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BENZODIAZEPINES AND CENTRAL GLYCINE RECEPTORS

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1 In cats, anaesthetized with pentobarbitone, intravenous diazepam (minimum dose 3.0 mg/kg) enhanced dorsal root potentials but did not significantly diminish the reduction by electrophoretic strychnine of the inhibitory action of electrophoretic glycine on dorsal horn interneurones.

2 In mice, intraperitoneal diazepam (2.5 mg/kg) had no appreciable effect on the potency of strychnine as a convulsant, although providing some protection against bicuculline.

3 These observations, together with the failure of chlordiazepoxide to either inhibit the firing of spinal interneurones or reduce antagonism between strychnine and glycine when administered locally, provide no support for the interaction between benzodiazepines and mammalian central glycine receptors which has been proposed on the basis of *in vitro* studies of strychnine binding.

Introduction

The inhibitory effect of glycine upon spinal and supraspinal neurones in the mammalian central nervous system is antagonized by strychnine (Curtis & Johnston, 1974). A number of recent publications, reviewed by Snyder (1975), have been concerned with the detection of postsynaptic glycine receptors on the basis of *in vitro* binding of tritiated strychnine to synaptic membrane fractions of central nervous tissue of rats (and monkeys, Young & Snyder, 1973). 'Specific' binding of the alkaloid to glycine receptors was defined as the amount of bound strychnine displaced by 10 mM glycine, and a remarkable correlation was found between the regional distribution of binding sites, the levels of endogenous glycine and the high affinity glycine uptake systems. Furthermore, strychnine binding was reduced by amino acids such as β-alanine, α-alanine, taurine and proline, considered 'glycine-like' on the basis of the sensitivity of their central inhibitory effects to strychnine (Curtis & Johnston, 1974), but not by amino acids structurally related to γ-aminobutyric acid (GABA).

A correlation between the 'clinical efficacy' of a series of benzodiazepines and the ability of these compounds to reduce the binding of strychnine to glycine receptors has led to the proposal that benzodiazepines, widely used therapeutically to control anxiety, seizures and muscle spasticity, 'exert their pharmacological activities by interacting with the glycine receptor' (Young, Zukin & Snyder, 1974; Snyder, 1975). Since the depressant effects of diazepam on spinal reflexes have largely been interpreted in terms of disturbed 'presynaptic' inhibition, a process involving GABA as a transmitter (see Schmidt, Vogel & Zimmermann, 1967; Schmidt, 1971; Polc, Möhler & Haefely, 1974; Costa, Guidotti, Mao & Suria, 1975), a study has been made in the cat and the mouse of the possible interaction between diazepam, strychnine and glycine *in vivo*. As a consequence no direct support can be offered to the proposal that benzodiazepines interact with glycine receptors in the spinal cord.

Methods

Cat spinal cord

Standard methods were used to record extracellular action potentials from dorsal horn interneurones of lumbar segments of cats (body temperature 37–38°C) anaesthetized with pentobarbitone sodium, using the centre barrel of seven barrel micropipettes as the recording electrode (Curtis, Duggan, Felix & Johnston, 1971). The outer barrels of these micropipettes contained aqueous solutions of DL-homocysteate (0.2 M, NaOH, pH 7.5); glycine (0.5 M, HCl, pH 3); GABA (0.5 M, HCl, pH 3); strychnine hydrochloride (0.002 M in 0.165 M NaCl); bicuculline methochloride (0.01 M in 0.165 M NaCl) and chlordiazepoxide hydrochloride (0.2 M), from which pharmacologically active ions were ejected electrophoretically.

Records were obtained from one dorsal horn interneurone in each of 3 cats before and for up to 30 min after the intravenous administration of diazepam. The commercially available solution of diazepam (Valium, Roche; 10 mg/2 ml benzoate

buffer) was diluted with 2 ml ethanol and 16 ml of 0.165M NaCl to provide a solution containing diazepam 0.5 mg/ml. Continuous monitoring of the amplitude of extracellular action potentials, and of the sensitivity of the neurones to DL-homocysteate, GABA and bicuculline methochloride, as well as to glycine and strychnine, provided a control for possible alterations in the distance between the neurones and micropipettes as a cause of changes in glycine or strychnine effects.

Dorsal root potentials were recorded from a caudal portion of the sixth lumbar dorsal root, in response to electrical stimulation of either hindlimb peripheral nerves (tibial, common peroneal) or the seventh lumbar dorsal root. In the doses used, diazepam reduced the systemic blood pressure by less than 10–15 mmHg; in all 3 animals dorsal root potentials were increased in amplitude and duration, and in view of the results of Polc *et al.* (1974) control experiments using the solvent alone were not performed.

Mice

In order to determine whether diazepam would protect against strychnine and bicuculline-induced convulsions and death, dose-response curves were prepared for these two drugs with and without the prior administration of diazepam (2.5 mg/kg). Four or five groups of 9 mice (body weight 30–37.5 g) were used to plot each curve. The solutions of strychnine (0.5 mg/ml), bicuculline (1 mg/ml) and diazepam (5 mg/ml, Valium; Roche) were administered intraperitoneally. Diazepam was injected 30 min before the administration of the convulsant, and mice were observed for a further 30 minutes. The convulsant end point used was either bilateral extensor spasms of the hind legs or opisthotonos. Dose-response curves were prepared by plotting linearly the number of animals convulsing or dying in each group against the dose of convulsant administered.

Results

Spinal interneurones

In preliminary experiments chlordiazepoxide could rarely be successfully administered electrophoretically, micropipettes containing solutions of this substance becoming 'noisy' with increased electrical resistance during the passage of even small electrophoretic currents. Chlordiazepoxide, ejected with currents of 2–5 nA did not reduce the firing of 5 interneurones, although glycine ejected with similar or even smaller currents clearly inhibited firing.

Attempts were made to administer chlordiazepoxide hydrochloride (0.1 M; 0.2 M; and 0.05 M in 0.165 M NaCl) by pressure (to 300 mmHg), either from one barrel of a 7 barrel micropipette or from another pipette (orifice 10–20 μm) cemented alongside. Although 3 of these neurones were readily excited by DL-homocysteate ejected with similar or even lower pressures from an adjacent barrel, the unreliability of this method of administration prevented a definite conclusion from the observed failure of chlordiazepoxide (3 neurones 0.05 M, 5 neurones 0.1 M, 1 neurone 0.2 M) either to reduce the effectiveness of strychnine as a glycine antagonist or to influence the firing of 9 neurones.

Results from one of the three experiments in which diazepam was administered intravenously are illustrated in Figure 1. In these experiments glycine and GABA were administered in amounts which did not completely suppress neuronal firing. Furthermore, the currents selected to administer strychnine and bicuculline methochloride were such that complete and rapid abolition of the actions of glycine and GABA did not occur. Thus both the rate of onset and the degree of antagonism could be used as parameters to assess the influence of diazepam. No consistent reductions in firing rate occurred after these doses of diazepam.

Control observations (Figure 1a) were made during excitation of the neurone by DL-homocysteate (8.5 nA). Strychnine (7 nA) progressively reduced the action of glycine (0 nA, removal of retaining current), that of the third dose being 27% of the effect observed beforehand. Similarly, and not illustrated, bicuculline methochloride (20 nA for 4.5 min) reduced the effectiveness of GABA to 30%. Seven minutes after intravenous diazepam (0.5 mg/kg) the effects of glycine, GABA, strychnine and bicuculline were unchanged. Twenty minutes later a further dose of 1.0 mg/kg was administered, and 8 min later the tracings of Figure 1b were recorded. The currents used to eject amino acids had been altered slightly to obtain responses similar to those of Figure 1a. Nevertheless the rate of antagonism of the inhibitory action of glycine by strychnine appeared not to be altered, and the effect of the third dose of glycine was approximately 17% of that occurring before strychnine was administered. At this time the dorsal root potential recorded from the caudal portion of the sixth lumbar dorsal root in response to stimulation of the seventh dorsal root was increased in amplitude and duration. The total area of the dorsal root potential was increased by 53% (see also Schmidt *et al.*, 1967; Polc *et al.*, 1974), and the short latency dorsal root reflex component was also enhanced.

Subsequently a further 1.5 mg/kg of diazepam was administered and 11 min later strychnine (7 nA) again reduced the action of glycine to 23% of that seen prior to strychnine. There was thus no evidence provided in this preparation that diazepam (3 mg/kg) reduced the effectiveness of strychnine as a glycine antagonist, and similar results were obtained in the other two cats with

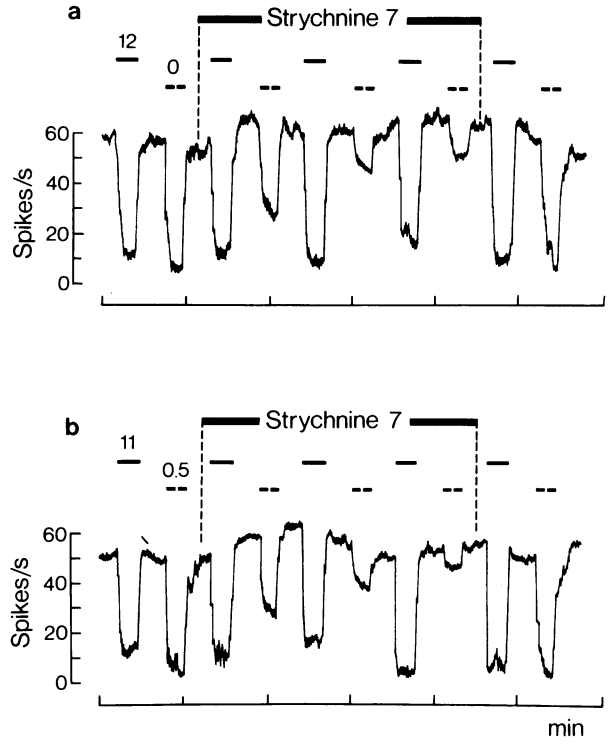

Figure 1 The effect of strychnine (a) before and (b) after the intravenous administration of diazepam, 1.5 mg/kg. The tracings plot the firing rate of a spinal dorsal horn interneurone excited by DL-homocysteate (a) 8.5 nA, (b) 9 nA. The firing was inhibited by periodic ejections of γ-aminobutyric acid (GABA, 7 s) and glycine (8 s) as indicated by the horizontal solid (GABA) and broken lines (glycine) and electrophoretic currents. Strychnine was administered with a current of 7 nA from a solution of strychnine hydrochloride 0.002 M in 0.165M NaCl.

increasing doses of diazepam to 3.0 and 3.85 mg/kg respectively.

In one of these cats the effect of bicuculline methochloride as a GABA antagonist appeared to be reduced by 1.5 mg/kg diazepam. However, the failure of increasing doses of diazepam (to 3 mg/kg) to decrease progressively the action of bicuculline methochloride, and the finding in the other two cats that diazepam did not significantly reduce the effect of bicuculline methochloride on the inhibitory action of GABA, suggests that the observation made in one animal probably arose from the vagaries of the techniques used rather than from an interaction between diazepam and bicuculline receptors.

Mice

Dose-response curves showing the convulsant activity of intraperitoneal strychnine and bicuculline are plotted in Figure 2. Diazepam had no appreciable effect on the potency of strychnine (Figure 2a) as a convulsant whereas it did afford protection against bicuculline-induced convulsions (Figure 2b). The most significant group was that receiving 6.3 mg/kg of bicuculline which convulsed 8 of 9 control mice and only 2 of 9 mice injected previously with 2.5 mg/kg diazepam. Using this data plotted on log-dose/probit scales the CD_{50} for bicuculline was 4.2 mg/kg in the control group and 8.5 mg/kg following diazepam; the CD_{50} for strychnine was 1.54 mg/kg in the control and 1.46 mg/kg in the diazepam-injected group.

The convulsant end points were easy to recognize with strychnine, but were slightly more difficult with bicuculline, especially since the convulsions appeared to be somewhat less severe in the diazepam-treated bicuculline animals. It was quite clear, however, when plotting dose-response curves for death (Figure 2c and d), rather than for convulsions, that diazepam offered far more protection against bicuculline than against strychnine.

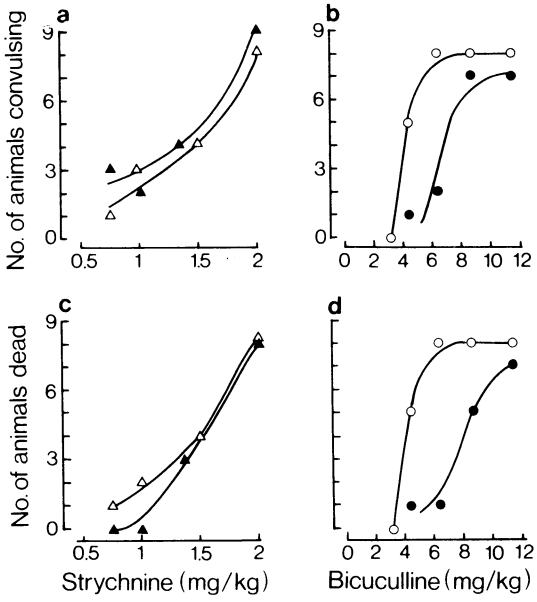

Figure 2 Effect of diazepam on the potency of strychnine and bicuculline as convulsants (a, b) and lethal agents (c, d) in mice. The curves were plotted from data obtained from groups of 9 mice following the intraperitoneal injection of (a, c) strychnine and (b, d) bicuculline with (▲, ●) and without (△, ○) the administration of diazepam (2.5 mg/kg) 30 min previously. Ordinates: number of animals convulsing (a, b) or dying (c, d) in each group. Abscissae: dose of convulsant, mg/kg.

Discussion

Despite uncertainties regarding the adequacy of electrophoretic or pressure ejection, no glycine-like effects of chlordiazepoxide, or impairment of the antagonism of glycine by strychnine, were observed in a small number of experiments. Furthermore, systemically administered diazepam, in relatively large doses which were sufficient to enhance and prolong dorsal root potentials, had no significant effect on the sensitivity of spinal interneurones to either glycine or GABA, and did not influence the effectiveness of strychnine or bicuculline methochloride as selective antagonists of these two amino acids. The total dose of diazepam tested was approximately 30 times that used clinically, for which the claim has been made that central nervous system and blood concentrations are similar to those required to displace strychnine from binding sites (Young *et al.*, 1974).

Although relatively high doses of diazepam (16 mg/kg orally) have been reported to inhibit strychnine convulsions in mice (see Zbinden & Randall, 1967), Costa *et al.* (1975) have recently reported that diazepam and chlordiazepoxide antagonize seizures induced in mice by picrotoxin more effectively than those produced by strychnine. The present results with mice indicate that at a dose level of 2.5 mg/kg diazepam had little or no influence on either the convulsant or lethal effects of strychnine, although providing some protection against these effects of bicuculline. Similar results have been reported by Schlosser, Zavatsky, Kappel & Sigg (1973). The failure of systemic diazepam to modify at a single cell level the effects of GABA or bicuculline suggests that the influence of benzodiazepines on GABA-mediated inhibitory processes may be more complex than mere interaction at GABA postsynaptic receptors.

The affinity of strychnine for its binding site *in vitro* is three orders of magnitude greater than that of glycine (Young & Snyder, 1973), and under the experimental conditions used (rat crude synaptic membrane fractions, 2 nM strychnine, 4°C) glycine and diazepam were of comparable potency in reducing strychnine binding, half maximum inhibition occurring with concentrations of 25 μM and 26 μM respectively (Young *et al.*, 1974). Thus if diazepam mimics the postsynaptic action of glycine (or strychnine) and interferes with strychnine binding the present investigation might have been expected to have revealed a modification in the effectiveness of strychnine as a glycine antagonist and as a convulsant. That this apparently is not so suggests that the receptors detected by *in vitro* strychnine binding may not be those directly involved in the inhibitory action of glycine. This conclusion may have implications for the interpretation of data regarding other central neurotransmitter receptors which depends upon membrane binding, under undoubtedly non-physiological conditions, rather than on the initiation or antagonism of a physiological response.

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