The effects of central myorelaxants on synaptically-evoked primary afferent depolarization in the immature rat spinal cord *in vitro*

¹R J Siarey, S.K. Long, M.T.H. Tulp & ²R.H. Evans

Department of CNS-Pharmacology, SOLVAY DUPHAR B V, P O Box 900, 1380 DA Weesp, The Netherlands and *The Department of Pharmacology, University of Bristol, School of Medical Sciences, University Walk, Bristol BS8 1TD

1 In the immature rat *in vitro* hemisected spinal cord preparation the dorsal root-evoked depolarizing potential recorded from an adjacent dorsal root DR-DRP had a mean peak amplitude ($\pm$ s.e.mean, $n = 27$) of 2.9 ± 0.2 mV and a mean latency to peak amplitude of 106 ± 3 ms. The DR-DRP amplitude was maximal with a stimulus intensity of four times the threshold intensity required to activate the lowest threshold fibres. The peak amplitude and/or integral over a time-source of 0.5 s were used to assess the effects of applied drugs.

2 The DR-DRP was abolished by baclofen (mean IC_{50} 190 ± 46 nM, $n = 7$). The depressant effect of baclofen was reversed by CGP35348 (1 mM). The mean apparent K_d value calculated from dose-ratios was 16.7 ± 6.4 μ M ($n = 3$).

3 At a maximally effective concentration, tizanidine (1 μ M) produced at the most only a 14% depression of the DR-DRP ($n = 4$). Clonidine (0.3 μ M) had an effect similar to that of tizanidine. These depressant effects were reversed by idazoxan (1 μ M).

4 The DR-DRP was potentiated by diazepam in a flumazenil (1 μ M)-reversible manner. A maximal potentiation of $23.2 \pm 2.7\%$ ($n = 5$) was produced by 1 μ M diazepam.

5 Diazepam (1 μ M) induced a mean bicuculline- (10 μ M, $n = 2$) and flumazenil- (1 μ M, $n = 8$) sensitive depolarization in the dorsal root of 0.25 ± 0.03 mV ($n = 8$). However, diazepam failed to depolarize dorsal roots ($n = 3$) which had been excised from the spinal cord.

6 Comparison of the above effects with previously reported depressant effects of these drugs on the synaptic output from ventral roots suggests that actions on presynaptic inhibition, as reflected in the DR-DRP, are of subsidiary importance in explaining the muscle relaxant actions of tizanidine or diazepam.

Keywords: Baclofen; diazepam; tizanidine; spinal cord; primary afferent depolarization

Introduction

In a previous study (Siarey *et al.*, 1992b) it was found that the myorelaxant drugs diazepam and tizanidine produced similar levels of depression of N-methyl-D-aspartate (NMDA) receptor-mediated synaptic excitation of motoneurons, as reflected in recordings from ventral roots of *in vitro* spinal cord preparations. These depressant actions were mediated respectively via flumazenil- and idazoxan-sensitive receptors. In the same study baclofen abolished the synaptic excitation of motoneurons and the effect of baclofen was reversed by the GABA_B receptor antagonist, CGP35348 (3-aminopropyl (diethoxymethyl)phosphinic acid) (Bittiger *et al.*, 1990).

The myorelaxant action of diazepam, unlike that of tizanidine or baclofen, is considered to be associated with the potentiation of presynaptic inhibition mediated at GABA_A receptors (Polc *et al.*, 1974). Presynaptic inhibition is reflected in the picrotoxin- or bicuculline-sensitive synaptic depolarization of dorsal root fibres (DR-DRP) evoked from electrical stimulation of an adjacent dorsal root (Eccles *et al.*, 1963). Thus, effects of drugs on this form of presynaptic inhibition can be monitored from their actions on the DR-DRP.

The present paper is an extension of the previous study (Siarey *et al.*, 1992b). In the present study the effects of the three myorelaxant drugs and the NMDA receptor antagonist 2-amino-5-phosphonopentanoate (AP5) have been compared on the DR-DRP.

The results suggest that the previously reported depression of output from motoneurons by tizanidine or diazepam

(Siarey *et al.*, 1992b) is independent of their actions on the DR-DRP.

Some of these results have been reported in preliminary form (Siarey *et al.*, 1992a).

Methods

The methods of superfusion, stimulation and recording were as described by Evans *et al.* (1982). Hemisected spinal cord preparations from rats of 2 to 5 days *post partum* age were superfused with medium containing (mM): MgSO₄ 1.25, CaCl₂ 1.5, NaCl 118, KCl 3, NaHCO₃ 24 and dextrose 12. Supramaximal electrical stimulation (0.5 ms square pulses at 10 to 20 times threshold) of a dorsal root (L4 or 5) at a rate of 0.0111 Hz was used to evoke synaptic potentials in the adjacent dorsal root (DR-DRP).

Effects of drugs on the electrically evoked responses were measured as the change in peak amplitude or area after integration over a time course of 0.5 s. In several preparations, for technical reasons, the size of synaptic responses decreased consistently over the period of the experiment. This is illustrated in Figure 1c. The effects of drugs were superimposed on such time-dependent changes.

Unless stated otherwise mean values are presented $\pm$ s.e.mean.

Results

The peak of the DR-DRP measured in 27 preparations had a mean latency to peak amplitude of 106 ± 3 ms, a mean amp-

¹ Present address: Institute of Physiology, University of Aarhus, Ole Worms Alle 160, Universitetsparken DK-8000, Aarhus C, Denmark.

² Author for correspondence.

Figure 1 (a) Plot of mean peak amplitude (■), integral over 0.5 s (□) and integral over 2 s (X) of DR-DRP against stimulus intensity in terms of threshold (T) for activation of the lowest threshold fibres. Values are mean $\pm$ s.e. mean of at least three observations. It can be seen that the peak amplitude is near maximal at 4T. (b) Effect of 2-amino-5-phosphonopentanoate (AP5, 100 μ M) on the DR-DRP. Time course of the effect, on individual values for peak amplitude from the preparation in (b), is plotted in (c). It can be seen that cumulative application of AP5 (μ M) preferentially abolished the later component of the potential. Calibration in (b), vertical 1 mV; horizontal 1 s.

litude of 2.9 ± 0.2 mV and a mean time to half decay of 274 ± 14 ms. The peak amplitude of the DR-DRP was near maximal at stimulus intensities of four times the threshold for the lowest threshold fibres (filled squares Figure 1). Stimulus intensities higher than four times threshold increased the amplitude and duration of the long latency (> 0.4 s) component of the DR-DRP (Figure 1). This profile of the DR-DRP is similar to that from a smaller sample described in a previous paper (Siarey *et al.*, 1991).

The depressant effect of the non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNOX) on the DR-DRP has been reported previously (Evans & Long 1990; Siarey *et al.*, 1991). The NMDA receptor antagonist AP5 produced a preferential depression of the long duration com-

ponent of the DR-DRP (Figure 1b,c) as indicated by respective mean EC_{25} values (μ M) of 23.7 ± 5.1 and 2.4 ± 0.4 ($n = 5$) for depression of peak amplitude and area of the DR-DRP.

Effect of baclofen

Figure 2a and b illustrate the depressant effect of baclofen on the DR-DRP. The potential was abolished at concentrations between 1 and 5 μ M. The mean ($n = 7$) EC_{50} for depression of the peak amplitude was 275 ± 62 nM. There was no significant difference, in the depressant effect of baclofen, between the peak amplitude and the integrated area of the DR-DRP. As illustrated in Figure 2c, CGP35348 (1 mM) produced a parallel rightward displacement of the concentration-effect plot for baclofen. The mean apparent K_d value calculated from this rightward displacement in three preparations was 16.7 ± 6.4 μ M.

Figure 2 Depressant effect of baclofen on the DR-DRP. It can be seen in (a) that baclofen (1 μ M) depressed all components of the DR-DRP; (b) shows the time course of the effect of cumulative (0.01, 0.03, 0.05, 0.1, 0.3, 1, 3, 10 and 30 μ M) concentrations of baclofen, as indicated above the plot, on the peak amplitude of the DR-DRP. The GABA_B antagonist, CGP35348 (1 mM) was introduced also as indicated above the plot. (c) Shows the concentration-effect plot of the percentage depressant action of baclofen with the rightward shift induced by CGP35348. Other details as for Figure 1.

Effect of α_2 -adrenoceptor agonist

Clonidine (EC_{50} 26 nM) and tizanidine (EC_{50} 135 nM) have been shown to be potent depressants of the synaptic output from motoneurons as recorded from ventral roots (Siarey *et al.*, 1992b). In the present study these two drugs were tested on the DR-DRP at concentrations higher than were required for the above actions. Figure 3 illustrates the weak depression of the DR-DRP produced by clonidine and tizanidine. Thus clonidine (0.3 μ M) and tizanidine (1 μ M) depressed the peak amplitude of the DR-DRP to mean levels of $92 \pm 2\%$ ($n = 3$) and $90 \pm 4\%$ ($n = 4$) of respective control values.

Figure 3 Effect of clonidine (1 μ M) (a) and tizanidine (1 μ M) (b) on the DR-DRP with the time-courses of the effect on peak amplitude plotted below. Clonidine (0.01, 0.02, 0.03, 0.05, 0.1, 0.3 and 1 μ M) was applied cumulatively as indicated on the plot. A small idazoxan (1 μ M)-reversible depressant action of both drugs can be seen. Other details as for Figure 1.

There was no significant difference between the effects of peak amplitude and integrated area. As previously observed with the depression of ventral root potentials (Siarey *et al.*, 1992b), the effect of clonidine was very slowly reversible (Figure 3a) but the depressant effects of clonidine and tizanidine were reversed quickly in the presence of idazoxan (1 μ M) (Figure 3).

Effect of diazepam

In the previous study from this laboratory, diazepam (1 μ M) depressed the integrated polysynaptic output from motoneurons by 50% (Siarey *et al.*, 1992b). In the present study application of diazepam (1 μ M) caused depolarization of the dorsal root (see Figure 4a). In eight preparations the mean level of the diazepam-induced depolarization was 0.25 ± 0.03 mV. As illustrated in Figure 4a the diazepam-induced depolarization was reversed in the presence of flumazenil (1 μ M) ($n = 6$) or bicuculline (10 μ M) ($n = 2$). Dorsal root fibres of the immature rat are very sensitive to depolarization by GABA_A receptor agonists (Allan *et al.*, 1980). However, in the present study diazepam failed to produce measurable depolarization of dorsal roots which had been excised from the spinal cord and to which the application of GABA (2 μ M) for 2 min produced depolarization of at least 0.1 mV.

In a series of five preparations from the present study the mean peak amplitude and integrated area of the DR-DRP were increased to levels of $112 \pm 2\%$ and $123 \pm 3\%$, respectively, in the presence of diazepam (1 μ M). In order to assess the effect of diazepam on the DR-DRP, the peak amplitudes and integrals recorded in the presence of diazepam were measured relative to the control baseline potential before diazepam was introduced. This eliminated the occluding effect of the diazepam-induced depolarization of the dorsal root on the synaptic potentials. In all the preparations the diazepam-induced potentiation of the DR-DRP was reversed to the control levels (100%) in the presence of flumazenil (1 μ M) (Figure 4b). Because of the small extent, slow induction and even slower reversal on washout of these diazepam-induced effects the application of flumazenil was particularly important in demonstrating that these effects were significant. This is illustrated in Figure 4b where the reduction in DR-DRP produced by the application of flumazenil is more apparent than the increase which followed the introduction of diazepam. Under similar conditions in this laboratory, potentiation of the DR-DRP to at least 120% was produced by pentobarbitone (50 μ M) (Wesselman *et al.*, 1991).

It was shown previously (Siarey *et al.*, 1992b) that diazepam is a potent and selective depressant of the NMDA receptor-mediated component of the long duration segmental excitatory reflex as recorded from a ventral root (DR-LVRP). It might be expected that this depressant effect of diazepam would be explained by potentiation of primary afferent depolarization (Polc *et al.*, 1974). Tizanidine has a selective depressant effect on the segmental reflex which is similar to that of diazepam (Siarey *et al.*, 1992b). However, as shown above, tizanidine depressed primary afferent depolarization. Therefore it was of interest to investigate the relationship between primary afferent depolarization and the DR-LVRP. In three hemisectioned spinal cord preparations, paired pulses were applied to a dorsal root. The first pulse of each pair was at an intensity sufficient to evoke a DR-DRP but insufficient to evoke a DR-LVRP from the corresponding ventral root. The second pulse of each pair was at higher intensity so that a DR-LVRP was evoked. The second pulse was timed to occur near the peak of the DR-DRP caused by the first pulse. The relative integrated areas of the DR-DRP caused by each pulse alone and in combination are shown in the first column of Table 1 as are the corresponding values for the DR-LVRP. It can be seen (as illustrated in Figure 5) that, despite the increased primary afferent depolarization of

Figure 4 (a) shows the depolarizing action of diazepam ($1\ \mu\text{M}$) on the dorsal root baseline potential of two separate preparations. The dashed line is the projection of the control base line recorded in the absence of diazepam. The diazepam-induced shift was reversed by flumazenil ($1\ \mu\text{M}$) applied to the upper preparation and bicuculline ($10\ \mu\text{M}$) applied to the lower preparation as indicated above the traces. Vertical calibration $2.0\ \text{mV}$ in the upper trace, $1.2\ \text{mV}$ in the lower trace. The regular vertical deflections are the DR-DRPs evoked following stimulation of an adjacent dorsal root at $90\ \text{s}$ intervals. (b) Shows the effect of diazepam ($1\ \mu\text{M}$) on the DR-DRP with the time-course of the effect on peak amplitude plotted in (c). Peak amplitudes were measured from the projected baseline d.c. level as depicted in (a). A small diazepam-induced potentiation can be seen which was reversed on introduction of flumazenil ($1\ \mu\text{M}$) as indicated above the plot. Calibration in (b), $2\ \text{mV}$ and $1\ \text{s}$.

Figure 5 Effect of a low intensity conditioning stimulus (S1) on the DR-LVRP evoked by a high intensity stimulus (S2). Both stimuli were applied to the same dorsal root. The lower traces show the DR-DRP evoked by the combination of stimuli used to evoke the DR-LVRP in the trace above. The DR-DRP was recorded from a dorsal root adjacent to the stimulated one. The lefthand traces are with S1 alone. The centre traces are with S2 alone and the righthand traces are with S1 + S2. It can be seen that S1 and S2 produced similar DR-DRPs. However, the increase in primary afferent depolarization caused by S1, in combination with S2 (at an interval of $70\ \text{ms}$), failed to inhibit the DR-LVRP evoked by S2. On the contrary the combination of stimuli produced a big enhancement of the DR-LVRP. Each trace is an average of six evoked potentials. The vertical scale is $0.2\ \text{mV}$ in the upper and $0.27\ \text{mV}$ in the lower traces. The corresponding horizontal scales are $10\ \text{s}$ and $300\ \text{ms}$.

Table 1 Integrated areas of the evoked potentials expressed as a percentage ($100 \times (S1 + S2)/S2$) of the potential evoked by a high intensity stimulus S2

Stimulus	DR-DRP		DR-LVRP Combined
	Separate	Combined	
	181	140	241
	140	90	125
	188	186	253

The first column is the sum of the areas of the two DR-DRPs when the low intensity stimulus (S1) was applied on a separate occasion from S2. The second column is the area of the DR-DRP when S1 and S2 were applied in combination with an interval of 50 to 90 ms between them. It is seen that the integrated area of the long duration DR-VRP (DR-LVRP) evoked by S2 was increased by S1 from 25 to 153% in each of the three preparations represented in each row. Each value is a mean of at least four responses obtained from each tissue at intervals of 90 s. S1 evoked no significant DR-LVRP.

up to 88%, the DR-LVRP was potentiated by as much as 150% by the first pulse in the pair.

Discussion

The primary afferent depolarization evoked following electrical stimulation of sensory nerves has a central latency of 2 to 3 ms in the anaesthetized cat and is considered therefore to be relayed through at least two interneurons (Eccles *et al.*, 1962). The present EC_{50} value for the depressant action of baclofen on the DR-DRP is not significantly different from the value of 237 nM reported for depression of the dorsal root-evoked monosynaptic population spike of motoneurons by baclofen (Siarey *et al.*, 1992b). We reported a value five fold lower for the depression of polysynaptic excitation. Thus the depressant action of baclofen on the DR-DRP appears to be dominated by depression of transmitter release from primary afferent terminals (Edwards *et al.*, 1989). A significantly lower EC_{50} value would have been expected if depression of the release of GABA and/or depression of polysynaptic excitatory pathways were involved. The apparent K_d value derived from the dose ratios produced by the GABA_B antagonist CGP35348 is not significantly different from previously reported values (Seabrook *et al.*,

1990; Siarey *et al.*, 1992b) which is again consistent with depression of the DR-DRP being mediated at the level of the primary afferent synapse.

The actions of the α_2 -adrenoceptor agonists and of diazepam contrast with their depressant actions on the output of the motoneurone (Siarey *et al.*, 1992b). The former agents produced only a small depression and the latter a small potentiation. Our previous study showed that the α_2 -adrenoceptor agonists and diazepam had no significant depressant action at primary afferent synapses. Thus the small effects observed in the present study are likely to have been mediated either at excitatory synapses between interneurons or at the axo-axonic synapses between GABAergic interneurons and primary afferents.

The maximal depression of the polysynaptic output of motoneurons produced by clonidine, tizanidine and diazepam, in the previous study, amounted to approximately 50% (Siarey *et al.*, 1992b). In the present experiments the maximal depressant effect of clonidine and tizanidine on the DR-DRP was approximately 10% and diazepam produced a maximal potentiation of approximately 20%. It is impossible to dissociate the primary afferent depolarizing action of afferent volleys in dorsal roots from their excitatory action. However, the experiments illustrated by Figure 5, show that the excitatory effect of dorsal root volleys on the DR-LVRP dominates any possible inhibitory action that might be associated with the DR-DRP. This is probably to be expected because the DR-LVRP outlasts the DR-DRP by several seconds. Therefore it would seem unlikely that potentiation of presynaptic inhibition (Polc *et al.*, 1974) plays a major role in depression of the DR-LVRP by diazepam (Akagi & Yanagisawa, 1987). Incidentally, Figure 5 illustrates also that the DR-LVRP is not exclusively caused by C fibre primary afferents.

The bicuculline-sensitive depolarization of dorsal roots induced by diazepam is unlikely to have been due to a direct GABA_A mimetic action as occurs with pentobarbitone (Evans, 1979) because the effect was absent from excised dorsal roots which are highly sensitive to GABA. The most likely cause of this effect is potentiation of the action of GABA which is released tonically from GABAergic terminals.

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