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GABAergic modulation of a substance P-mediated reflex of slow time course in the isolated rat spinal cord

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1 The effects of γ-aminobutyric acid (GABA) and other drugs which interact with GABA receptors were studied on a reflex of slow time course in the spinal cord preparation isolated from the neonatal rat.

2 A single shock to a dorsal root (L3–L5) elicited a stereotyped series of reflexes, consisting of fast and slow components, recorded from the contralateral ventral root of the corresponding segment. The slow component, i.e. the contralateral slow ventral root potential (v.r.p.) had a time-to-peak of 2–5 s and lasted 20–30 s.

3 Bath-application of GABA (5–20 μM) or muscimol (0.05–0.5 μM) caused a decrease in the amplitude of the contralateral slow v.r.p. without producing any change in the d.c. potential recorded from the ventral root. The monosynaptic reflex recorded from the ipsilateral ventral root was not changed by the drugs at these concentrations.

4 Diazepam (0.1–1 μM) potentiated the depolarizing response of the dorsal root to GABA and markedly depressed the contralateral slow v.r.p. Neither the d.c. potential of the ventral root nor the dorsal root was changed by diazepam. The monosynaptic reflex was also unaffected by the drug.

5 Bicuculline (1 μM) suppressed the GABA-induced depolarization recorded from the dorsal root whilst it markedly potentiated the contralateral slow v.r.p.

6 Baclofen at concentrations from 0.01 to 0.1 μM reduced the contralateral slow v.r.p. The inhibitory action of baclofen on the contralateral slow v.r.p. was more marked than on the monosynaptic reflex.

7 The depolarization of the ventral root induced by a brief application of substance P (SP) was depressed by muscimol, diazepam and baclofen, whereas the depolarization was potentiated by bicuculline.

8 The present results suggest that an intraspinal GABAergic inhibitory mechanism plays a role in the modulation of certain slow spinal reflexes. They also support the hypothesis that SP released from certain primary afferent fibres is a neurotransmitter involved in the contralateral slow v.r.p.

Introduction

In the isolated spinal cord of the neonatal rat, a single shock to a dorsal root (L3–L5) elicits a stereotyped series of reflexes from the contralateral ventral root of the corresponding segment. These include a fast ventral root potential (v.r.p.) which peaks at a latency of about 70 ms, and a subsequent slow v.r.p., which has a time-to-peak of 2–5 s and lasts 20–30 s (Akagi *et al.*, 1985). Previous reports showed that the slow component, referred to as the contralateral slow v.r.p., but not the fast component, required activation of high-threshold and slow-conducting (therefore presumably small-diameter) primary afferent fibres. Furthermore, the contralateral slow v.r.p. was abolished by *in vitro* or *in vivo* treatment with capsaicin and depressed by a substance P (SP) antagonist, [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP (Yanagisawa *et al.*, 1982; Otsuka *et al.*, 1984; Akagi *et al.*, 1985). We therefore suggested that SP (and other tachykinins such as neurokinin A) released from certain primary afferent fibres, may be involved as a neurotransmitter in the contralateral slow v.r.p.

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γ-Aminobutyric acid (GABA) has a widespread distribution in the central nervous system and is thought to act as a neurotransmitter mediating both pre- and postsynaptic inhibition (Levy, 1977; Fagg & Foster, 1983; Nistri, 1983). In the mammalian spinal cord, high concentrations of GABA or glutamic acid decarboxylase (GAD, a GABA-synthesizing enzyme) are found in the dorsal area of gray matter, especially in the superficial layers (laminae I–III; Miyata & Otsuka, 1975; McLaughlin *et al.*, 1975). Axon terminals containing GAD make axo-axonic or axo-dendritic contact with other neurones in these areas (McLaughlin *et al.*, 1975). Furthermore, the distribution pattern of nerve terminals containing GAD within superficial layers is similar to that of terminals containing SP (Hunt *et al.*, 1981). In view of these morphological and histochemical findings, it seems reasonable to suppose that a GABAergic inhibitory mechanism might operate in these regions of the spinal cord to regulate SP-mediated synaptic transmission.

The present study attempts to examine the effects of GABA and other drugs, which interact with the GABA receptor e.g. muscimol, diazepam, bicuculline and baclofen, on the contralateral slow v.r.p. and on the SP-induced depolarizing response recorded from the ventral root in the rat isolated spinal cord.

Methods

Electrophysiological experiments were carried out on the isolated spinal cord of 1–4 day Wistar rats, as previously described (Otsuka & Yanagisawa, 1980; Akagi *et al.*, 1985). Briefly, animals were anaesthetized with ether and the spinal cord below thoracic level was isolated without hemisection. In some experiments, hemisected spinal cords were also used. The preparation was superfused with artificial cerebrospinal fluid (CSF, for composition of the solution, see Akagi *et al.*, 1985) at 27°C, at a rate of 4–6 ml min^{-1}. The solution was equilibrated with a mixture of 95% O_2 and 5% CO_2. A dorsal root (L4 or L5) of one side was stimulated with a suction electrode, and the evoked reflex responses were recorded from ipsi- and con-

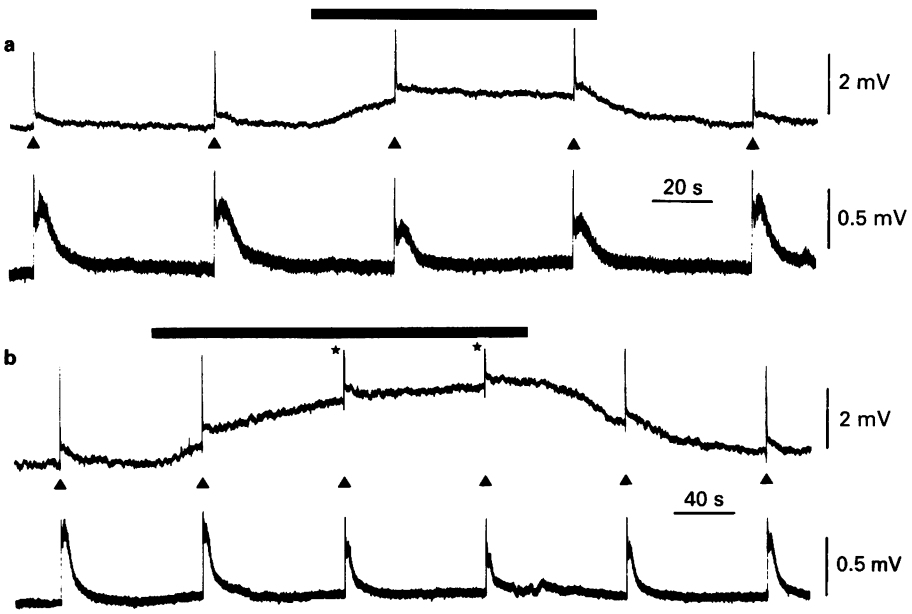

Figure 1 Effects of γ-aminobutyric acid (GABA) and muscimol on dorsal and ventral root potentials of isolated spinal cord. A single stimulus (10 V, 0.5 ms) was given to L4 dorsal root of the right side every 60–90 s (▲) and the evoked potentials were recorded simultaneously from the ipsilateral dorsal root (L3) and the contralateral ventral root (L4) with suction electrodes. In (a) and (b), upper tracings show the dorsal root potentials (d.r.ps) and lower tracings show the ventral root potentials, both of which were displayed on a pen recorder. (a) GABA was dissolved in artifical CSF in a final concentration of 20 μM and applied by superfusion during the period indicated by a black horizontal bar. (b) Muscimol at a concentration of 0.3 μM was applied during the period marked with a black horizontal bar. The peaks of d.r.ps marked with asterisks were truncated by the pen recorder.

tralateral ventral roots of the corresponding segment with suction electrodes. Dorsal root potential (d.r.p.) was recorded from a root (L3 or L4) adjacent to the stimulated dorsal root (Suzue & Jessell, 1980).

The drugs used were: GABA (Sigma), muscimol (Sigma), diazepam (Sumitomo Chemical, Osaka), bicuculline (Sigma), (±)-baclofen (CIBA-Geigy) and substance P (Peptide Institute, Osaka). Diazepam was dissolved in 95% ethanol at a concentration of 1 mM and added to the superfusion medium in final concentrations of 0.1–3 μM. Other drugs were dissolved in artificial CSF. GABA and SP were injected into the superfusion system as brief pulses of 0.3–0.8 s duration using solenoid valves. Other drugs were applied by superfusion for periods of 3–10 min.

Results

Effects of GABA and muscimol on spinal reflexes

Bath-application of GABA at concentrations of 5 to 20 μM produced depolarization of the dorsal root in a concentration-dependent manner, but these concentrations of GABA did not cause any noticeable change in d.c. potential of the ventral root (Figure 1a). The contralateral slow v.r.p. was depressed by GABA (20 μM), its amplitude being reduced to 60–70% of the control response (Figure 1a). The time course of the inhibition of the contralateral slow v.r.p. produced by GABA paralleled that of the depolarizing potential change of the dorsal root induced by GABA (Figure 1a). The contralateral fast v.r.p. was slightly depressed by GABA (20 μM, Figure 1a), but the monosynaptic reflex recorded from the ipsilateral ventral root was not affected by GABA at 5 to 20 μM (data not shown).

Low concentrations of muscimol, a potent GABA agonist, had a similar action on the slow spinal reflex. As shown in Figure 1b, the amplitude of the contralateral slow v.r.p. was reduced to 50% of control size by muscimol (0.3 μM), which markedly depolarized the dorsal root but not the ventral root. The contralateral fast v.r.p. was slightly depressed by 0.3 μM muscimol (reduced to 80% of control; Figure 1b), but the monosynaptic reflex was not affected by the drug at this concentration (Figure 2). Muscimol inhibited the contralateral slow v.r.p. in a concentration-dependent manner between 0.05 to 0.5 μM (Figure 2). When higher concentrations were applied, the ventral root was depolarized, and both the monosynaptic reflex and the contralateral fast v.r.p. were depressed (Figure 2). Thus, the contralateral slow v.r.p. is more susceptible to the actions of GABA and muscimol at low concentrations than other reflexes such as the monosynaptic reflex and the contralateral fast v.r.p.

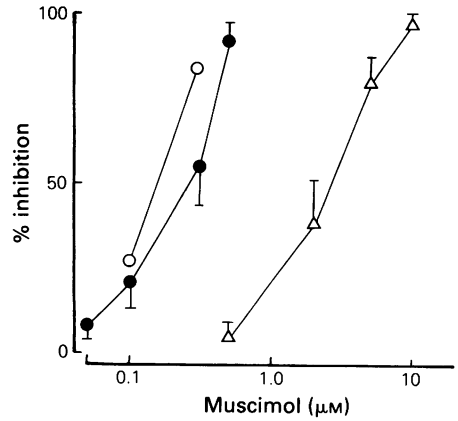

Figure 2 Dose-response relationship for inhibitory actions of muscimol on monosynaptic reflex (Δ), contralateral slow ventral root potential (●) and the depolarizing response of the ventral root to substance P (○). The monosynaptic reflex and the contralateral slow v.r.p. evoked by stimulation of L4 dorsal root (10 V, 0.5 ms) were recorded simultaneously from ipsi- and contralateral L4 ventral roots. The depolarizing response to substance P (1 μM, pulses of 0.7 s duration) was recorded from L4 ventral root of a hemisected spinal cord. Muscimol was applied by superfusion for 4–7 min at various concentrations. The percentage inhibition by muscimol of each response was expressed as: (1 − response amplitude in presence of drug/amplitude of control response) × 100. Each point represents the mean obtained from two (○) or three (●, Δ) different experiments. Vertical lines show s.e.mean.

Effects of diazepam and bicuculline on spinal reflexes and the response of dorsal root to GABA

We examined the effect of diazepam, a benzodiazepine derivative, on the spinal reflexes and the depolarizing response of the dorsal root to GABA. It has been reported that several benzodiazepine agonists potentiate the GABA-induced response in mammalian central and peripheral nervous systems (Squires & Braestrup, 1977; Korobath, 1979; Hulliham *et al.*, 1983). In the isolated spinal cord of the neonatal rat, diazepam at a low concentration (0.5 μM) potentiated the depolarizing response of the dorsal root to GABA (Figure 3a). The same concentration of diazepam caused a marked depression of the contralateral slow v.r.p., but only slightly depressed the contralateral fast v.r.p. (Figure 3b). The monosynaptic reflex was unaffected by the drug (see Figure 4), and neither the d.c. potential of the ventral root nor the dorsal root were altered by 0.1–3 μM diazepam (Figure 3a and b).

Figure 4 shows the dose-response relationship and the time course of action of diazepam on the various types of response. Diazepam (0.1 to 3 μM) enhanced

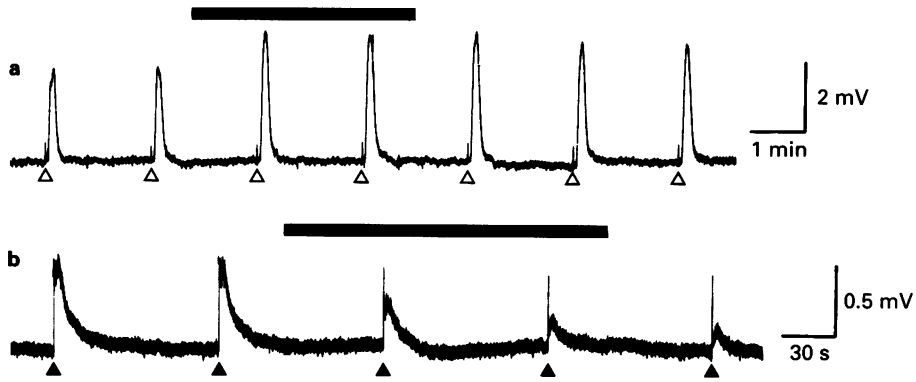

Figure 3 Effects of diazepam on the responses of the dorsal root to γ-aminobutyric acid (GABA) and on the contralateral ventral root potentials (v.r.p.). (a) GABA (1 mM) was applied using pulses of 0.5 s duration every 2 min (△) and responses recorded from L4 dorsal root of a hemisected spinal cord. (b) Contralateral fast and slow v.r.ps were recorded from L4 ventral root by stimulation of L4 dorsal root of opposite side (10 V, 0.5 ms) at (▲) in whole spinal cord. Diazepam (0.5 μM) was applied by superfusion during the periods indicated by black horizontal bars (a and b).

the response to GABA of the dorsal root and inhibited the contralateral slow v.r.p. in a dose-dependent manner, whereas it had little effect on the monosynaptic reflex. The effect of diazepam on both these responses was relatively long lasting (Figure 4b, see also Figure 3). The depression of the contralateral slow v.r.p. persisted for more than 30 min after washing out diazepam. However, the sustained effect of diazepam was immediately abolished after adding 1 μM bicuculline (a GABA antagonist) to the superfusion medium (Figure 4b).

Figure 5 shows the effect of bicuculline on responses to GABA and on the spinal reflexes. While bicuculline at a concentration of 1 μM depressed the depolarizing response of the dorsal root to GABA (Figure 5a), the contralateral slow v.r.p. was progressively enhanced to about twice the size of the control response (Figure 5b). The contralateral fast v.r.p. was also potentiated by bicuculline (150% of the control; Figure 5b) but the monosynaptic reflex was not affected by the drug (data not shown).

Effects of baclofen on spinal reflexes

Figure 6 shows the effect of baclofen on the spinal reflexes. As previously reported, baclofen at concentrations of 1–2 μM markedly reduced the amplitude of the monosynaptic reflex in the isolated spinal cord of newborn rat (Otsuka & Yanagisawa, 1978; 1980). However, baclofen was about 10 times more potent in inhibiting the contralateral slow v.r.p. than on the monosynaptic reflex (IC_{50} value for the contralateral slow v.r.p. was 42 ± 8 nM, and for the monosynaptic reflex, 560 ± 73 nM; means ± s.e.mean of 4 experiments), and the action was not blocked by bicuculline (Figure 6a). The action of baclofen on the contralateral slow v.r.p., as well as on the monosynaptic reflex, was reversible and these reflexes readily recovered to control levels within 5 min after the removal of baclofen.

Effects of drugs on the response of ventral root to substance P

The actions of muscimol, diazepam, bicuculline and baclofen on the depolarizing response of ventral root to SP applied by brief pulses were investigated in the hemisected spinal cord and were compared to those on the contralateral slow v.r.p. Muscimol at concentrations of 0.1 to 0.3 μM depressed the response to SP (Figure 7a, see also Figure 2). The inhibitory action of muscimol was reversible and the response to SP recovered immediately after removal of the drug (Figure 7a). The SP-induced depolarization of the ventral root was also depressed by diazepam at concentrations of 0.2 to 3 μM (Figures 4 and 7b). The reduction of the SP-induced response by diazepam persisted for more than 20 min after removal of the drug, but the inhibitory action of diazepam was immediately abolished after adding 1 μM bicuculline. Furthermore, the amplitude of the response to SP became larger than the control size (Figure 7b). The depolarizing response of the ventral root to SP was also depressed by baclofen at low concentrations (0.05 to 1 μM; data not shown, see Saito *et al.*, 1975; Otsuka & Yanagisawa, 1980).

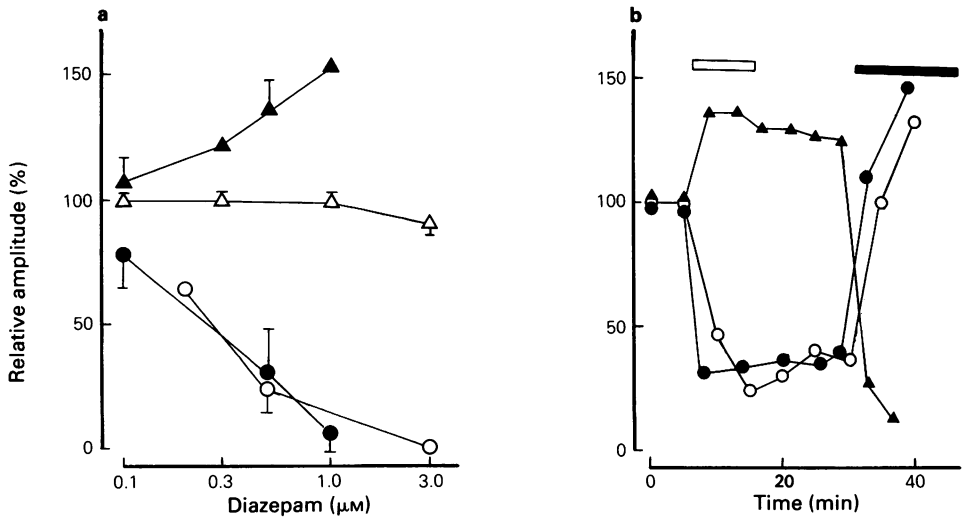

Figure 4 Dose-response relationships and time-courses of action of diazepam on spinal reflexes and responses to substance P (SP) and γ-aminobutyric acid (GABA). (a) The contralateral slow ventral root potential (●) and the response of dorsal root to GABA (▲) were obtained as described in Figure 3. The monosynaptic reflex (△) evoked by the stimulation of L4 dorsal root, and the depolarizing response to SP (1 μM), applied using 0.7 s pulses (○), were recorded from L4 ventral root of hemisected spinal cord. The amplitude of the responses was expressed as a percentage of the control. Each point represents the mean (with s.e.mean) of two or three experiments, plotted against varying concentrations of diazepam. (b) The relative amplitudes of the responses were plotted against time. (●) Contralateral slow v.r.p.; (○) depolarizing response of ventral root (L4) to SP; (▲) depolarizing response of dorsal root (L4) to GABA. Each response was obtained in a different spinal cord preparation. Diazepam (0.5 μM) was added to the superfusion medium during the period indicated by the open horizontal bar. Fifteen min after washing out diazepam, bicuculline (1 μM) was administered by superfusion (closed horizontal bar). The sustained effect of diazepam on the three types of response was observed in 6 other preparations.

Thus, the pharmacological actions of drugs used in the present study on the depolarizing response to SP paralleled those on the contralateral slow v.r.p.

Discussion

In the present study, the effects of GABA and other GABAergic drugs on the contralateral slow v.r.p. were examined in the isolated spinal cord of neonatal rat, and were compared with those on the depolarizing response to SP of the ventral root. The results indicate that GABA, muscimol, diazepam and baclofen depress both the contralateral slow v.r.p. and the SP-induced depolarization, whereas bicuculline potentiates the two types of response.

The release of GABA from certain intraspinal neurones produces a depolarization in primary afferent terminals and this is associated with the reduction of spinal reflexes of fast time course, such as the monosynaptic reflex (Eccles & Willis, 1962; Eccles *et al.*, 1963; Levy, 1977; Nistri, 1983). In the neonatal rat spinal cord, the dorsal root potential evoked by stimulation of an adjacent root (d.r.–d.r.p.) was blocked by bicuculline (Otsuka & Konishi, 1976; Seno & Saito, 1985). Thus, a GABAergic inhibitory mechanism appears to function in the spinal cord, even at an early postnatal stage. In the present study, we found that low concentrations of GABA and muscimol depressed the contralateral slow v.r.p., even though they did not reduce the amplitude of the monosynaptic reflex. This suggests that slow spinal reflexes such as the contralateral slow v.r.p. are also regulated by the GABAergic inhibitory mechanism.

It has been reported that benzodiazepines including diazepam act on central and peripheral neurones by potentiating the action of GABA (Korobath, 1979). This potentiation does not seem to be caused by a direct GABA-agonist action of the benzodiazepines, since benzodiazepines exert no inhibitory action in the

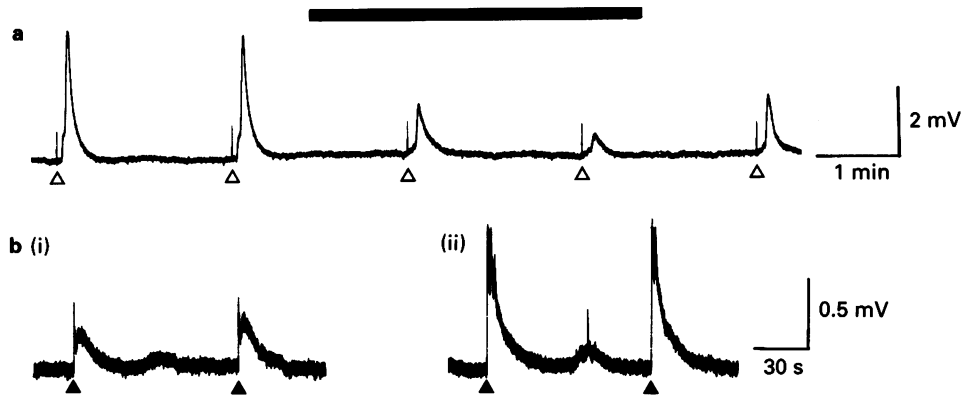

Figure 5 Effects of bicuculline on the response of dorsal root to γ-aminobutyric acid (GABA) and on the contralateral ventral root potentials (v.r.ps). (a) Recording from L4 dorsal root. GABA (1 mM) was applied using pulses of 0.3 s duration with 150 s intervals (△). Bicuculline (1 μM) was applied by superfusion during the period marked with the black horizontal bar. (b) In whole spinal cord, the L4 dorsal root of the right side was stimulated with a single shock (10 V, 0.3 ms) (▲) every 90 s and evoked responses recorded from the contralateral ventral root of the same segment. Bicuculline (1 μM) was applied by superfusion for 10 min. (i) Control responses; (ii) responses in the presence of bicuculline.

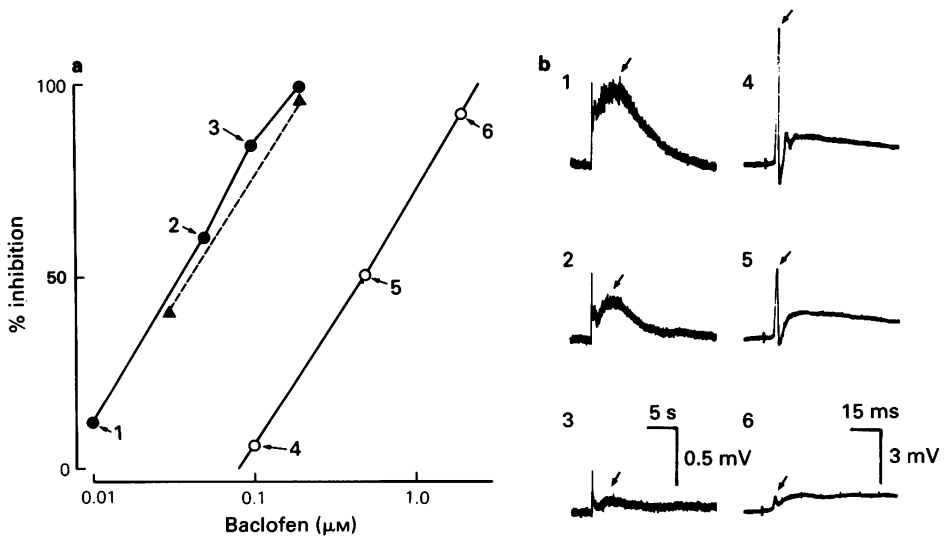

Figure 6 Effects of baclofen on spinal reflexes. A single stimulus (10 V, 0.5 ms) was given to L4 dorsal root every 90 s and reflex responses recorded simultaneously from L4 ventral roots of both sides. Baclofen (0.01–2 μM) was applied for 4–6 min by superfusion. (a) Percentage inhibition of the contralateral slow ventral root potential (v.r.p.) and monosynaptic reflex are expressed as in Figure 2, and plotted against concentrations of baclofen. (● and ▲) Contralateral slow v.r.p. in the absence and presence of bicuculline (1 μM), respectively; (○) monosynaptic reflex. (b) Sample records of contralateral slow v.r.p. (shown by arrows in 1–3) and the monosynaptic reflex (shown by arrows in 4–6). Each response corresponds to the point indicated by arrow in (a). All records were derived from a single preparation.

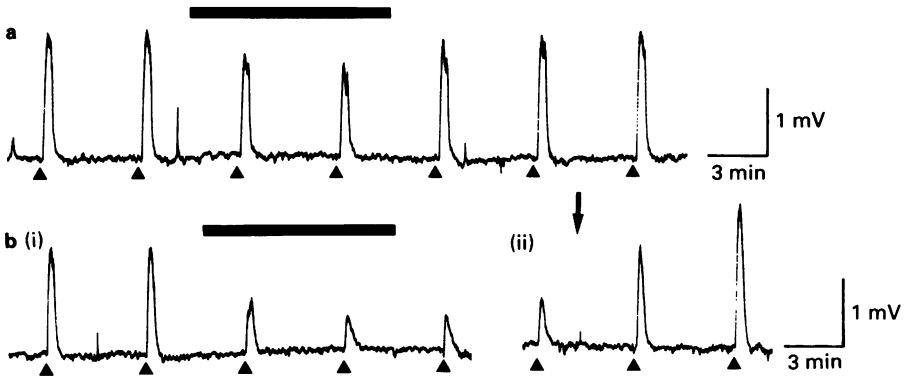

Figure 7 Effects of muscimol, diazepam and bicuculline on the substance P (SP)-induced depolarization of ventral root. Extracellular recording from L4 ventral root of a hemisected spinal cord which was superfused with artificial CSF containing 1.25 mM Ca^{2+} and 2 mM Mg^{2+}. SP (1 μM) was applied using 0.7 s pulses (▲) at 4 min intervals. (a) Muscimol at a concentration of 0.1 μM was applied by superfusion (black horizontal bar). (b(i)) Diazepam at a concentration of 0.3 μM was applied by superfusion (black horizontal bar); (ii) the responses 15 min after washing out diazepam. At the arrow, superfusion with artificial CSF containing 1 μM bicuculline was started. Note that the amplitude of the response induced by SP in the presence of bicuculline is larger than that of the control response in normal medium.

absence of endogenous or exogenous GABA (Korobath, 1979). Therefore, our finding that diazepam depressed the contralateral slow v.r.p. suggests that the GABAergic inhibitory mechanism is activated by stimulation of a dorsal root. Alternatively, it is conceivable that diazepam may cause the suppression of the contralateral slow v.r.p. by a different mechanism from its potentiating effect on GABA action. Cherubini and North (1985) reported that benzodiazepines such as midazolam and diazepam, at low concentrations of 100–300 pM, decrease both amplitude and duration of calcium action potentials in guinea-pig myenteric neurones. However, the action of benzodiazepines on the calcium spike was insensitive to bicuculline (Cherubini & North, 1985), whereas that of diazepam on the contralateral slow v.r.p. was reversed by bicuculline (Figure 4b), suggesting that the latter effect is associated with the GABAergic system. Furthermore, that activation of the GABAergic inhibitory mechanism occurs during the slow spinal reflex is supported by the finding that the contralateral slow v.r.p. was markedly potentiated by bicuculline (Figure 5b). Thus, it is likely that a GABAergic inhibitory system is involved in the neural circuits for the contralateral slow v.r.p. in the spinal cord. Stimulation of a dorsal root may evoke the release of neurotransmitters, including SP, from central terminals to activate GABAergic interneurones, so that released GABA causes pre- and/or postsynaptic inhibition in the neural pathways involved in the contralateral slow v.r.p. Thus, in normal condition, the contralateral slow v.r.p. appears to be kept at a relatively inhibited level by tonic or phasic GABAergic inhibition.

In this connection, Kawasaki & Matsushita (1981; 1982) reported that contraction of quadriceps muscle, elicited by stimulation of the contralateral sciatic nerve in adult rat (the crossed extensor reflex), was depressed by intraventricular administration of muscimol and systemic injection of some benzodiazepines including diazepam, and was potentiated by intravenous injection of bicuculline. These results agree with the results obtained in the present study. Although it is unclear whether the sites of action of these drugs on the crossed extensor reflex is at a spinal or supraspinal level, it is conceivable that a common GABAergic inhibitory mechanism is involved in both the contralateral slow v.r.p. and the crossed extensor reflex.

It has been proposed that GABA receptors in the central and peripheral nervous system are not homogeneous, but can be divided into $GABA_A$ and $GABA_B$ subtypes (Bowery *et al.*, 1980; Hill & Bowery, 1981; Bowery *et al.*, 1981). Muscimol, bicuculline and diazepam are thought to act on $GABA_A$ receptors, whereas baclofen is postulated to be a $GABA_B$ receptor agonist. We observed that baclofen at very low concentrations effectively inhibited the contralateral slow v.r.p. This result is consistent with the findings of Price *et al.* (1984) who reported that most $GABA_B$ receptors in the spinal cord are concentrated in the superficial layers of dorsal horn and about half are located on small-diameter primary afferent terminals, because the contralateral slow v.r.p. is elicited by activation of high-threshold and slow-conducting primary afferent fibres (Akagi *et al.*, 1985). Thus, baclofen may act on $GABA_B$ receptors located on

small-diameter primary afferent terminals and on dorsal horn neurones to exert an inhibitory effect on the contralateral slow v.r.p. It remains unclear, however, whether endogenous GABA released by stimulation of primary afferent fibres could inhibit the contralateral slow v.r.p. through $GABA_B$ receptors, because no selective antagonist for the $GABA_B$ receptor has been developed.

Previous reports from our group suggested that SP is involved as a neurotransmitter in the contralateral slow v.r.p. Specifically, SP released from central terminals of certain (probably small-diameter) primary afferent fibres may produce slow excitatory postsynaptic potentials in dorsal horn neurones, which via intraspinal neural pathways can evoke the generation of a slow depolarization in the ventral root (Yanagisawa *et al.*, 1982; Otsuka *et al.*, 1984; 1985; Akagi *et al.*, 1985). If this hypothesis is correct, the depolarizing response of ventral root to exogenous SP would be influenced by drugs such as muscimol, diazepam, bicuculline and baclofen in a similar manner to that of the contralateral slow v.r.p. This was found to be the case in the present experiments, thus supporting the hypothesis that SP is involved in the contralateral slow v.r.p.

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