potentials. Among several divalent cations only Sr^{2+} and to some extent Ba^{2+} have been shown to replace Ca^{2+} in the process of transmitter release by nerve impulses. The evidence comes from experiments at the vertebrate neuromuscular junction, the giant synapse in the

stellate ganglion of the squid and synapses in sympathetic ganglia (Miledi, 1966; Dodge, Miledi & Rahamimoff, 1969; Katz & Miledi, 1969*a*, *b*; McLachlan, 1977). However, the action of these cations on vertebrate central synaptic transmission was still unknown.

It was found that Sr^{2+} (2–11 mM) but not Ba^{2+} (2–10 mM) restored synaptic transmission which had been blocked by superfusion of the cord with Ca^{2+}-free Ringer containing 2 mM-EGTA (procedure I). Following equimolar replacement of Ca^{2+} with Sr^{2+}, composite e.p.s.p.s could always be evoked in response to dorsal root

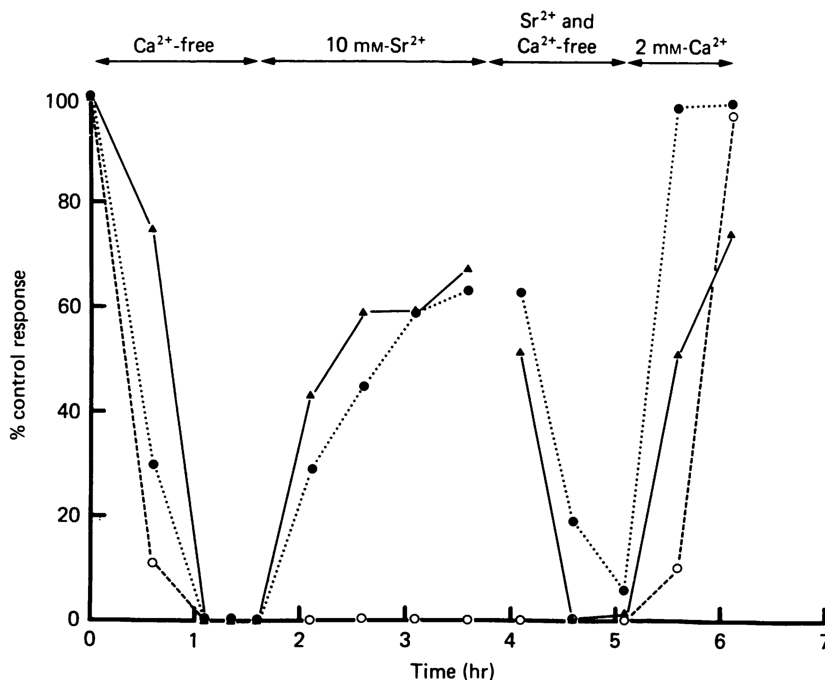

Fig. 10. Effects of Ca^{2+} and Sr^{2+} on peak amplitude of root potentials. Abscissa: time in hours. Ordinate: peak amplitude of root potential expressed as percentage of control responses (i.e. responses obtained in normal Ringer). ○, dorsal root potential evoked by stimulation of the segmental ventral root; ●, dorsal root potential evoked by stimulation of an adjacent dorsal root; ▲, ventral root potential evoked by stimulation of the segmental dorsal root. Changes in composition of the perfusion fluid are indicated by arrows on the top (temp. 8 °C).

stimulation. The composite dorsal root e.p.s.p.s generated in Sr^{2+} Ringer differed from those in normal Ringer in their slower time course. For instance, in the experiment illustrated in Fig. 9, the half-width and total duration of the composite e.p.s.p. in normal Ringer (Fig. 9*A*) were 0·6 and 1·8 sec respectively, while in 2 mM-Sr^{2+} (Fig. 9*B*) the same parameters were increased to 1·6 and 4·2 sec. The rising phase of the potentials generated in Sr^{2+} was also slower, as can be seen by comparing Fig. 9*C* and *D*.

The slow hyperpolarization which follows the antidromic spike could also be restored superfusing the cord with the Ca^{2+}-free Ringer containing 2 mM-Sr^{2+}. However, the amplitude of the after-potential was smaller than in normal Ringer.

Resting membrane potentials sampled from motoneurones superfused with Sr^{2+} (2 mM) were not significantly different from those measured in normal Ringer.

As expected, Sr^{2+} but not Ba^{2+} restored the root potentials which had been abolished by Ca^{2+}-free Ringer. The efficacy of Sr^{2+} as a substitute for Ca^{2+} was not the same for all the root potentials studied. Practically complete recovery of the dorsal root potential evoked by stimulation of an adjacent dorsal root was seen in most experiments with solutions containing 2–11 mM-Sr^{2+}, while only partial recoveries (50–70 % of control) were obtained for the ventral root potential elicited by dorsal root stimulation. On the other hand, the dorsal root potential evoked by stimulation of the segmental ventral root showed no sign of recovery. Only in one out of twenty experiments did the latter potential recover 5–10 % of its control amplitude while the cord was in Sr^{2+}-Ringer. The three root potentials recovered their amplitude to control values after Sr^{2+} was washed out, and the cords were superfused with normal Ringer. The results of one of these experiments are shown in Fig. 10.

Root potentials generated in Sr^{2+}-containing solutions rose and declined more slowly than those in normal Ringer. Besides these changes in the time course, the ventral root potential elicited by dorsal root stimulation showed many asynchronous spikes superimposed. On the other hand, the spikes which are usually superimposed on the rising phase of the dorsal root potential evoked by stimulation of an adjacent dorsal root in normal Ringer, were not present in Sr^{2+}.

In contrast with Sr^{2+}, Ba^{2+} (2–10 mM) did not restore any of the root potentials. However superfusion with Ba^{2+} Ringer produced 'spontaneous' spikes which were particularly prominent in fequency on the ventral roots.

DISCUSSION

Components of the dorsal root e.p.s.p. which are generated by chemical synapses

The present results show that in frog motoneurones, supramaximal stimuli to dorsal roots (segments 7–9) evoke a composite depolarizing potential consisting of a short latency e.p.s.p. followed by a series of two or three delayed e.p.s.p.s (cf. Araki, Otani & Furukawa, 1953; Machne, Fadiga & Brookhart, 1959; Katz & Miledi, 1963). In contrast to the short latency e.p.s.p., the delayed components of the dorsal root e.p.s.p. can be reversibly abolished either by removing the external Ca^{2+} and substituting it for Mg^{2+} (6–10 mM) or Co^{2+} (4–5 mM), or superfusing the cord with Ringer containing both Ca^{2+} (1 mM) and Co^{2+} (4 mM). This blockage of delayed synaptic events does not appear to be due to changes in the electrical properties of motoneuronal membrane, because these cells could support antidromic action potentials. Nor does it appear to result from failure of impulse propagation along intraspinal afferent fibres. The delayed components of the dorsal root e.p.s.p. are currently attributed to polysynaptic activity (Araki, 1960; Katz & Miledi, 1963). If this is so, failure of impulse propagation along axons of putative interneurones responsible for the generation of these e.p.s.p.s cannot be completely ruled out. However, this seems very unlikely since neither the antidromic spike, nor the action potential recorded at intraspinal dorsal root afferents were blocked. In addition, we have shown that Sr^{2+} but not Ba^{2+} can act as a substitute for Ca^{2+} at these synapses.

Thus, the actions of Ca^{2+}, Mg^{2+}, Co^{2+}, Sr^{2+} and Ba^{2+} at these central synapses are similar to their action at chemical synapses such as the neuromuscular junction (Del Castillo & Engbaek, 1954; Katz & Miledi, 1965*d*; Miledi, 1966; Dodge *et al.* 1969; Weakly, 1973), the giant synapse of the squid (Miledi & Slater, 1966; Katz & Miledi, 1969*a*, *b*), and synapses at sympathetic ganglia (McLachlan, 1977). Therefore, it seems very likely that the synapses involved in the generation of the delayed components of the dorsal root e.p.s.p.s operate by releasing a chemical transmitter (see also Katz & Miledi, 1963; Dambach & Erulkar, 1973).

The abolition of chemical transmission presumably arises because Co^{2+} and Mg^{2+} not only are unable to act as substitutes for Ca^{2+} in the release process (Miledi, 1973), but also because, as in other excitable cells (Hagiwara, 1973; Baker & Reuter, 1975) or synapses (Katz & Miledi, 1965*d*, 1969*a*, *b*), Co^{2+} and Mg^{2+} probably block voltage-sensitive Ca^{2+} channels in the terminals of these central synapses. A similar explanation holds for the reversible abolition of the slow after-hyperpolarization which follows the antidromic action potential. This after-potential is probably generated by an increase in K^+ conductance induced by calcium influx across the motoneurone membrane (Krnjević & Lisiewicz, 1972; Barrett & Barrett, 1976; Krnjević, Puil & Werman, 1978*a*, *b*). The fact that this after-potential could be restored in Ca^{2+}-free Ringer containing 2 mM-Sr^{2+}, is consistent with the idea that Sr^{2+} can pass through Ca^{2+} channels located in the motoneurone membrane, and trigger an increase in K^+ conductance either directly, or by displacing Ca^{2+} from intracellular sites (Krnjević, Lamour, MacDonald & Nistri, 1978).

The fact that synaptic transmission could also be restored by Sr^{2+} in the virtual absence of external Ca $^{2+}$ suggests that, as at the neuromuscular junction and squid synapses (Miledi, 1966; Katz & Miledi, 1969*a*, *b*), Sr^{2+} can replace external Ca^{2+} in the process of transmitter release. It is possible that Sr^{2+} pass through the Ca^{2+} channels of these terminals, triggering the release of transmitter (Miledi, 1973), either directly, or by releasing Ca^{2+} from an intracellular store. The failure to detect any significant recovery of transmission with Ba^{2+} is in agreement with previous observations showing that this cation is a very poor substitute for Ca^{2+} in the process of release of transmitter evoked by nerve impulses (Miledi, 1966; Dodge *et al.* 1969; Silinsky, 1978).

Possible electrotonic coupling between dorsal root afferents and motoneurones

In contrast to the delayed components of the dorsal root e.p.s.p., and the Ca^{2+}-dependent slow after-hyperpolarization; the short latency e.p.s.p. evoked by dorsal root stimulation persisted even more than 8 hr after superfusion with Ca^{2+}-free Ringer containing either Mg-$^{2+}$ (6–10 mM) or Co^{2+} (4–5 mM). These short latency e.p.s.p.s could be elicited by stimulation of segmental or adjacent dorsal roots, implying convergence of heterosegmental afferent fibres onto motoneurones, and exhibited temporal and spatial summation. They could be evoked by low threshold dorsal root fibres; it is possible therefore, that their principal source is the large group of fibres from muscle spindles (see also Shapovolov *et al.* 1978). The fact that these e.p.s.p.s persist in the virtual absence of external Ca^{2+} (estimated to be $\leqslant$ 10 μM) and in the presence of Co^{2+} or Mg^{2+}, suggests that synaptic transmission between some dorsal

root afferent fibres and motoneurones involves an electrical component (cf. Shapovalov *et al.* 1978; Shapovalov & Shiriaev, 1978). An alternative explanation is that these synapses are chemical; but in this case the synapses would have an unusually short synaptic delay and would need to be protected by a physical barrier impermeable to divalent ions; or what is even less likely they would need to have a transmitter release process capable of being activated by Ca^{2+}, Co^{2+} and Mg^{2+}.

The effect of EGTA deserves here some comment. When EGTA was added to a divalent ion free solution all synaptic activity in the spinal cord was abolished. Presumably this was due to abolition of transmitter release at chemical synapses plus failure of propagation of nerve impulses, and eventual membrane inexcitability caused by the complete absence of divalent ions in the external medium. It should be noted that this state is reversible, and presynaptic impulses reached the terminals soon after external Ca^{2+} was restored, as evidenced by the recovery of synaptic activity in both ventral and dorsal roots (cf. Fig. 10).

When Mg^{2+}, instead of Ca^{2+}, was used to restore the external divalent ions, chemical synapses did not recover as should be expected, since Mg^{2+} does not substitute for Ca^{2+} in the process of transmitter release. But, since Mg^{2+} does substitute for Ca^{2+} in maintaining propagation of impulses into nerve terminals, even in the presence of EGTA (Miledi & Thies, 1971), electrical synapses would be expected to recover. This was indeed what we observed but the 'electrically mediated' e.p.s.p.s recovered only after a relatively long delay. As the e.p.s.p.s had not yet recovered when the intraspinal presynaptic potentials, and the motoneurones ability to generate action potentials, was back to normal it seems that the lack of electrical synapse activity, at this time, is probably not due to failure of impulse propagation or decrease in motoneuronal membrane resistance. A plausible explanation of the temporary loss of Co^{2+}, Mg^{2+}-resistant e.p.s.p.s is that there is a transient uncoupling of an electrical synapse, similar to the uncoupling of electrototonic junctions in the crayfish septate axon produced by exposing abdominal cords to solutions free of divalent ions containing chelating agents and then to normal saline (Asada & Bennett, 1971; Peracchia & Dulhunty, 1976; see also Loewenstein, Nakas & Socolar, 1967; Rose & Loewenstein, 1971).

With prolonged exposure to divalent ion free-EGTA Ringer the e.p.s.p. recovered partially or not at all. It may be that after such treatment the uncoupling of the electrical synapse is more permanent. It would be interesting to see if the axodendritic gap junctions, which have been found in the frog's spinal cord (Charlton & Gray, 1966; Sotelo & Taxi, 1970; Sotelo & Grofova, 1976) and which may be the morphological substrate underlying the Co^{2+}, Mg^{2+}-resistant e.p.s.p. are disrupted by similar treatment.

The synaptic delay of the Co^{2+}, Mg^{2+}-resistant e.p.s.p.

One characteristic which distinguishes chemical transmission from electrical transmission is the synaptic delay. Such a delay is almost absent (Furshpan & Potter, 1959) or very brief at electrical synapses (Bennett, 1966, 1976). The minimum delay at the frog neuromuscular junction, measured between the negative peak of the presynaptic spike and the beginning of the focal end-plate potential at 10 °C is 1·3–1·4 msec (see Fig. 4 in Katz & Miledi, 1965*a*). The present results show that the

minimum delay, measured as the time interval between the negative peak of the intraspinal presynaptic spike recorded extracellularly and the onset of the intracellularly recorded Co^{2+} or Mg^{2+} resistant e.p.s.p. was $\leqslant$ 0·3 msec at 10 °C. This value probably represents an over-estimate of the 'genuine' synaptic delay, as defined for the frog neuromuscular junction (Katz & Miledi, 1965*c*), and includes time lags arising from conduction of impulses to the terminals and delays introduced by electrotonic propagation of the e.p.s.p.s (Rall, 1967). These appear to be generated mainly at distal dendritic branches (Liu & Chambers, 1957; Szekely, 1976) and thus the start of local current through the dendritic post-synaptic membrane, probably precedes the onset of the e.p.s.p. intracellularly recorded at the motoneurone soma. Thus it appears that the minimum delay for transmission between primary afferents and motoneurones is at least 3–4 times shorter than expected for a chemical synapse at 10 °C.

A mixed synapse?

It is still not clear whether all the delayed components of the dorsal root e.p.s.p. are generated through polysynaptic pathways. The minimum delay for the first of the late components was 2·6 msec at 10 °C. This delay is not incompatible with the idea that this component may be generated through a monosynaptic pathway operating by release of a chemical transmitter. Therefore, transmission between primary afferents and motoneurones could be mediated through a mixed synapse, i.e. electrical and chemical, whose morphological counterpart could be the axodendritic contacts at which gap junctions and active zones with vesicles coexist in the same presynaptic terminal (Charlton & Gray, 1966; Sotelo & Taxi, 1970; Sotelo & Grofova, 1976).

It is concluded that the monosynaptic e.p.s.p. set up by dorsal root stimuli is in part generated by electrical coupling between primary afferents and motoneurone dendrites. An alternative, but less attractive explanation for the present results is that a chemical synapse is enveloped by a 'physical barrier', relatively impermeable to divalent cations, and which can be made transiently and reversibly 'leaky' with EGTA. If that were the case, it would still be necessary to explain, among other findings, the briefness of the synaptic delay.

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