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Depression of A and C fibre-evoked segmental reflexes by morphine and clonidine in the *in vitro* spinal cord of the neonatal rat

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1 Population synaptic responses of motoneurones were recorded from a ventral root following electrical stimulation of the corresponding lumbar dorsal root in neonatal rat hemisected spinal cord preparations *in vitro*. Two levels of electrical stimulation were used to elicit dorsal root compound action potentials that contained either an A fibre component alone or both A and C fibre components. The effects of centrally acting analgesics and an N-methyl-D-aspartate (NMDA) receptor antagonist were tested on synaptic responses produced by these two levels of stimulation.

2 At stimulus intensities below four times threshold (T) there was no C fibre component in the dorsal root compound action potential. Responses to a single pulse at 3T (the low intensity excitatory postsynaptic potential (e.p.s.p.)), a train of five pulses at 2T (the train e.p.s.p.) and a single supramaximal pulse (the high intensity e.p.s.p.) were used to compare the depressant actions of morphine, clonidine and the competitive NMDA antagonist CGP40116 (D-(E)-2-amino-4-methyl-5-phosphono-pentenoic acid). The train e.p.s.p. (mean half-time to decay 5 ± 0.6 s, $n=6$) had a similar profile to the high intensity e.p.s.p. (mean half-time to decay 6.8 ± 0.7, $n=8$).

3 The monosynaptic compound action potential of motoneurones (MSR) was resistant to all three drugs irrespective of the intensity of dorsal root stimulation. The low intensity e.p.s.p., the train e.p.s.p. and the high intensity e.p.s.p. were depressed by all three drugs. The EC_{50} values for depression by morphine were 79 ± 1 nM ($n=8$) for the high intensity e.p.s.p. and 99 ± 1 nM ($n=4$) for the low intensity e.p.s.p. The corresponding values for clonidine were 25 ± 1 nM ($n=8$) and 9 ± 1 nM ($n=4$) and those for CGP40116 were 860 ± 1.3 nM ($n=4$) and 76 ± 1.1 nM ($n=4$).

4 The depressant profile of the NMDA antagonist, having the least depressant activity on the C fibre-mediated response, was different from that of the two analgesics. CGP40116 (3 μM) depressed the high intensity e.p.s.p. to $62 \pm 8\%$, the low intensity e.p.s.p. to $22 \pm 4\%$ and the train e.p.s.p. to $16 \pm 2\%$ of control values.

5 The depressant actions of morphine were fully reversed by naloxone (1 μM) and those of clonidine were fully reversed by atipamezole (1 μM).

6 These results show that, in contrast to previous findings, activation of primary afferent C fibres in dorsal roots is not required for generation of morphine- or clonidine-sensitive synaptic responses in ventral roots of this *in vitro* preparation.

Keywords: Spinal cord; ventral root potentials; centrally acting analgesics; clonidine; morphine; CGP40116

Introduction

The spinal cord of the neonatal rat is a robust *in vitro* preparation (Otsuka & Konishi, 1974) which is convenient for the analysis of the spinal actions of known concentrations of centrally acting analgesic drugs. In this preparation supramaximal electrical stimulation of a lumbar dorsal root and recording from the corresponding ipsilateral ventral root gives rise to reflexes of a slow time-course lasting tens of seconds. These long lasting reflexes are thought to reflect a nociceptive reflex for several reasons; the threshold of activation corresponds to that of C fibre primary afferents (Akagi *et al*., 1985); they can be depressed by opioids (Yanagisawa *et al*., 1985; Nussbaumer *et al*., 1989) and α_2-adrenoceptor agonists (Kendig *et al*., 1991) and a similar response can be evoked by peripheral noxious stimulation (Yanagisawa *et al*., 1985). The primary afferent A fibre-mediated monosynaptic reflex was shown to be unaffected by application of morphine (Yanagisawa *et al*., 1985) and clonidine (Kendig *et al*., 1991). Low threshold primary afferent A fibres were therefore considered not to be involved in the generation of analgesic-sensitive reflexes in this *in vitro* preparation (Akagi *et al*., 1985; Kendig *et al*., 1991).

Contrasting with these *in vitro* studies are *in vivo* studies in cats (McLane & Martin, 1967; Krivoy *et al*., 1973), rabbits (Clarke *et al*., 1988) and rats (Dickenson & Sullivan, 1986; Sullivan *et al*., 1987) that show depression of spinal reflexes elicited from low threshold A fibres by morphine and clonidine. The present experiments were designed to investigate this apparent discrepancy between *in vitro* and *in vivo* effects of morphine and clonidine on spinal segmental transmission. Therefore responses of *in vitro* hemisected spinal cord preparations to either low (no activation of C fibres) or supramaximal (activation of C fibres) stimulation of a dorsal root were tested for sensitivity to morphine or clonidine.

Previous experiments have shown that clonidine selectively depressed the NMDA receptor-mediated component of synaptic responses (Siarey *et al*., 1992). Therefore, in the present experiments, the effects of morphine and clonidine have been compared with those of the competitive N-methyl-D-aspartate (NMDA) receptor antagonist, CGP40116 (D-(E)-2-amino-4-methyl-5-phosphono-pentenoic acid) (Fagg *et al*., 1990). The results show that the long latency components of low threshold primary afferent A fibre-mediated reflexes of *in vitro* spinal preparations are sensitive to both analgesics.

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Methods

Spinal cords were removed with attached lumbar spinal dorsal roots from neonatal Sprague-Dawley rats (aged between 3 and 6 days, 9–14 g in weight). The spinal cord was hemisected and placed in a chamber with the L4 or L5 dorsal root in contact with the stimulating electrode and the corresponding ventral root in contact with the recording electrode. Electrical isolation of the electrodes was achieved with grease gaps. The hemisected spinal cords were superfused with artificial cerebral spinal fluid (ACSF) at a rate of 2 ml min^{-1} and were maintained at a temperature of 25–27°C. The ACSF consisted of (mM): NaCl 118, $NaHCO_3$ 24, glucose 12, $CaCl_2$ 1.5, KCl 3, $MgCl_2$ 1.25 and was gassed with O_2/CO_2 (95%/5%) pH 7.4. The preparation was allowed to equilibrate for at least 60 min before recordings were made. Drugs were applied to the preparation in known concentrations by adding them to the superperfusate.

Electrical stimulation of the dorsal root of the neonatal rat hemisected spinal cord evokes a population ventral root potential (DR-VRP) in the corresponding ipsilateral ventral root. There are three distinct components of the DR-VRP which can be measured over progressively longer time sweeps. Low intensity stimulation activates the A primary afferent fibre-mediated components of the DR-VRP; an initial monosynaptic compound action potential (MSR, Figure 2a) (Otsuka & Konishi, 1974) superimposed on a population excitatory postsynaptic potential (e.p.s.p.) (the low intensity e.p.s.p., Figure 2b) which lasts up to two seconds. High intensity stimulation evokes a polysynaptic presumed C fibre-mediated response which has a duration of up to forty seconds, the high intensity e.p.s.p. (Figure 2c, Akagi *et al*., 1985; Garcia-Arraras *et al*., 1986).

The A fibre-mediated MSR and the low intensity e.p.s.p. were evoked by a single pulse 0.5 ms in duration at three times threshold, where threshold was the intensity at which a discernable response first appeared in the ventral root. The high intensity e.p.s.p. was evoked by a single pulse 0.5 ms in duration at sixteen times threshold. An additional long duration response similar to that elicited by a single supramaximal pulse could also be evoked following stimulation of the dorsal root with a train of five stimuli (20 Hz) at two times threshold (Figure 2d). This ventral root potential was therefore called the train e.p.s.p. In order to ensure that following low intensity stimulation there was no C fibre input in the ventral root potentials, the activation thresholds for A and C primary afferent fibres were measured from the compound action potentials (CAP) in the dorsal root. These were evoked by stimulation of the sciatic nerve with a single pulse of 0.5 ms duration.

The action of drugs on each of these population responses was assessed by measuring the peak amplitude of the MSR, the areas under the curve of the low intensity e.p.s.p., the high intensity e.p.s.p. and the train e.p.s.p. and the peak amplitude of the A and C waves of the dorsal root CAP. Responses were amplified, monitored and analysed by use of commercially available software (Scope v3.3, MacLab). The results are expressed as mean ± s.e.mean. The EC_{50} values were calculated as the concentration required to produce a 50% depression of the maximum effect of the drug. Some of the data have previously been presented in an abstract form (Evans, 1995a).

Drugs and chemicals

Naloxone hydrochloride was obtained from Sigma, atipamezole hydrochloride from SmithKline Beecham, clonidine hydrochloride from Boehringer Ingelheim, morphine hydrochloride was obtained from Evans Medical Ltd. CGP40116 was synthesized in the Pharmaceuticals Division of CIBA-GEIGY Ltd, Basel, Switzerland.

Results

Dorsal root CAP

Figure 1 shows typical dorsal root CAPs evoked by electrical stimulation of the sciatic nerve. Electrical stimulation at two and four times threshold evoked only an A fibre-mediated wave ($n=4$). A stimulus intensity of eight times threshold was required in order to evoke a near maximal C wave ($n=4$), as identified by its sensitivity to capsaicin (Figure 1b). Capsaicin (2 μM) abolished the C wave, but had no effect on the amplitude of the A wave ($n=4$). Sixteen times threshold was chosen as the intensity to evoke the high intensity e.p.s.p., whereas low intensity responses were evoked at stimulus intensities of three times threshold or less so that they would contain no C fibre-mediated components.

At stimulus intensities insufficient to activate C fibres the DR-VRP consisted of the MSR superimposed on the low intensity population e.p.s.p. (Figure 2a, b). At maximal intensity (16T) a long duration population e.p.s.p. was evoked, the high intensity e.p.s.p. (Figure 2c). In the present experiments it was discovered that a brief train of pulses, at an intensity insufficient

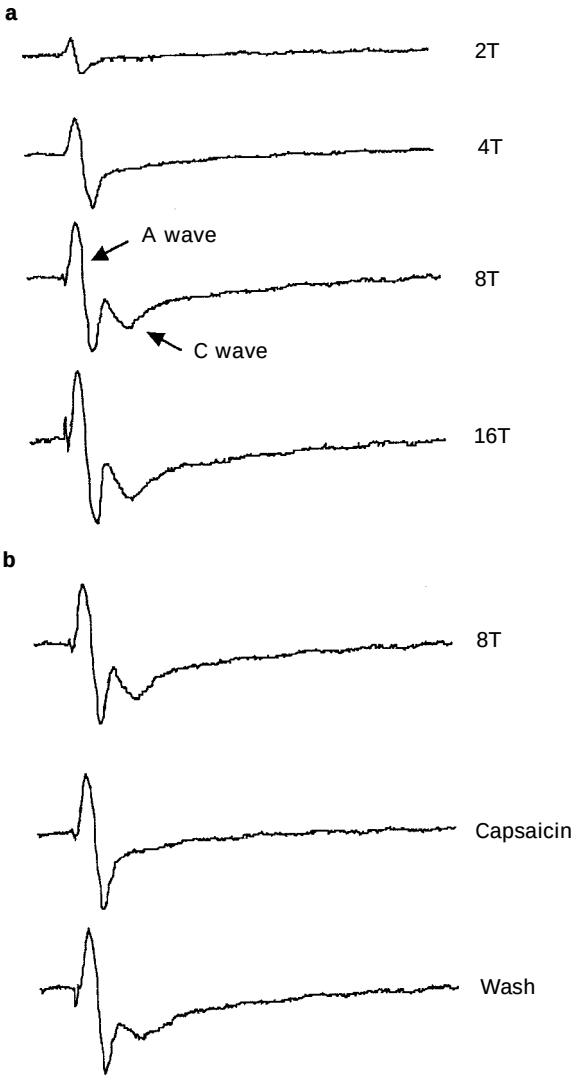


Figure 1 Effect of increasing intensities of stimulation on the dorsal root compound action potentials (CAP). (a) Only at stimulation intensities at or above eight times threshold (T) is a C wave apparent. This was depressed reversibly by capsaicin (2 μM, b) applied for 2 min. Recovery is shown in the lowest trace 20 min following return to capsaicin-free medium.

for activation of C fibres, elicited a long duration population e.p.s.p., the train e.p.s.p. (mean half time to decay 5 ± 0.6 s, $n=6$, Figure 2d), having some similarity in profile to the high intensity e.p.s.p. (mean half time to decay 6.8 ± 0.7, $n=8$).

Effects of morphine and clonidine

As previously shown (Yanagisawa *et al*., 1985; Kendig *et al*., 1991) the MSR was insensitive to morphine (Figures 2a and 3a) and clonidine (Figure 4a), but the low intensity e.p.s.p. was depressed by both drugs in a concentration-dependent manner (Figures 2b, 3 and 4). The mean EC_{50} values for this depression were 99 ± 1 nM for morphine ($n=4$, Figure 3b) and 9 ± 1 nM for clonidine ($n=4$, Figure 4b). The long duration C fibre-mediated high intensity e.p.s.p. was also depressed by morphine (Figures 2c and 3a) and clonidine (Figure 4). The mean EC_{50} values for the depression of this component were 79 ± 1 nM for morphine ($n=8$, Figure 3b) and 25 ± 1 nM ($n=8$, Figure 4b). The depressant actions of morphine reached steady state within 10 min of application (Figure 3a) and those of clonidine within 25 min (Figure 4a). There was no significant difference between the EC_{50} values for depression of the high intensity e.p.s.p. and the low intensity e.p.s.p. by either morphine ($P>0.05$, Mann-Whitney non-parametric test) or clonidine ($P>0.05$, Mann-Whitney non-parametric test). However, the maximum depressant actions of morphine and clonidine on the low intensity e.p.s.p. were at least 20% less than on the high intensity e.p.s.p. (see below).

The train e.p.s.p. showed a similar sensitivity to the depressant actions of morphine and clonidine as did the low and high intensity e.p.s.ps. Morphine (1 μM) depressed the area under the curve of the low intensity e.p.s.p. to $45 \pm 4\%$ ($n=4$,

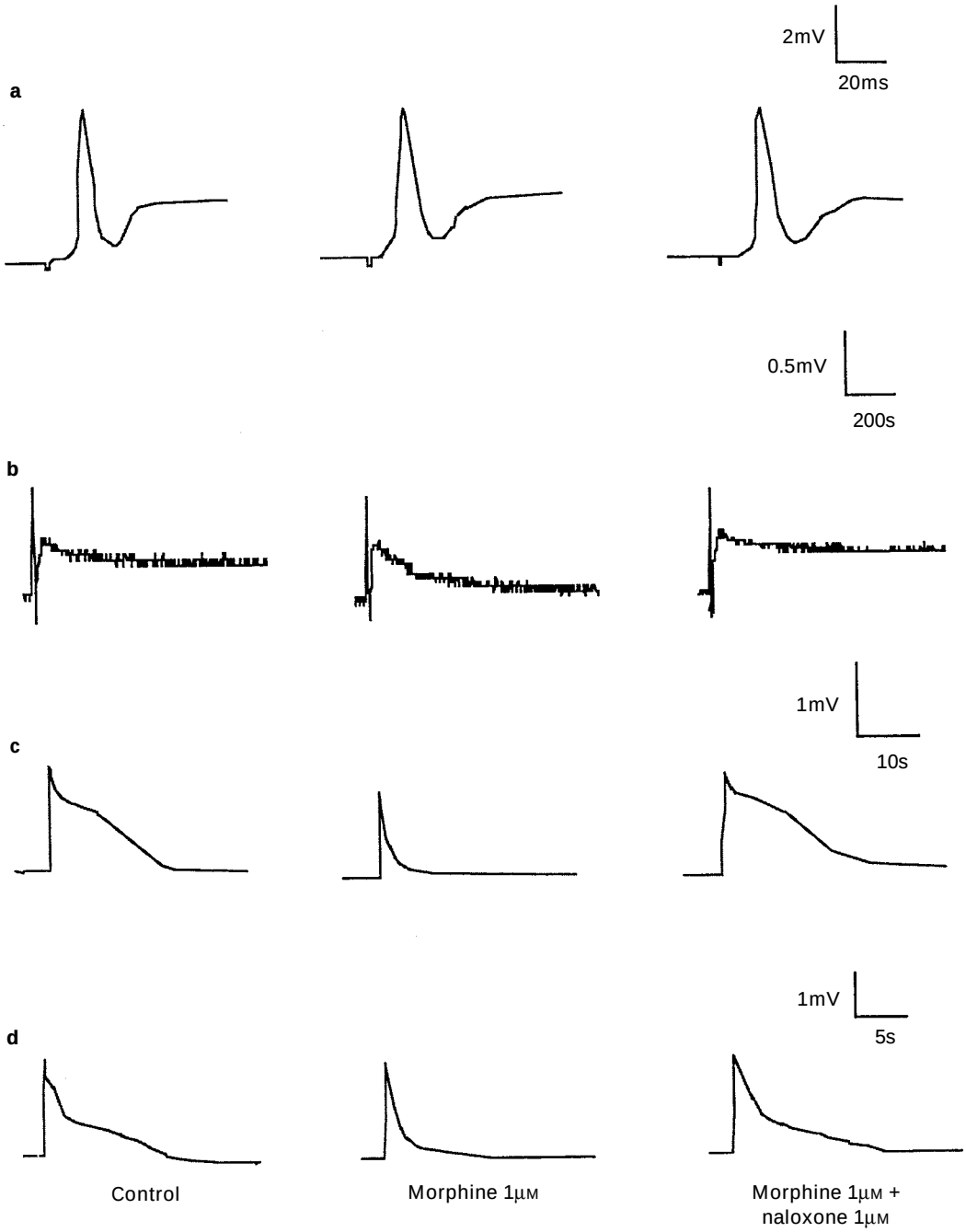


Figure 2 Effects of morphine on the population ventral root potential (DR-VRP). (a) Morphine (1 μM) had no effect on the amplitude of the monosynaptic compound action potential (MSR), but depressed the low intensity e.p.s.p. (b), the high intensity e.p.s.p. (c) and the train e.p.s.p. (d). Each of these effects was reversed by naloxone (1 μM, right panels).

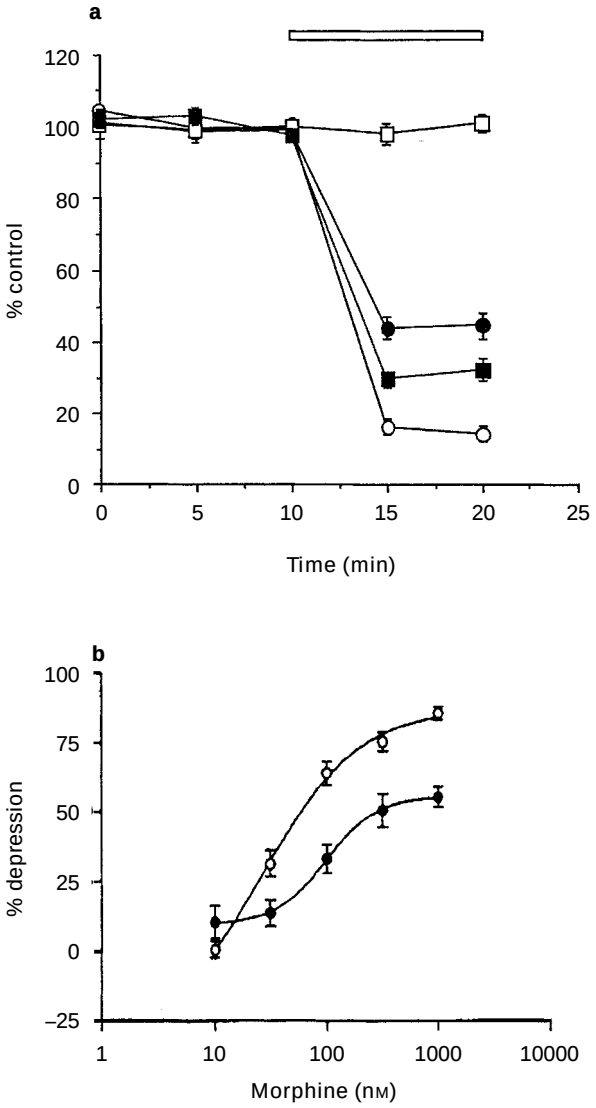


Figure 3 Effects of morphine on the population ventral root potentials (DR-VRP). (a) Time course showing the depressant action of morphine on the high threshold e.p.s.p. (○, $n=8$), the low threshold e.p.s.p. (●, $n=4$), the train e.p.s.p. (■, $n=5$) and the lack of effect on the monosynaptic compound action potential (MSR) (□, $n=8$). The horizontal bar indicates the application of 1 μM morphine. (b) Morphine depressed the high intensity e.p.s.p. (○) with an EC_{50} of 79 nM ($n=8$) and the low intensity e.p.s.p. (●) with an EC_{50} of 99 nM ($n=4$). Vertical lines show s.e.mean.

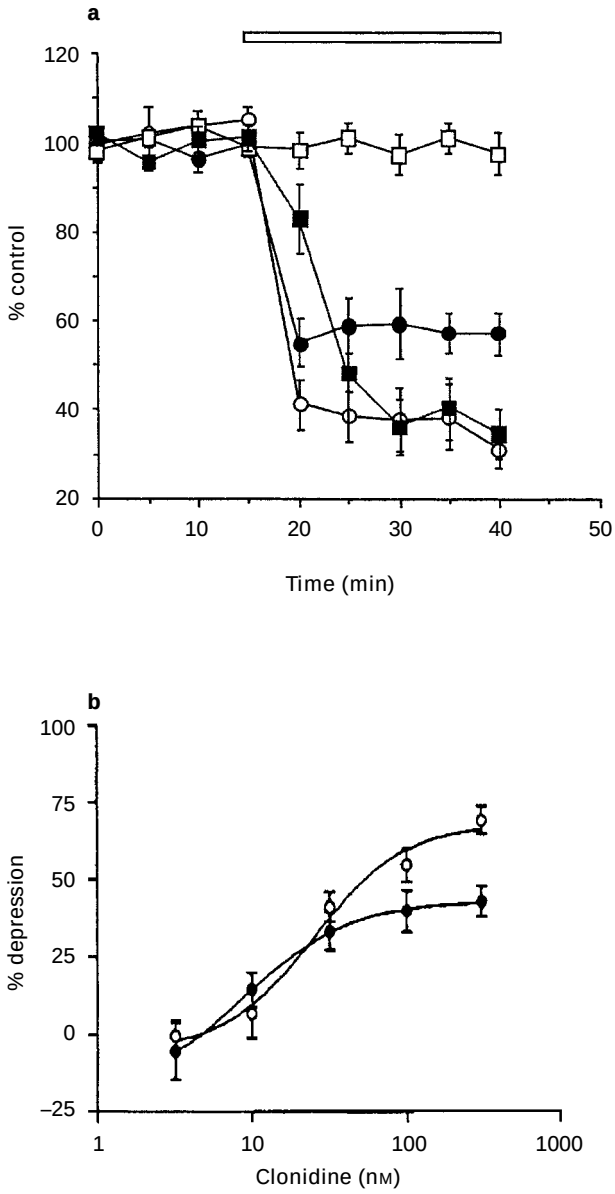


Figure 4 Effects of clonidine on the population ventral root potentials (DR-VRP). Time course showing the depressant action of clonidine on the high threshold e.p.s.p. (○, $n=8$), the low threshold e.p.s.p. (●, $n=4$), the train e.p.s.p. (■, $n=5$) and the lack of effect on the monosynaptic compound action potential (MSR) (□, $n=8$). The horizontal bar indicates the application of 300 nM clonidine. (b) Clonidine depressed the high threshold e.p.s.p. (○) with an EC_{50} of 25 nM ($n=8$) and the low threshold e.p.s.p. (●) with an EC_{50} value of 9 nM ($n=4$). Vertical lines show s.e.mean.

Figure 2b and 3a) that of the high intensity e.p.s.p. to $14\pm2\%$ ($n=8$, Figures 2c and 3a) and that of the train e.p.s.p. to $30\pm2\%$ ($n=5$, Figures 2d and 3a) of the control values. In each case the depressant actions of morphine were reversed by naloxone (1 μM, Figure 2, righthand panels). Clonidine (300 nM) depressed the low intensity e.p.s.p. to $57\pm5\%$ ($n=4$), the high intensity e.p.s.p. to $31\pm5\%$ ($n=8$) and the train e.p.s.p. to $35\pm6\%$ ($n=5$) of the control values (Figure 4a). Each of these depressant actions of clonidine was reversed by atipamezole (1 μM).

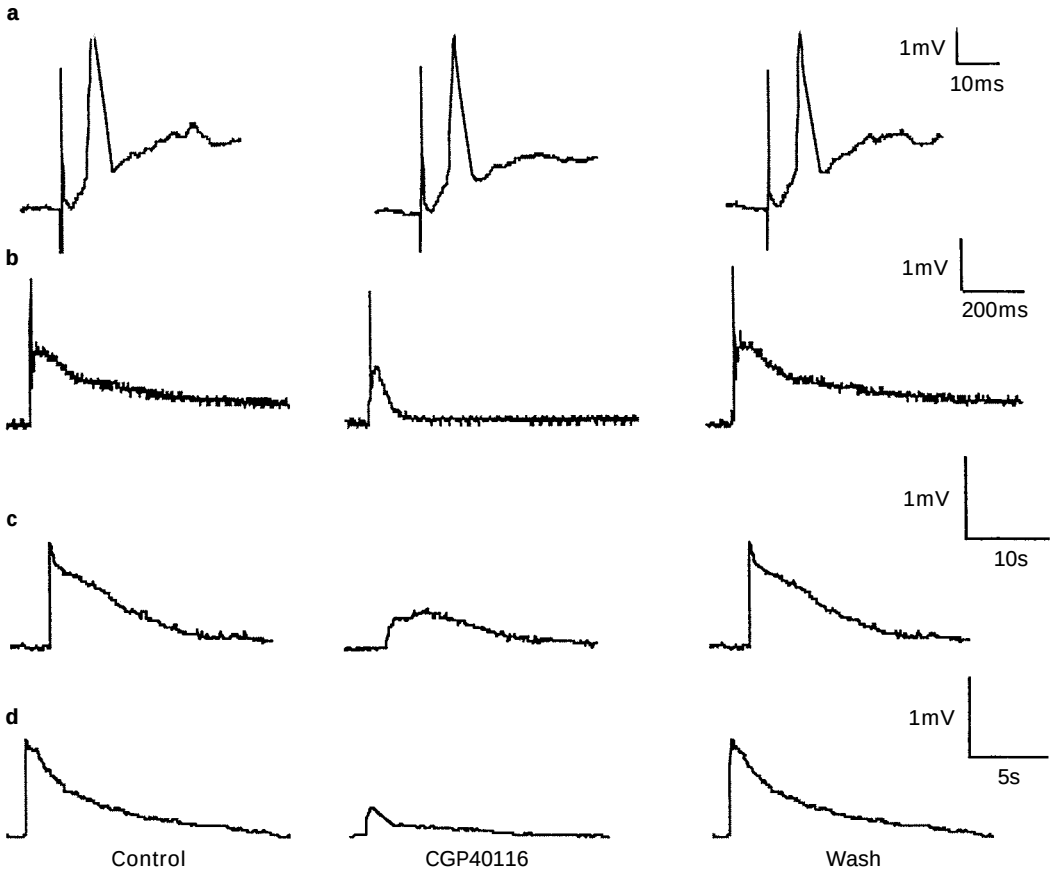
Effects of CGP40116

Siarey *et al*. (1992) showed that the high intensity e.p.s.p., but not the MSR, was depressed by the NMDA antagonist AP5 (2-amino-5-phosphonopentanoate). In the present experiments it was found that apart from the MSR (Figure 5a) the other synaptic components of the DR-VRP were depressed by the competitive NMDA receptor antagonist CGP40116 (Figure 5b, c and d). However, the two types of low intensity response were more sensitive than the high intensity e.p.s.p. to CGP40116. CGP40116 (3 μM) depressed the low intensity e.p.s.p. (Figure 5b) to $22\pm4\%$, EC_{50} 76 ± 1.1 nM ($n=4$), the high intensity e.p.s.p. (Figure 5c) to $62\pm8\%$, EC_{50} 860 ± 1.3 nM ($n=4$) and the train e.p.s.p. (Figure 5d) to $16\pm2\%$ ($n=4$) of the control values. Each of these depressant actions was reversed on washout of the drug. The difference between the EC_{50} values for depression of the low intensity e.p.s.p. and the high intensity e.p.s.p. was statistically significant ($P<0.05$, Mann-Whitney non-parametric test). It can be seen from Figure 5e that, over the range of concentrations tested, there was a clear maximum depressant effect of CGP40116 on the low intensity e.p.s.p. The high intensity

e.p.s.p. was depressed progressively by increasing concentrations of CGP40116 and a maximum depressant effect was not attained at the highest concentration tested. The EC_{50} of 860 nM for the depression of the high intensity e.p.s.p. by CGP40116 is therefore an underestimate.

Discussion

The present experiments show that centrally acting analgesic-sensitive segmental reflexes can be evoked in the *in vitro* neonatal rat spinal cord preparation in the absence of primary



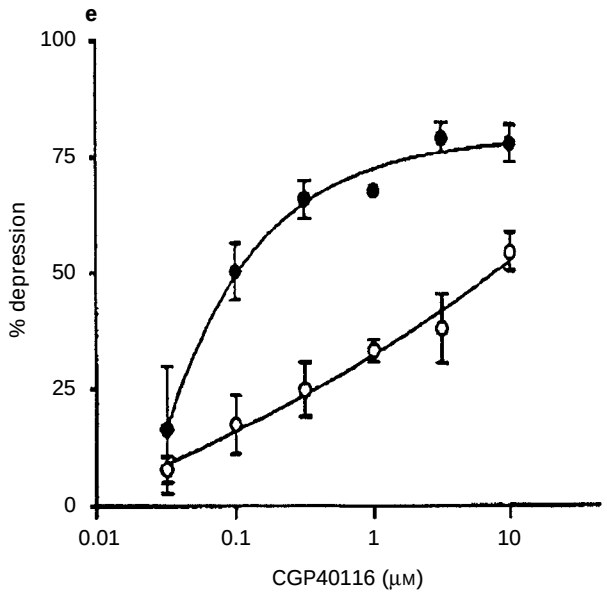


Figure 5 Effects of CGP40116 on the population ventral root potentials (DR-VRP). CGP40116 (3 μM) had no significant effect on the monosynaptic compound action potential (MSR) (a) but depressed the low intensity e.p.s.p. (b), the high intensity e.p.s.p. (c) and the train e.p.s.p. (d). (e) Concentration-effect plot showing that CGP40116 depressed the high intensity e.p.s.p. (○) with an EC_{50} of 860 nM ($n=4$) and the low intensity e.p.s.p. (●) with an EC_{50} of 76 nM ($n=4$).

afferent C fibre activation. This is to be expected from *in vivo* studies (McLane & Martin, 1967; Krivoy *et al*., 1973; Dickenson & Sullivan, 1986; Sullivan *et al*., 1987; Clarke *et al*., 1988).

Involvement of A and C fibres

Previous investigations on the *in vitro* preparation have not employed stimulus intensities low enough to activate exclusively A fibres (Akagi *et al*., 1985; Kendig *et al*., 1991). Thus, apart from the monosynaptic reflex, all components of reflexes would have been a mixture of responses from A and C afferent fibres, so in previous studies it was not possible to separate the A and C fibre mediated components of evoked reflexes.

Sensory A fibres comprise α, β and δ components. Myelination is incomplete in neonatal rats; myelination of A$\alpha\beta$ afferent fibres occurs at an earlier stage than that of Aδ fibres (Fitzgerald, 1985) and conduction speeds in A fibres are approximately ten fold lower than in the adult (Agrawal & Evans, 1986). In the present experiments two waves were observed in the dorsal root compound action potential (Figure 1). The first of these waves corresponds to the A$\alpha\beta$ component and the second capsaicin-sensitive wave to the Aδ and C fibre component (Fitzgerald, 1985; 1987). Thus the low intensity stimuli used in the present study would have activated only A$\alpha\beta$ afferent fibres. Therefore in the present experiments there was no C or Aδ fibre input to either the MSR, the low intensity e.p.s.p. or the train e.p.s.p.

Duration of synaptic response

In earlier studies on the *in vitro* neonatal rat spinal cord preparation it has been suggested that the long duration reflexes of these preparations are a consequence of primary afferent C fibre activation (Akagi *et al*., 1985). The present experiments show that a brief train of low intensity impulses can evoke a synaptic response which is similar in duration to one evoked by a single supramaximal shock. This is in agreement with Thompson *et al*. (1994) who found that A fibre-mediated long duration responses could be elicited after inflammatory conditioning. It should therefore not be assumed that long lasting spinal reflexes in this *in vitro* preparation are necessarily a consequence of C fibre activation. The repetitive firing of A fibres in response to a single high intensity shock (Gasser, 1950) may be important in this respect. It is not clear whether the long duration responses in the present preparation are a feature of immaturity or if similar events can occur in a fully mature spinal cord. Intervals of at least forty seconds would be required between stimuli in order to see such responses. However, Price *et al*. (1971) showed long lasting discharges of dorsal horn neurones in decerebrate spinal cat preparations that would be consistent with the possibility of such long duration responses.

Effects of morphine and clonidine

Morphine and clonidine depressed the high intensity e.p.s.p., the low intensity e.p.s.p. and the train e.p.s.p.; these effects were reversed by naloxone (1 μM) and atipamezole (1 μM), respectively, indicating an action mediated through opioid receptors and α_2-adrenoceptors. These findings differ from previous data which have implied that in the *in vitro* spinal cord preparation these centrally acting analgesics depress only the C fibre-mediated high intensity e.p.s.p. (Yanagisawa *et al*., 1985; Kendig *et al*., 1991).

The concentration-effect curves for clonidine and morphine (Figure 3b) show that the maximal depression of the high intensity e.p.s.p. is greater than the maximal depression of the low intensity e.p.s.p., suggesting that the A fibre-mediated low intensity e.p.s.p. is less susceptible to depression by opioids and α_2-adrenoceptor agonists than is the C fibre-mediated high intensity e.p.s.p. The low intensity e.p.s.p. has a larger non-NMDA receptor-mediated component than the high intensity e.p.s.p. as indicated by the plateau of resistance of the low intensity e.p.s.p. to CGP40116 shown in Figure 5e. Therefore the relative resistance of the low intensity e.p.s.p. to clonidine and morphine would follow from the finding of Siarey *et al*. (1992) that non-NMDA receptor-mediated components of transmission were resistant to clonidine. The train e.p.s.p. was also depressed by morphine and clonidine to a level (30%) intermediate between the depressant effects on the low and high intensity e.p.s.ps. This is also to be expected from the recruitment of NMDA receptor-mediated synaptic activity which occurs with paired stimuli (Evans, 1995b).

Effects of CGP40116

The NMDA antagonist CGP40116 also depressed the A fibre-mediated low intensity e.p.s.p. as well as the C fibre-mediated high intensity e.p.s.p. This suggests that, regardless of the primary afferent fibre type, these responses appear to be relayed to motoneurones via interneurones in excitatory pathways dominated by NMDA receptors. The present finding that the EC_{50} values for depression of the low intensity e.p.s.p. and the high intensity e.p.s.p. by morphine and clonidine were not significantly different shows that such interneurones are presumably not exclusive to C fibre input and are subject to modulatory control by opioid receptors and α_2-adrenoceptors. However, the EC_{50} values for depression of the low intensity e.p.s.p. and the high intensity e.p.s.p. by CGP40116 were significantly different, with the high intensity e.p.s.p. being apparently more resistant to depression than the low intensity e.p.s.p. (Figure 5e). This could be due to the high intensity e.p.s.p. having a significant neuropeptide component compared to the low intensity e.p.s.p. However, the concentration-effect plot (Figure 5e) for the high intensity e.p.s.p. showed no tendency toward a plateau of resistance, which would have indicated a non-NMDA receptor-mediated component. The train e.p.s.p. was also very sensitive to CGP40116. This is consistent with the previous findings of Brugger *et al*. (1990) that long duration responses in this preparation are mediated largely through NMDA receptors. CGP40116 is a competitive antagonist. Thus it is to be expected that the responses to supramaximal stimulation of dorsal roots, which presumably causes a maximal concentration of synaptic glutamate, should be more resistant to a competitive antagonist than is a low intensity response.

The relative potencies for depression of the low intensity e.p.s.p. and the high intensity e.p.s.p. by clonidine and morphine correlate well with the effects of these analgesics *in vivo*. The potency ratio between clonidine and morphine from *in vivo* analgesic tests with electrical stimulation of adult rat tails has been found to be 3:1 (Skingle *et al*., 1982), compared to the ratio of 3.2:1 for the depressant action on the high intensity e.p.s.p. in the neonatal rat hemisected spinal cord preparation. This appears to validate the *in vitro* hemisected spinal cord preparation as a predictor of *in vivo* analgesic activity. The low intensity e.p.s.p. appeared less susceptible to clonidine with a ratio of 11:1 for the potencies of clonidine: morphine.

In summary, the present findings show, as demonstrated by the depression of the low threshold e.p.s.p. by clonidine and morphine, that it is not necessary to activate C fibre primary afferents in order to see a depressant action of centrally acting analgesics in the *in vitro* spinal cord preparation. These results also show that activation of A$\alpha\beta$ fibres alone can elicit long duration synaptic responses, similar to those considered to be evoked by C fibre primary afferents, and that these responses, which are mediated largely by NMDA receptors, are also sensitive to morphine and clonidine. These findings are consistent with observations of the actions of opiates and α_2-adrenoceptor agonists on spinal reflexes *in vivo* (McLane & Martin, 1967; Krivoy *et al*., 1973; Dickenson & Sullivan, 1986; Sullivan *et al*., 1987; Clarke *et al*., 1988).

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