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THE EFFECTS OF Ca^{2+}, Mg^{2+} AND KYNURENATE ON PRIMARY AFFERENT SYNAPTIC POTENTIALS EVOKED IN CAT SPINAL CORD NEURONES *IN VIVO*

BY BRUCE WALMSLEY AND MADELEINE JANE NICOL

From the Neural Research Laboratory, School of Anatomy, University of New South Wales, PO Box 1, Kensington, NSW 2033, Australia

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SUMMARY

1. A technique was developed for perfusing the central canal of the cat spinal cord over a defined region to alter the extracellular environment and examine the effects of various ions and pharmacological agents on synaptic transmission *in vivo*.

2. Monosynaptic excitatory postsynaptic potentials (EPSPs) evoked by hindlimb muscle nerve stimulation were recorded intracellularly from dorsal spinocerebellar tract (DSCT) neurones in Clarke's column, in close proximity to the central canal.

3. The effects of central canal perfusion of solutions containing Ca^{2+}, Mg^{2+} and kynurenate on the monosynaptic afferent EPSP were examined.

4. Perfusion of the central canal with solutions containing a high Mg^{2+} concentration completely and reversibly blocked the monosynaptic EPSP, while perfusion with solutions containing a high Ca^{2+} concentration produced up to a fourfold increase in the peak amplitude of the EPSP. This large increase in the EPSP indicates that the pool of quanta available for release is considerably greater than estimated from previous quantal analysis studies at this synaptic connection.

5. Perfusion of the central canal with kynurenate, an antagonist at excitatory amino acid receptors, resulted in a complete block of the monosynaptic EPSP in DSCT neurones. This provides direct evidence that an excitatory amino acid, such as glutamate, is released from primary muscle afferent terminals in Clarke's column of the cat spinal cord *in vivo*.

INTRODUCTION

Over the past several years we have investigated a number of physiological and anatomical aspects of excitatory synaptic transmission at the monosynaptic connection between primary muscle afferents and dorsal spinocerebellar tract (DSCT) neurones in the cat spinal cord (Tracey & Walmsley, 1984; Walmsley, Wieniawa-Narkiewicz & Nicol, 1985, 1987*b*; Walmsley, Edwards & Tracey, 1987*a*, 1988; Walmsley & Stuklis, 1989; Walmsley, 1989; Walmsley & Nicol, 1990). Our anatomical results have shown that DSCT neurones receiving monosynaptic muscle afferent input are large cells with extensive dendritic trees oriented primarily in a rostrocaudal direction (Walmsley & Nicol, 1990; see also Randic, Miletic & Loewy,

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1981; Houchin, Maxwell, Fyffe & Brown, 1983). These DSCT neurones lie within Clarke's column, immediately adjacent to the central canal. Intracellular recordings have demonstrated that excitatory postsynaptic potentials (EPSPs) evoked in DSCT neurones by impulses in single primary muscle afferents are generally large and exhibit uniformly rapid rise times (Kuno & Miyahara, 1968; Eide, Fedina, Jansen, Lundberg & Vyklický, 1969*a*, *b*; Walmsley, 1989). Analysis of the electrotonic properties of DSCT neurones has indicated that these single-fibre EPSPs are generated at synaptic contacts on, or close to, the soma of these cells (Walmsley, 1989).

Single-fibre EPSPs evoked in DSCT neurones fluctuate in amplitude from trial to trial. Analysis of these fluctuations has indicated that EPSPs fluctuate in amplitude between discrete levels separated by equal, or quantal, increments. These quantal fluctuations cannot be described by an underlying simple binomial process (Walmsley *et al.* 1987*a*, 1988). However, the data can be well fitted by a compound binomial quantal model exhibiting a high degree of non-uniformity in the underlying quantal release probabilities (Walmsley *et al.* 1988). An important parameter in the compound binomial quantal model (and other quantal models) is the total number (N) of quanta available for release. Because of the uncertainties associated with quantal analysis it is usually only possible to determine a minimum value for N, as shown by the results of our compound binomial analysis at this synapse (Walmsley *et al.* 1988). Because of this uncertainty, further independent evidence relating to the value of N is required.

In the present study, we have attempted to gain such evidence by altering the extracellular Ca^{2+} concentration in the spinal cord, thus facilitating transmitter release. We have taken advantage of the close anatomical proximity of Clarke's column to the central canal to alter the ionic environment *in vivo* of the DSCT neurones which lie within Clarke's column. We have developed a technique in which the central canal may be perfused over the spinal segment (or segments) of interest, by micro-cannulation. We have used this technique in the present study to examine the effects of extracellular changes in Ca^{2+} and Mg^{2+} concentration on the monosynaptic EPSPs evoked in DSCT neurones by stimulation of primary muscle afferent fibres.

Although the effects of Ca^{2+} and Mg^{2+} on primary afferent EPSPs generated in DSCT neurones confirm conventional chemical transmission at this connection, the identity of the neurotransmitter remains unknown. We have made further use of our ability to change the extracellular environment of DSCT neurones to study the effects of kynurenic acid on the primary afferent EPSP. Previous experiments using *in vitro* preparations have demonstrated that kynurenate blocks the action of excitatory amino acids, such as glutamate, in the spinal cord (Jahr & Yoshioka, 1986). In the present experiments, we have shown that kynurenate completely blocks primary muscle afferent transmission to DSCT neurones. This result provides direct *in vivo* evidence that an excitatory amino acid, such as glutamate, is the transmitter released by primary muscle afferents in Clarke's column in the cat spinal cord.

METHODS

Experiments were performed on adult cats weighing 2·5–3·5 kg. Cats were anaesthetized with sodium pentobarbitone (35 mg/kg I.P.) and maintained with supplementary doses (5 mg, I.V.). Mean arterial pressure and end-tidal CO_2 were monitored. Paralysing agents were not used and the animals respired naturally.

The lumbosacral spinal cord was exposed by laminectomy from L2 to L7. Muscle nerves in the left hindlimb were exposed, cut and placed on bipolar stimulating electrodes. These included the nerves to medial gastrocnemius (MG), lateral gastrocnemius (LG), soleus (Sol), plantaris (Pl) and posterior biceps–semitendinosus (PBST) muscles. The remaining branches of the sciatic nerve in the left hindlimb were then cut. Nerves were stimulated at approximately 2 × threshold for group I excitation in all experiments.

Micro-cannulation of the spinal cord

A small rostrocaudal slit, approximately 3–4 mm in length, was carefully made through the dorsal columns at the L2 spinal level, down to the central canal. The central canal was easily located by observing the sudden outflow of cerebrospinal fluid. Care was taken not to disrupt surface blood vessels, and the slit was usually made with no apparent bleeding. A small flexible plastic tube (drawn down to a diameter of approximately 0·1 mm) was then carefully inserted into the central canal, and pushed several millimetres along the central canal in the caudal direction (as illustrated in Fig. 1). The tube was located and sealed in place by suturing the dura over the slit. Outflow was achieved by making another slit at the L4 spinal level, down to the central canal, through the contralateral (right) dorsal columns. Care was taken not to damage the left dorsal column, which carries the primary afferents from the muscles of interest.

Flow was achieved by gravity feed, and adjusted to approximately 100–200 μl/min. A multi-way tap was used to select different perfusates. The central canal was perfused with different solutions to examine their effect on the monosynaptic EPSPs evoked in DSCT neurones by stimulation of the hindlimb muscle nerves. In the present experiments, solutions contained $CaCl_2$, $MgCl_2$ or kynurenic acid (Sigma, adjusted to pH 7·2 with NaOH) dissolved in sterile saline (0·9 % NaCl w/v).

Intracellular recordings

Intracellular recordings were obtained from DSCT neurones within Clarke's column using conventional capillary glass micropipettes filled with 2 M-potassium methylsulphate (Walmsley *et al.* 1987*b*). As previously described, DSCT neurones that receive monosynaptic excitatory input from the hindlimb ankle extensor muscles are located within the rostrocaudal limits defined by the L3 dorsal root entry zone, and this is the region in which all recordings were obtained in the present study (Walmsley & Nicol, 1990).

RESULTS

Results were obtained from experiments on nineteen adult cats weighing 2·5–3·5 kg.

During initial experiments, the dura was slit along the entire exposed length of the spinal cord (L2 to L7) to allow access for the central canal cannula and for the intracellular microelectrode. In later experiments, the dura was left intact except for small slits in the inflow (cannula) and the outflow regions, and a very small slit for the recording electrode. This seemed to improve both the viability of the spinal cord and the stability of the intracellular penetrations.

The central canal was continuously perfused with sterile saline (0·9 % NaCl w/v) during electrode tracking. This was found to be necessary for the maintenance of intracellular penetrations, since an abrupt flow through the central canal usually dislodged the microelectrode from the cell. Presumably, this sudden flow caused a local movement of the tissue, especially in the region immediately adjacent to the

central canal. However, in one experiment we were able to maintain an intracellular penetration before perfusion of the central canal with any solution, and subsequently during perfusion with sterile saline. Perfusion of the central canal with saline had no measurable effect on monosynaptic EPSP evoked by stimulation of the MG nerve in this cell.

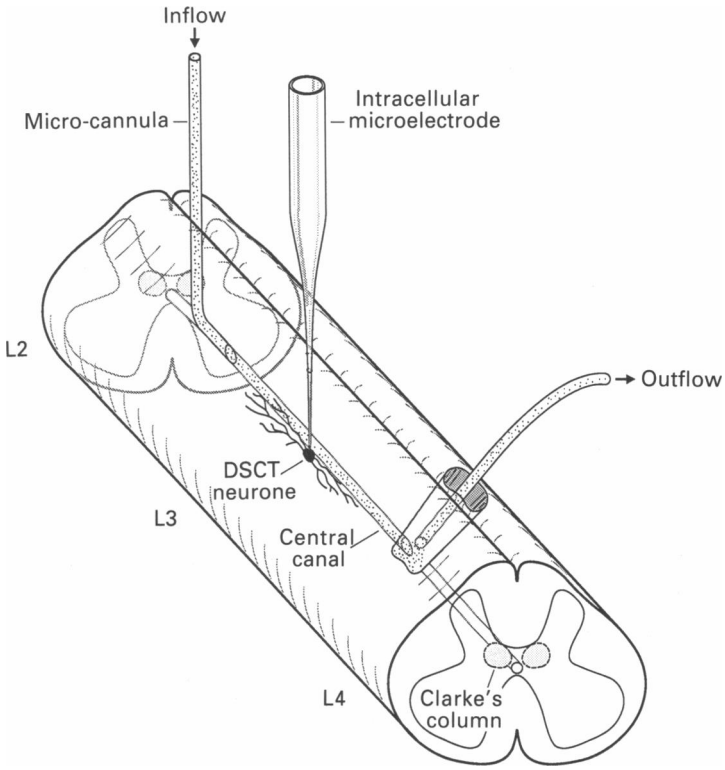

Fig. 1. Schematic diagram illustrating the cannulation and perfusion of the central canal (see Methods for details).

Apart from the problems caused by abrupt flow through the central canal, no further difficulties were encountered which could be directly attributed to central canal perfusion. In accordance with our previous experience, the greatest difficulty encountered during this series of experiments was associated with maintaining stable intracellular penetrations of DSCT neurones, due to blood pressure pulsations and respiratory movements. The normally low yield of results due to instability of intracellular penetrations was made worse in the present experiments by the fact that only one cell per experiment could be tested. This restriction ensured that each cell was not affected by prior perfusion of ions or drugs which may not have completely washed out of the spinal cord, and thus allowed comparison with previous data obtained in unperfused spinal cords. Despite these difficulties, successful results were obtained in four out of ten experiments on the effects of Mg^{2+} and Ca^{2+}, and three out of nine experiments on the effects of kynurenate on synaptic potentials evoked in DSCT neurones.

Effects on Ca^{2+} and Mg^{2+} on the monosynaptic EPSP

Figure 2 illustrates the effects of central canal perfusion with solutions containing $MgCl_2$ and $CaCl_2$ on a monosynaptic EPSP evoked by stimulation of the LG–Sol nerve. The central canal was first perfused with a high concentration (100 mM) of

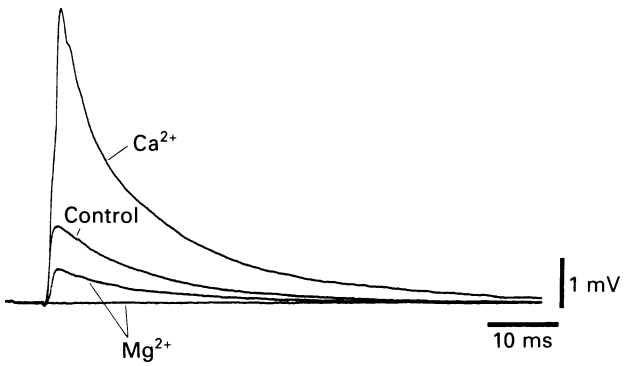

Fig. 2. The effect of central canal perfusion of solutions containing Ca^{2+} and Mg^{2+} ions (100 mM) on the monosynaptic EPSP evoked in a DSCT neurone by stimulation of the LG–Sol muscle nerve.

$MgCl_2$, so that a progressive block of the EPSP could be observed. The effect of this perfusion on the EPSP was rapid, and a complete block (lower trace) was achieved within minutes. Following wash-out with isotonic saline, during which the EPSP slowly recovered, the central canal was perfused with a 100 mM-$CaCl_2$ solution. A rapid increase in the amplitude of the EPSP occurred, and this increase continued over the following 15 min to reach a plateau. During this time, a noticeable increase in the occurrence of spontaneous EPSPs and IPSPs was observed. The peak amplitude of the monosynaptic EPSP attained a final value of 5·75 mV, which is 385 % of the control EPSP amplitude. The membrane potential of the cell was steady throughout these changes at approximately −65 mV.

Shape indices of the evoked EPSP during partial Mg^{2+} block were determined, and compared to control values. The control EPSP had a peak amplitude of 1·49 mV, a 10–90 % rise time of 0·86 ms and half-width of 10·5 ms. The membrane time constant was determined from the linear region of the log plot of the decay phase of the EPSP and gave a value of 13·3 ms. The EPSP illustrated in Fig. 2 during partial Mg^{2+} block has a peak amplitude of 0·66 mV, a 10–90 % rise time of 0·92 ms and a half-width of 9·5 ms. The decay time constant of the EPSP was calculated to be 13·2 ms. These values do not reveal any significant change in the time course of the EPSP or in the membrane time constant of the cell during partial Mg^{2+} block of the EPSP (see Discussion).

Figure 3 illustrates the results obtained during another experiment, and shows the effects of $CaCl_2$ perfusion on three different monosynaptic EPSPs evoked in the same DSCT neurone by stimulation of the Sol, LG and Pl muscle nerves. (As noted in a previous study, many DSCT neurones receive monosynaptic afferent input from more than one ankle extensor muscle; Walmsley & Nicol, 1990). In this case

we were able to monitor the increase in amplitude of all three EPSPs during perfusion of 50 mM-$CaCl_2$ through the central canal. A noticeable increase in all EPSPs was observed within minutes of $CaCl_2$ perfusion. The facilitated EPSPs continued to increase, and attained a steady level after about 15 min. Measurements

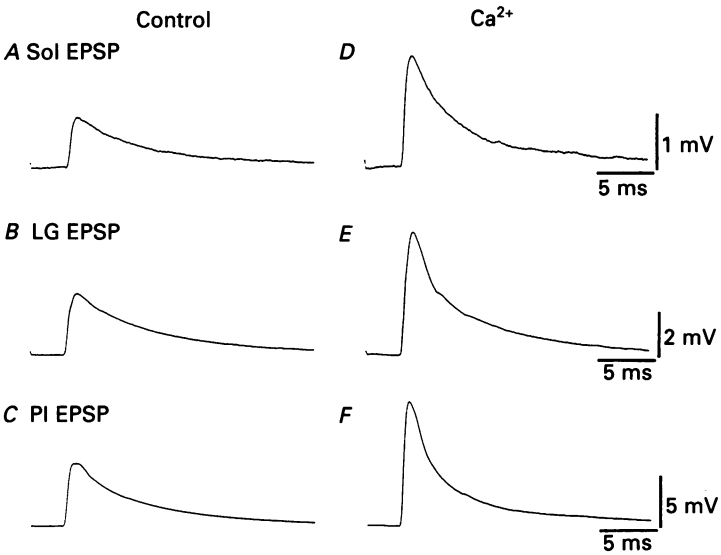

Fig. 3. Monosynaptic EPSPs evoked in the same DSCT neurone by stimulation of the Sol, LG and PI muscle nerves (*A*, *B* and *C*). Perfusion of a Ca^{2+}-containing solution (50 mM) through the central canal produced facilitation of these EPSPs as illustrated in *D*, *E* and *F*. Calibration in *D*, *E* and *F* also applies to the control EPSPs shown in *A*, *B* and *C*.

of the peak amplitude of the control and Ca^{2+}-facilitated EPSPs illustrated in Fig. 3 revealed increases of 221, 201 and 198% for the Sol, LG and PI EPSPs respectively. Following perfusion with 50 mM-$CaCl_2$, the perfusate was changed to 100 mM-$CaCl_2$ solution. The EPSP evoked by PI nerve stimulation increased further to reach a final value of 237% of the control EPSP amplitude. However, no further increase was observed in the amplitude of the EPSPs evoked by the Sol and LG muscle nerves, indicating that these EPSPs were at maximal facilitation.

These observations were repeated during a further experiment in which EPSPs evoked by stimulation of Sol, LG and PI nerves were recorded in the same DSCT neurone. Following central canal perfusion with 50 mM-$CaCl_2$ these EPSPs increased in amplitude by 200, 203 and 179% of their respective control amplitudes. The average increase in peak amplitude of the seven EPSPs measured in three different experiments, following Ca^{2+} facilitation, was 232 ± 65% (mean $\pm$ S.D.) of the control amplitudes.

Occasionally, obvious polysynaptic responses could be observed on the decay phase of the evoked EPSP. Figure 4 shows a polysynaptic EPSP (Fig. 4*A*) and an IPSP (Fig. 4*B*) observed in two different DSCT neurones. In both cases the monosynaptic EPSP was evoked by stimulation of the MG nerve. The effect of central canal perfusion of 50 mM-$CaCl_2$ on both the monosynaptic and polysynaptic potentials was observed as an increase in the amplitude of all components. This

observation is consistent with a Ca^{2+} facilitation of transmitter release from the synaptic terminations of both monosynaptic and polysynaptic pathways onto these cells.

Antagonism of the monosynaptic EPSP by kynurenate

Figure 5 illustrates the effect of central canal perfusion of 10 mM (Fig. 5*A* and *B*) and 2 mM (Fig. 5*C*) kynurenate. The EPSPs illustrated in Fig. 5*A* and *B* were

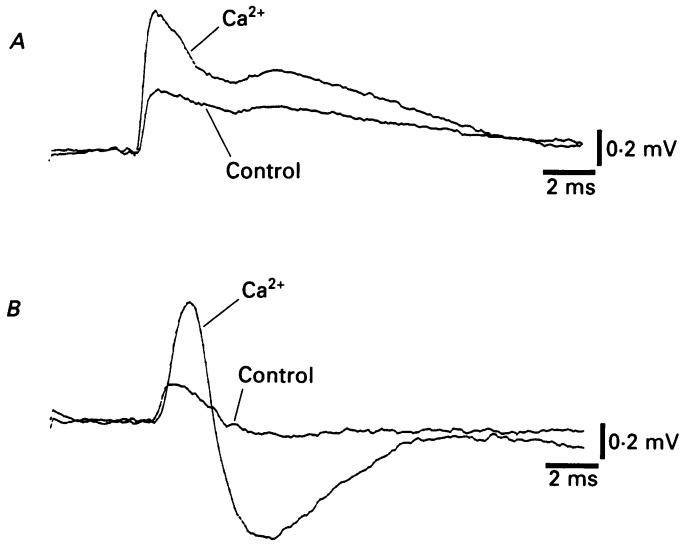

Fig. 4. The effect of Ca^{2+} perfusion (50 mM) on monosynaptic and polysynaptic potentials recorded in two different DSCT neurones (*A* and *B*) evoked by stimulation of the MG muscle nerve.

recorded in the same DSCT neurone. Perfusion of the central canal with 10 mM-kynurenate completely abolished the EPSP evoked by stimulation of both muscle nerves. The result illustrated in Fig. 5*C* was obtained in another DSCT neurone, during perfusion with 2 mM-kynurenate. This resulted in a decrease of the monosynaptic EPSP to 48 % of the control EPSP amplitude. It should be noted that this significant reduction in EPSP amplitude was obtained using a kynurenate concentration comparable to that required to block the monosynaptic EPSP in motoneurones *in vitro* (see Fig. 3 of Jahr & Yoshioka, 1986).

The time of nerve stimulation for all EPSPs illustrated in Fig. 5 is at the beginning of each trace. Approximately 3 ms following nerve stimulation, a small pre-potential, indicated with 'P', can be clearly observed just preceding the rising phase of all three EPSPs. This pre-potential represents the compound field potential arising from the arrival of the action potential in the collateral branches of the afferent nerves, following stimulation of the muscle nerve. As Fig. 5 shows, there is no measurable change in this pre-potential for all three EPSPs following block of the EPSPs with kynurenate. This observation indicates that the kynurenate did not interfere with the normal propagation of the presynaptic action potential into the collateral branches of the afferent fibres. Following EPSP block with kynurenate, attempts

were made to follow the recovery of the EPSPs during wash-out of the central canal with isotonic saline. Recovery of the EPSPs occurred extremely slowly. We were not able to maintain stable penetrations of cells for a long enough period to observe complete recovery, although we did observe significant partial recovery of these

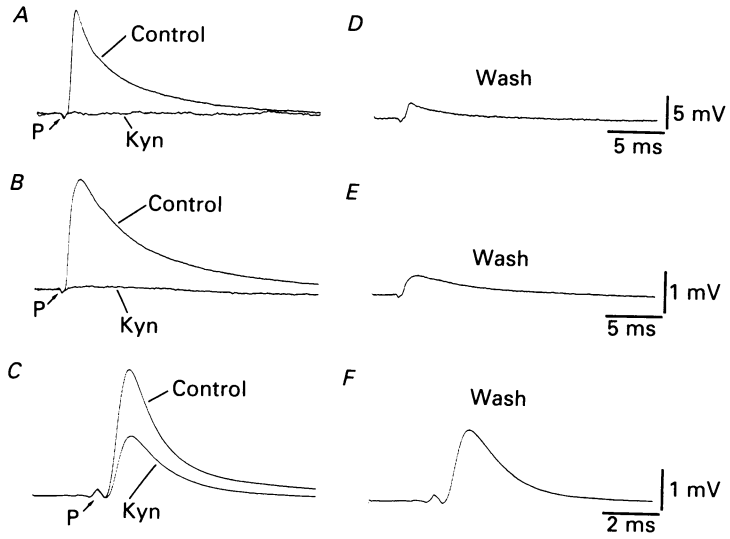

Fig. 5. Block of the monosynaptic EPSP by kynurenate (at a concentration of 10 mM in *A* and *B*, and 2 mM in *C*). The EPSPs illustrated in *A* and *B* were recorded in the same neurone by stimulation of LG–Sol and Pl nerves respectively. The EPSP illustrated in *C* was evoked in another DSCT neurone by stimulation of the PBST nerve.

EPSPs. The EPSPs illustrated in Fig. 5*D* and *E* illustrate the partial recovery of the EPSPs shown in Fig. 5*A* and *B* respectively. We were able to record from this cell for over 3 h, and the EPSPs illustrated in Fig. 5*D* and *E* were recorded 1 h 50 min after the start of the wash-out. The EPSP illustrated in Fig. 5*F* shows partial recovery 10 min after the start of wash-out, for the EPSP illustrated in Fig. 5*C*.

DISCUSSION

Manipulation of the extracellular environment to study synaptic transmission *in vivo* is difficult to achieve and these difficulties are usually overcome by the use of *in vitro* preparations (e.g. Kuno & Takahashi, 1986; Forsythe & Coates, 1988). In the present study, we required a means of altering the environment of adult cat spinal cord neurones *in vivo*, in order to directly relate the results to our previous studies in Clarke's column. Previous studies have attempted to alter the extracellular environment using an extracellular micropipette. The results of such experiments are often complicated by the local nature of ionophoretic application, as reported by Flatman, Durand, Engberg & Lambert (1987). In the present study, we have developed a means of perfusing the central canal to alter the environment of the cells within Clarke's column over a more extensive region than could be achieved using ionophoresis. Although a diffusion gradient within the spinal cord would still

obviously exist with the central canal perfusion, no difficulty was encountered in obtaining rapid and large effects on all cells studied in the present experiments. It is therefore possible that this method would also be useful in studying the effect of ions and drugs on groups of spinal cord neurones other than DSCT neurones.

In the present study, we have used this technique to study the effects of Ca^{2+}, Mg^{2+} and kynurenate on the monosynaptic EPSP evoked in DSCT neurones by impulses in primary muscle afferents.

Block of the monosynaptic EPSP by Mg^{2+} and facilitation by Ca^{2+}

Classical studies at the frog neuromuscular junction originally revealed the role of Mg^{2+} to block Ca^{2+} entry and subsequent release of transmitter from the presynaptic terminal (Katz, 1969). The present results have confirmed this action *in vivo* by showing that an increase in extracellular Mg^{2+} readily blocks the monosynaptic EPSP evoked in DSCT neurones.

Previous electrophysiological studies at this connection have revealed that EPSPs evoked in DSCT neurones by impulses in single group I muscle afferents are large and exhibit uniformly rapid rise times (Kuno & Miyahara, 1968; Eide *et al.* 1969*a*, *b*; Tracey & Walmsley, 1984; Walmsley *et al.* 1978*a*, 1988; Walmsley, 1989). Analysis of single-fibre EPSPs in conjunction with electrotonic measurements of DSCT neurones indicates that these EPSPs are generated by synaptic contacts at, or very close to, the soma of these neurones (Walmsley, 1989). Single group I afferent EPSPs fluctuate in peak amplitude from trial to trial. In a previous study we have demonstrated that these fluctuations can be described by a compound binomial quantal model with extremely little variance in the quantal unit amplitude (Walmsley *et al.* 1988). One of the major conclusions from this study was that there is a high degree of non-uniformity in the quantal release probabilities between release sites at this connection. The compound binomial quantal model describes the overall EPSP fluctuation pattern in terms of a quantal release probability at each release site, and a total number, N, of quanta available for release. Unfortunately, in such a quantal analysis it is only possible to calculate a minimum value for N (Walmsley *et al.* 1988). A knowledge of the value of N is extremely important to our interpretation of the mechanisms underlying the EPSP fluctuation pattern and release site probabilities. It would be very useful to know how close to the maximal possible quantal release level a given synaptic connection normally operates. In the present study, we have attempted to gain some insight into this issue by facilitating transmitter release using an increase in the extracellular Ca^{2+} concentration. Our results indicate that the monosynaptic connection between primary afferents and DSCT neurones is normally operating well below maximal release. The results of our previous compound binomial quantal analysis indicate that the minimum possible value of N corresponds to an amplitude level of 122% (on average) above the mean peak amplitude for the EPSP (Walmsley *et al.* 1988). Our present results indicate that the amplitude of the monosynaptic EPSP may be facilitated up to a value of 385% (average value 232%). Therefore, N appears to be 2–3 times greater than the minimum possible values calculated in our previous study (Walmsley *et al.* 1988). Furthermore, the minimum degree of non-uniformity in underlying quantal release probabilities in that study was calculated on the basis of the minimum

possible value of N. Since greater values of N correspond to higher levels of non-uniformity (Walmsley *et al.* 1988), the present results indicate that the level of non-uniformity between release site probabilities is even greater than our previous results suggest.

Block of the monosynaptic EPSP by kynurenate

Kynurenate has been shown in a number of studies to block the actions of excitatory amino acids (e.g. Ganong, Lanthorn & Cotman, 1983; Robinson, Anderson & Koerner, 1984; Jahr & Jessell, 1985; Jahr & Yoshioka, 1986; Forsythe & Coates, 1988). In the *in vitro* neonatal rat spinal cord, kynurenate has been shown to selectively antagonize the monosynaptic afferent excitation of motoneurones (Jahr & Yoshioka, 1986). In the present study, we have shown that kynurenate is capable of completely abolishing the monosynaptic EPSP evoked in DSCT neurones by stimulation of primary muscle afferents. This provides direct evidence that an excitatory amino acid, such as glutamate, acts as a neurotransmitter at this connection. Glutamate has been shown to act as an agonist at a variety of receptor types (see recent reviews by Mayer & Westbrook, 1987; Evans, 1989). One of the important receptor subtypes is activated by the amino acid *N*-methyl-D-aspartate (NMDA). The kinetics of the postsynaptic current generated by NMDA have been shown to be considerably slower than the non-NMDA-activated component (Forsythe & Westbrook, 1988). In addition, it has been shown that Mg^{2+} acts to block the NMDA-activated current in a voltage-dependent manner.

The synaptic current underlying single Ia fibre EPSPs evoked in motoneurones in the cat spinal cord has been measured by Finkel & Redman (1983) using a single-electrode voltage clamp. The current time course was found to be extremely rapid (decay time constant 0·3–0·4 ms), even at membrane potentials sufficiently depolarized to relieve Mg^{2+} block of NMDA receptor/channels. In addition, Flatman, Durand, Engberg & Lambert (1987) found that the specific NMDA receptor antagonist, APV, did not affect the fast phase of composite Ia EPSPs evoked in spinal motoneurones. (A lack of effect of non-NMDA antagonists on the slower phase of the EPSPs was attributed to problems associated with local ionophoresis.) This evidence indicates that that NMDA receptors do not appear to be involved in synaptic transmission at the Ia motoneurones synapse (see also Davies & Watkins, 1985).

In the present experiments we have demonstrated that there is no change in the time course of the monosynaptic EPSP evoked in DSCT neurones during partial block of the EPSP with raised extracellular Mg^{2+}. This implies that NMDA receptor/channels do not contribute significantly to the synaptic current at resting membrane potential because either (a) there are no NMDA receptors at this synapse, or (b) the NMDA receptor/channels are completely blocked at resting potential by the normal extracellular Mg^{2+} concentration. (It should be noted, however, that Flatman *et al.* (1987) demonstrated that motoneurones could be readily depolarized from resting potential by ionophoresis of NMDA in the cat spinal cord *in vivo*.) We hope to obtain further evidence on this issue using central canal perfusion of specific NMDA and non-NMDA antagonists in conjunction with voltage-clamp measurements of the synaptic current.

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