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Effects of non-sedative anxiolytic drugs on responses to GABA and on diazepam-induced enhancement of these responses on mouse neurones in cell culture

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1 Intracellular microelectrode recording techniques were performed on mouse spinal cord and cerebral hemisphere neurones grown in primary dissociated cell culture. The effects of several anxiolytics applied by local pressure ejection on responses to γ-aminobutyric acid (GABA) evoked by iontophoresis were investigated. Responses to GABA were depolarizing since intracellular chloride ion concentration was increased by injection from potassium chloride (3 M)-filled recording micropipettes and neurones were held at large negative membrane potentials (−70 to −90 mV). The agents studied were six 'non-sedative anxiolytics', CL 218,872 (3-methyl-6-(3-trifluoromethyl-phenyl) 1,2,4-triazolo(4,3-b) pyridazine), PK 8165 (2-phenyl-4-(2-(4-piperidinyl)ethyl)-quinoline), PK 9084 (2-phenyl-4-(2-(3-piperidinyl)ethyl)-quinoline), CGS 9896 (2-(4-chlorophenyl)-2,5-dihydropyrazolo(4,3-c)quinoline-3(3H)-one), ZK 91296 (ethyl 5-benzyloxy-4-methoxymethyl-β-carboline-3-β-carboxylate), buspirone (8-4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl-8-azaspiro[4.5]decane-7,9-dione), and two sedative anxiolytics, diazepam and zopiclone ([6-(5-chloro-2-pyridyl)-6,7-dihydro-7-oxo-5 H-pyrrolo [3,4-b] pyrazin-5-yl] 4-methyl-1-piperazinecarboxylate).

2 Direct effects on responses to GABA were studied for all drugs applied in varying concentrations. For the drugs which significantly altered responses to GABA, the effects of the benzodiazepine receptor antagonists Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5a)-(1,4)benzodiazepine-3-carboxylate) and CGS 8216 (2-phenylpyrazolo(4,3-c)-quinolin-3(5H)-one) were evaluated. For the drugs devoid of significant direct effect on responses to GABA, the influence on diazepam-induced enhancement of responses to GABA was evaluated.

3 Diazepam, zopiclone and CL 218,872 concentration-dependently and reversibly enhanced responses to GABA. Maximal enhancement was 82% for diazepam (500 nM), 64% for zopiclone (10 μM) and 20% for CL 218,872 (10 μM). PK 8165 effects varied with concentration, enhancing responses to GABA (up to 18%) at nM concentrations and reducing responses to GABA (up to 90%) at μM concentrations. CGS 9896, ZK 91296, PK 9084 and buspirone, in concentrations ranging from 1 nM to 10 μM, lacked significant direct effects on responses to GABA.

4 The enhancing effects of diazepam, zopiclone, CL 218,872 and PK 8165 were antagonized by Ro 15-1788. However, the reducing effect on responses to GABA of PK 8165 at μM concentrations was not antagonized by CGS 8216. CGS 9896 and ZK 91296 concentration-dependently blocked the diazepam-induced enhancement of responses to GABA. However, PK 9084 and buspirone did not antagonize the diazepam-induced enhancement of responses to GABA.

5 These results indicate that diazepam and zopiclone may be full agonists, CL 218,872 and PK 8165 are partial agonists, and CGS 9896 and ZK 91296 are pure antagonists at benzodiazepine receptors. On the other hand, PK 9084 and buspirone do not interact with benzodiazepine receptors.

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Introduction

The benzodiazepines, in addition to their anxiolytic and anticonvulsant properties, have sedative effects (Fink & Swinyard, 1962; Haefely *et al.*, 1981), and the use of these drugs is associated with the development of tolerance and dependence (Allgulander, 1978). As a result there has been a continuing effort to develop selective, non-sedative anxiolytic drugs. Several benzodiazepine receptor ligands and non-benzodiazepine receptor ligands have been proposed in this respect. Some of these compounds have been suggested to be devoid of induction of tolerance and/or dependence (Lamb & Griffiths, 1984; Gerhardt *et al.*, 1985).

Six novel compounds, suggested to be anxiolytic drugs, are considered in this study: CL 218,872 (3-methyl-6-(3-trifluoromethylphenyl)1,2,4-triazolo(4,-3-b) pyridazine), PK 8165 (2-phenyl-4-(2-(4-piperidinyl) ethyl)-quinoline), PK 9084 (2-phenyl-4-(2-(3-piperidinyl)ethyl)-quinoline), CGS 9896 (2-(4-chlorophenyl)-2,5-dihydropyrazolo(4,3-c)quinoline-3(3H)-one), ZK 91296 (ethyl 5-benzyloxy-4-methoxymethyl-β-carboline-3-β-carboxylate) and buspirone (8-4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl-8-azaspiro[4.5] decane-7,9-dione). All of these compounds have been demonstrated to display a non-sedative anxiolytic behavioural profile. CL 218,872 was shown to be a central benzodiazepine receptor ligand (Squires *et al.*, 1979; Lippa *et al.*, 1979a,b). This triazolopyridazine was found to be relatively free of ataxic and depressant side-effects, to increase punished responding in a conflict procedure and to protect against pentylenetetrazol-induced seizures (Squires *et al.*, 1979; Lippa *et al.*, 1979b). However, the initial results indicating that CL 218,872 is non-sedative have not been confirmed (Oakley *et al.*, 1984; File *et al.*, 1985; McElroy *et al.*, 1985). PK 8165 and PK 9084 are both phenylquinolines and were found to have a non-sedative anxiolytic profile (Le Fur *et al.*, 1981). However, subsequent investigators have failed to substantiate that the phenylquinolines are non-sedative (File & Lister, 1983; File, 1983; Keane *et al.*, 1984). Both phenylquinolines have been suggested to be partial agonists at the benzodiazepine receptor (Morelli *et al.*, 1982; Gee *et al.*, 1983; Benavides *et al.*, 1984). CGS 9896, a pyrazoloquinoline, (Yokoyama *et al.*, 1982) and ZK 91296, a β-carboline, (Petersen *et al.*, 1984; Pellow & File, 1986) were shown to be non-sedative anxiolytics and to protect against pentylenetetrazol-induced seizures. Both CGS 9896 (Yokoyama *et al.*, 1982; Gee & Yamamura, 1982) and ZK 91296 (Petersen *et al.*, 1984) are known to be benzodiazepine receptor ligands. Buspirone, a piperazinyl pyrimidine, is a non-benzodiazepine receptor ligand with anticonflict activity (Riblet *et al.*, 1982; Geller & Hartmann, 1982). Several clinical trials have confirmed the non-sedative anxiolytic profile of this compound (Goldberg & Finnerty, 1979; Rickels *et al.*, 1982; Newton *et al.*, 1982).

Diazepam and zopiclone ([6-(5-chloro-2-pyridyl)-6,7-dihydro-7-oxo-5 H-pyrrolo [3,4-b] pyrazine-5-yl] 4-methyl-1-piperazinecarboxylate) were included as sedative anxiolytic drugs. Diazepam is a benzodiazepine receptor agonist (Haefely *et al.*, 1981), displaying all benzodiazepine-like activities, i.e., anxiolytic, anticonvulsant, sedative and muscle relaxant effects. The pyrrolopyrazine zopiclone, believed to be a benzodiazepine receptor ligand (Blanchard *et al.*, 1979; 1983), has a similar benzodiazepine-like pharmacological profile in animal models and in clinical trials ((Duriez *et al.*, 1979; Julou *et al.*, 1983). It has been suggested that Zopiclone interacts allosterically with the benzodiazepine receptor complex (Trifiletti & Snyder, 1984).

Various lines of evidence support the hypothesis that facilitation of the postsynaptic responses to γ-aminobutyric acid (GABA) is involved in many of the actions of the benzodiazepines (Choi *et al.*, 1977; Macdonald & Barker, 1978; Haefely *et al.*, 1979; Costa & Guidotti, 1979), the barbiturates (Nicoll *et al.*, 1975; Macdonald & Barker, 1979; Haefely *et al.*, 1979) and related central nervous system depressants. In this study we evaluated the effects of six 'non-sedative' and two 'sedative' anxiolytic agents on responses to GABA (Macdonald & Barker, 1979; Nowak *et al.*, 1982), and on diazepam-induced enhancement of responses to GABA, on mouse spinal cord and cerebral hemisphere neurones grown in primary dissociated cell culture. In addition, the effects of the benzodiazepine receptor antagonists Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5a)(1,4)-benzodiazepine-3-carboxylate) and CGS 8216 (2-phenylpyrazolo(4,3-c)quinolin-3(5H)-one) on the drug-induced alteration of responses to GABA were studied in mouse spinal cord neurones.

Methods

Primary dissociated cell culture

Cultures of spinal cord neurones were prepared from dissected spinal cords and attached dorsal root ganglia from 12–14 day old foetal mice as described previously (Ransom *et al.*, 1977). The tissue was minced and then mechanically dissociated by trituration in Ca^{2+}-, and Mg^{2+}-free balanced salt solution to a suspension of single cells and small clumps. The dissociated cells were suspended in culture medium (90% Eagle's minimal essential medium supplemented with 5.5 g l^{-1} of glucose and 1.5 g l^{-1}

of $NaHCO_3$, 5% heat-inactivated horse serum and 5% Nu-Serum II (Collaborative Research Inc., Lexington, MA, U.S.A.), 325 mOsmol) and then plated on sterile collagen-coated 35 mm dishes. Each cord produced 6 to 8 cultures. The cultures were maintained in an incubator with an atmosphere of 93% room air and 7% CO_2 at 35°C. The bicarbonate/CO_2 buffer maintained pH at 7.4. 5-Fluoro-2'-deoxyuridine was added to the cultures on days 6 to 8 in order to suppress the growth of rapidly dividing non-neuronal cells. Medium was changed twice weekly. Cultures were maintained for 4 to 9 weeks before electrophysiological experiments.

Cerebral hemisphere cultures were prepared from 14- to 18-day gestational mice after transection of the cervical cord. The cerebral hemispheres were dissected away from the brainstem. The pieces of lateral cortical mantle were pooled separately in Ca^{2+}-, Mg^{2+}-free balanced salt solution. After replacement of the balanced salt solution with culture medium, the pieces were dissociated mechanically, plated on collagen-coated dishes (12 to 14 cultures per-hemisphere) and maintained as described above.

Experimental procedures

Solutions All recordings were made in a Dulbecco's phosphate buffered saline after removal of growth medium. The recording solution, with elevated magnesium ion concentration in order to suppress spontaneous activity, contained in mM: NaCl 137, Na_2HPO_4 8.06, KCl 2.68, KH_2PO_4 1.47, $CaCl_2$ 1.0, $MgCl_2$ 10 and glucose 5.6 (pH 7.3–7.4). Heavy paraffin oil was applied to the surface of the bathing solution to retard evaporation.

Solutions of the tested drugs were always prepared on the day of the experiment in the following manner. The dry drugs were dissolved in dimethylsulphoxide to form a 1 or 10 mM stock solution. Aliquots were removed and diluted in bathing medium to give concentrations between 1 nM and 100 μM. These final solutions contained 0.1% or less dimethylsulphoxide.

Experimental apparatus For experiments, the culture dish containing the bathing solution was placed on a stage heated by a Pellitier device with temperature regulated at 34–35°C. The stage was mounted on a Leitz inverted microscope fitted with phase contrast optics to facilitate micropipette placement (using Leitz micromanipulators) and to penetrate cells under direct visual control.

Electrophysiological recordings Intracellular recordings were made from the somata of spinal cord ($\geq 20\,\mu m$) and hemisphere ($\pm 10\,\mu m$) neurones with glass micropipettes filled with 3 M potassium chloride. Micropipettes with different resistances were used for cortical (40–80 MΩ) and spinal cord (25–50 MΩ) neurones. Use of an active bridge circuit (Model 8100, Dagan, Inc., Minneapolis, MN, U.S.A.) allowed simultaneous recording of membrane potential and injection of current (for steady-state polarization or periodic stimulation) with a single micropipette. The preamplifier output signal was led to a 6-channel polygraph, (Model 2600, Gould Instruments, Inc., Cleveland, OH, U.S.A.) for continuous recording.

Responses to GABA GABA (0.5 M, pH 3.4) was applied iontophoretically using 500 ms duration rectangular positive current pulses at 5 s intervals. Iontophoretic pipettes were positioned to within 2 μm of neuronal somata. The use of 3 M KCl-filled micropipettes shifted the chloride equilibrium potential from about −65 mV to about −20 mV. Under these conditions, an increase of chloride conductance resulted in an outward chloride current (Nishi *et al.*, 1974), giving depolarizing responses to GABA (Nowak *et al.*, 1982). Responses to GABA of about 10 to 15 mV in amplitude were evoked following membrane hyperpolarization (to −70 to −90 mV) to avoid saturation at or near the chloride equilibrium potential. In studies of drug effects on responses to GABA, data were accepted only if responses to GABA returned to control amplitude within 5 min of removal of the drug-containing micropipette. In studies of antagonists, data were accepted only if the original direct effect of the agonist returned after removal of the tested putative antagonist.

Drug application For evaluation of drug effects on responses to GABA, all drugs were applied by pressure ejection. A blunt tipped (5–10 μm) micropipette, filled with the test solution, was positioned 15–30 μm from the soma of the cell under study. The open end of each pressure ejection micropipette was connected by tight fitting polyethylene tubing to a pressure regulator, set between 0.4 and 0.8 pounds per square inch (psi). Pressure pulse duration, regulated by a voltage-activated 3-way valve, was 10 s. Under these conditions, application of control solutions was virtually free of effects. Pressure and position of the pressure ejection micropipettes were adjusted to attain a maximal drug response. We have demonstrated previously that the concentration-dependency of drug action determined using pressure ejection or by superfusion are equivalent if this experimental procedure is used (Heyer *et al.*, 1982). For assessment of possible antagonistic effects at the benzodiazepine receptor, the putative antagonist was applied by diffusion from a large tipped (10–20 μm) micropipette, before application by pressure ejection of the anxiolytic drug. As a control, record-

ing solution alone or with vehicle was applied by diffusion and was without effect. To decrease leakage of drugs into the bathing medium, the tips of the pressure ejection micropipettes were kept in the oil phase between drug application trials. They were lowered into the aqueous phase only when drug application was desired.

Drugs

Buspirone HCl was obtained from Bristol-Myers Co., Evansville, Indiana, U.S.A. CGS 8216 and CGS 9896 were provided by Ciba-Geigy Corp., Summit, New Jersey, U.SA. CL 218,872 was provided by Lederle Laboratories, New York, NY, U.S.A. Diazepam and Ro 15-1788 were obtained from Hoffman-LaRoche, Nutley, New Jersey, U.S.A. GABA was purchased from Sigma Chemical Company, St Louis, Missouri, U.S.A. PK 8165 and PK 9084 were provided by Dr Sandra File, School of Pharmacy, University of London, U.K. ZK 91296 was provided by A/S Ferrosan, Denmark. Zopiclone was provided by Rhône-Poulenc Research, Vitry-sur-Seine, France.

Algebraic and statistical methods

For the different drugs at all applied concentrations, mean values and standard deviations were calculated for the direct effects on responses to GABA and antagonistic effects on diazepam-induced enhancement of responses to GABA. The direct effects were expressed as % change of responses to GABA. The antagonistic effects were expressed as % enhancement of responses to GABA produced by diazepam (100 nM) in the presence of the antagonist when compared to initial enhancement produced by diazepam (100 nM). The statistical significance of differences between controls and drug groups was calculated by use of Student's two-tailed t test; $P < 0.05$ was considered to be statistically significant.

Results

Direct effects on responses to GABA recorded from spinal cord neurones

Pressure ejection of the studied drugs did not alter resting membrane potential or conductance. Pressure ejection of recording solution alone or with vehicle ($n = 10$) gave non-significant enhancement ($0.8 \pm 1.9\%$) of responses to GABA. Maximal effects of the different drugs are shown in Figure 1. Diazepam (500 nM) (Figure 1a) and zopiclone (10 μM) (Figure 1b) reversibly enhanced responses to GABA. PK 8165 at a low concentration (10 nM) reversibly enhanced responses to GABA (Figure 1c), but at a high concentration (100 μM), PK 8165 reversibly reduced responses to GABA (Figure 1d). CL 218,872 (10 μM) reversibly enhanced responses to GABA (Figure 1e).

Effects of diazepam, zopiclone, PK 8165 and CL 218,872 on responses to GABA recorded from spinal cord neurones were concentration-dependent (Figure 2). For diazepam, enhancement of responses to GABA was produced at 10 nM and peak enhancement occurred at 500 nM. A lesser enhancement was observed at higher concentrations. For zopiclone, enhancement was observed at 50 nM, and the enhancement was concentration-dependent up to 10 μM. PK 8165 enhanced responses to GABA at 10, 100 and 500 nM. However, at a concentration of 1 μM, PK 8165 was without effect. In contrast, at concentrations higher than 1 μM, PK 8165 reversibly reduced responses to GABA. For CL 218,872, enhancement was observed at 100 nM, and the enhancement was concentration-dependent up to 10 μM.

CGS 9896, ZK 91296, PK 9084 and buspirone, however, had no significant direct effect on responses to GABA at concentrations ranging from 1 nM to 10 μM (Table 1).

Antagonism of diazepam-induced enhancement of responses to GABA

Attempts were made to antagonize the diazepam (100 nM)-induced enhancement of the response to GABA by the compounds that did not display a significant direct effect on the response to GABA. These drugs were: CGS 9896, ZK 91296, PK 9084 and buspirone. The % increases of responses to GABA by diazepam with and without co-application of control or drug-containing solution were compared. Diazepam-induced enhancement of responses to GABA was unaffected by co-application of recording solution alone or with vehicle ($96 \pm 7.1\%$ ($n = 8$)). CGS 9896 (1 μM, $n = 5$) (Figure 3a) and ZK 91296 (1 μM, $n = 5$) (Figure 3b) almost completely antagonized diazepam-induced enhancement of responses to GABA. However, PK 9084 (10 μM, $n = 6$) (Figure 3c) and buspirone (10 μM, $n = 5$) (Figure 3d) did not antagonize diazepam-induced enhancement of responses to GABA. The antagonistic effects of the benzodiazepine receptor ligands CGS 9896 and ZK 91296 on diazepam-induced enhancement of responses to GABA were concentration-dependent (Figure 4). CGS 9896 antagonized diazepam-induced enhancement of responses to GABA at a threshold concentration of 10 nM ($n = 8$, $P < 0.001$). ZK 91296 antagonized diazepam-induced enhancement of responses to GABA at a threshold concentration of 1 nM ($n = 6$, $P < 0.001$). The estimated IC_{50} values were 17 nM for CGS 9896 and 9 nM for ZK 91296.

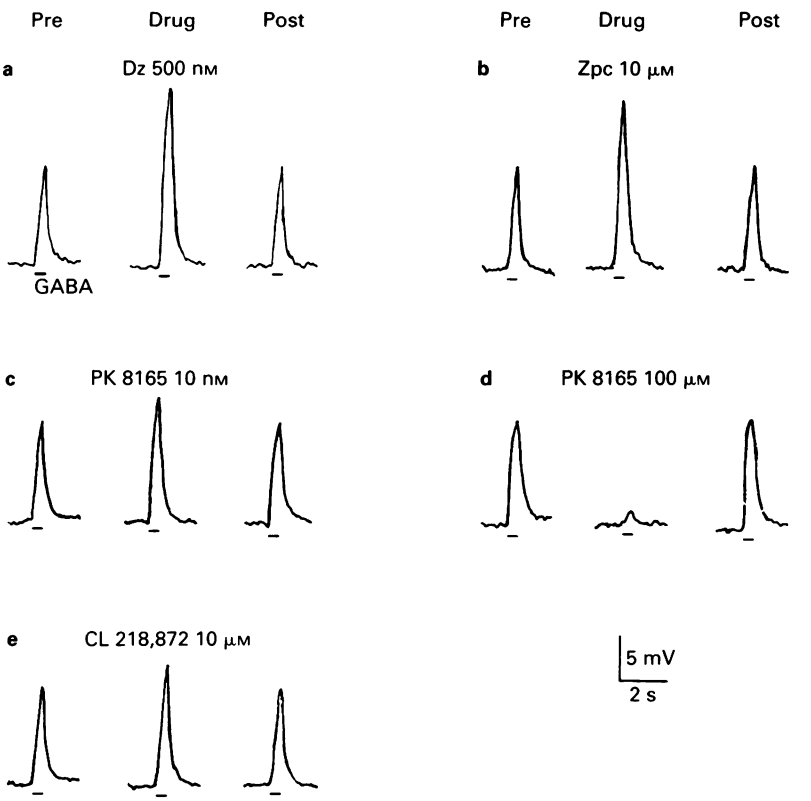

Figure 1 Maximal, reversible direct effects on responses to γ-aminobutyric acid (GABA) on spinal cord neurones of diazepam (Dz) 500 nM (a), zopiclone (Zpc) 10 μM (b), PK 8165 10 nM (c), PK 8165 100 μM (d) and CL 218,872 10 μM (e). Pre shows stable responses to GABA before drug application. Drug shows the effect of each drug applied by pressure ejection. Responses to GABA returned to control values (Post) within 2 min following removal of diazepam, PK 8165 (10 nM) and CL 218,872; within 10 min following removal of PK 8165 (100 μM). For each drug application illustrated, a different neurone was used. Iontophoretic GABA application (500 ms) is indicated by a horizontal bar. GABA was applied at 5 s intervals. Neurones were held at −70 to −90 mV and 3 M KCl-filled recording micropipettes were used. Diazepam (500 nM), zopiclone (10 μM), PK 8165 (10 nM) and CL 218,872 (10 μM) enhanced responses to GABA. PK 8165 (100 μM) reduced responses to GABA.

Complete antagonism of the action of diazepam was obtained with 2 μM CGS 9896 and 10 μM ZK 91296. PK 9084 and buspirone in concentrations ranging from 1 nM to 10 μM did not antagonize the diazepam-induced enhancement of responses to GABA (Figure 4).

Effects of Ro 15-1788 and CGS 8216 on drug-induced enhancement of responses to GABA in spinal cord neurones

The effects of Ro 15-1788, a weak partial agonist at the benzodiazepine receptor (Skerritt & Macdonald, 1983), or CGS 8216, a weak partial inverse agonist at the benzodiazepine receptor (DeDeyn & Macdonald, 1987) on enhancement of responses to GABA produced by diazepam, zopiclone, CL 218,872 and PK 8165 were studied. Diazepam (100 nM) enhanced responses to GABA by 43.4% (Figure 5a, Table 2). Ro 15-1788 enhanced responses to GABA by 7.5% at 100 nM and by 15.1% at 500 nM (Figure 5, Table 2). In the presence of Ro 15-1788 (500 nM), diazepam (100 nM)-induced enhancement of responses to GABA was significantly reduced to 9.4% (Table 2).

Zopiclone (500 nM) enhanced responses to GABA by 43.8% (Figure 5b, Table 2). In the presence of Ro 15-1788 (100 nM), the zopiclone (500 nM)-induced increase of the response to GABA was significantly reduced to 19.3% (Figure 5b, Table 2).

CL 218,872 (1 μM) enhanced responses to GABA by 10.9% (Figure 5c, Table 2). However, CL 218,872

Table 1 Lack of direct effects of CGS 9896, ZK 91296, PK 9084 and buspirone on responses to GABA in mouse spinal cord neurones

Drugs and concentrations		*Number of neurones studied*	*Responses to GABA* (% control)
CGS 9896	1 nM	7	103.2 ± 0.8
	10 nM	9	98.5 ± 2.2
	50 nM	8	102.0 ± 4.1
	100 nM	7	100.2 ± 4.7
	200 nM	7	101.5 ± 3.0
	300 nM	7	101.9 ± 3.7
	500 nM	7	101.5 ± 3.0
	1 μM	9	100.2 ± 4.9
	2 μM	15	100.3 ± 1.5
	10 μM	7	101.2 ± 2.1
ZK 91296	1 nM	11	98.5 ± 3.0
	10 nM	11	100.9 ± 2.7
	100 nM	8	100.4 ± 0.9
	1 μM	6	100.3 ± 2.7
	10 μM	7	98.7 ± 1.3
PK 9084	1 nM	5	99.6 ± 3.8
	10 nM	6	98.7 ± 3.3
	100 nM	5	102.3 ± 3.1
	500 nM	4	99.0 ± 2.5
	1 μM	5	99.5 ± 1.4
	10 μM	4	100.6 ± 0.8
Buspirone	1 nM	5	100.2 ± 1.6
	10 nM	5	97.7 ± 2.5
	100 nM	6	98.7 ± 2.2
	1 μM	5	99.5 ± 1.5
	10 μM	5	97.1 ± 2.7

Data shown are mean ± s.d.

was without significant effect on the response to GABA after diffusion of Ro 15-1788 (500 nM) (Figure 5c, Table 2). At a low concentration (10 nM) PK 8165 enhanced responses to GABA by 25.6% but had no significant effect on responses to GABA when applied after diffusion of Ro 15-1788 (500 nM) (Figure 5d, Table 2). At a high concentration (10 μM), PK 8165 reduced responses to GABA by 38.8% (Table

Table 2 Effects of Ro-1788 and CGS 8216 upon diazepam-, zopiclone-, CL 218,872- and PK 8165-induced changes of responses to GABA of mouse spinal cord neurones

	Drugs and concentrations		*Number of neurones studied*	*Response to GABA* (% control)
**	Diazepam	100 nM		143.4 ± 7.5*
	Diazepam	100 nM + Ro 15-1788 500 nM	8	109.4 ± 4.1*
**	Zopiclone	500 nM		143.8 ± 7.3*
	Zopiclone	500 nM + Ro 15-1788 100 nM	3	119.3 ± 7.4*
**	CL 218,872	1 μM		110.9 ± 3.1*
	CL 218,872	1 μM + Ro 15-1788 500 nM	5	99.3 ± 4.7
**	PK 8165	10 nM		125.6 ± 5.9*
	PK 8165	10 nM + Ro 15-1788 500 nM	4	101.3 ± 1.7
	PK 8165	10 μM		64.2 ± 10.5*
	PK 8165	10 μM + CGS 8216 100 nM	4	56.7 ± 15.1*
	Ro 15-1788	100 nM	12	107.5 ± 2.9*
	Ro 15-1788	500 nM	11	115.1 ± 5.2*
	CGS 8216	100 nM	6	90.6 ± 3.0*

Data shown are mean ± s.d. The vertical line to the left of pairs of drugs indicates that the data were paired observations. A double asterisk indicates a significant reduction of the drug-induced enhancement of GABA responses by either Ro 15-1788 or by CGS 8216 ($P < 0.05$). A single asterisk indicates a significant drug-induced change in the GABA response ($P < 0.05$).

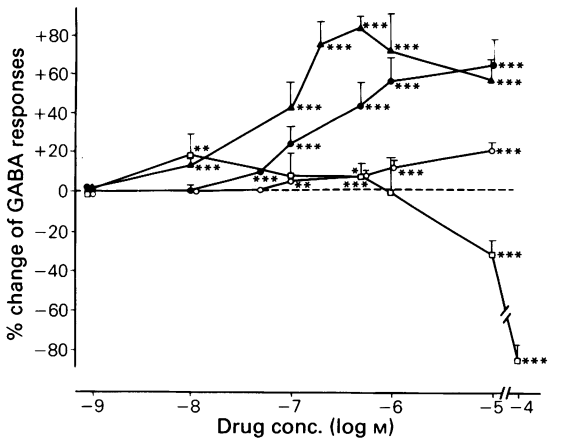

Figure 2 Concentration-dependent effects of diazepam (▲, $n = 7$–86), zopiclone (●, $n = 5$–12), PK 8165 (□, $n = 6$–13) and CL 218,872 (○, $n = 5$) on the responses to γ-aminobutyric acid (GABA) of spinal cord neurones. Effects are expressed as % change of the original response to GABA. Data shown are mean and vertical lines indicate s.d. If the s.d. is smaller than the symbol it is not shown; s.d.'s are shown only in one direction. n = number of cells for all concentrations tested. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ from control responses to GABA. Diazepam, zopiclone, PK 8165 and CL 218,872 concentration-dependently enhanced responses to GABA. At high concentrations, PK 8165 reduced responses to GABA.

2). Application of CGS 8216 (100 nM) did not significantly alter the reduction of the responses to GABA induced by PK 8165 (100 nM) (Table 2). CGS 8216 (100 nM) had a direct effect, reducing responses to GABA by 9.4% (Table 2).

Effects of CGS 9896 and ZK 91296 on cerebral hemisphere neurones

CGS 9896 (2 μM), without having any direct effect on responses to GABA, completely antagonized the diazepam (100 nM)-induced enhancement of responses to GABA on cerebral hemisphere neurones (Table 3). Similar findings were obtained for ZK 91296 (2 μM) (Table 3). The simultaneous application of diazepam (100 nM) and CGS 9896 (2 μM) and diazepam (100 nM) and ZK 91296 (2 μM) gave no significant effect (Table 3).

No significant difference in the enhancement of responses to GABA by diazepam (100 nM) was observed between spinal cord neurones (41.9 ± 15%, $n = 86$) and cerebral hemisphere neurones (37.3 ± 11.2%, $n = 10$).

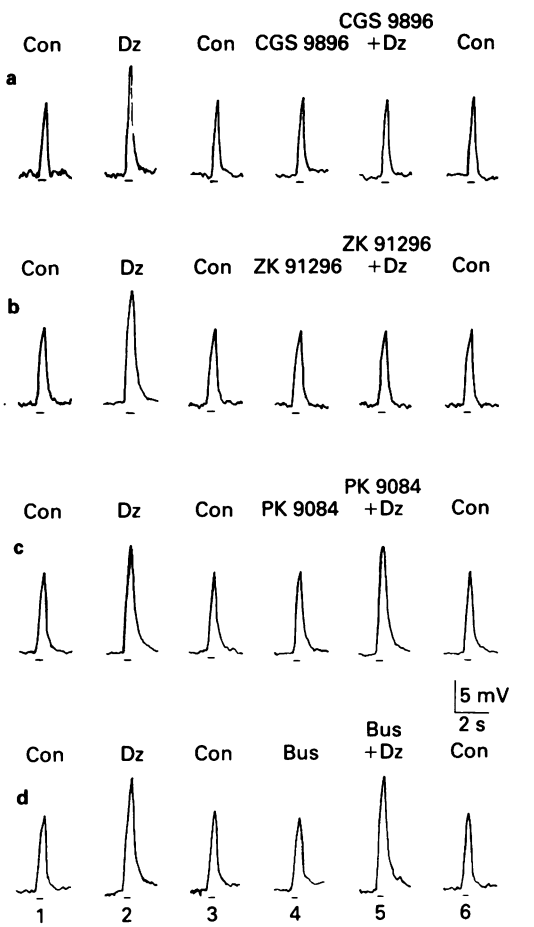

Figure 3 Effect of CGS 9896 1 μM (a), ZK 91296 1 μM (b), PK 9084 10 μM (c) and buspirone (Bus) 10 μM (d) on diazepam (Dz, 100 nM)-enhancement of responses to γ-aminobutyric acid (GABA) on spinal cord neurones. Each row represents responses obtained on one neurone. Column 2 shows the effect of diazepam. Column 4 shows the direct effect of the putative antagonists, and column 5 shows the effects of the simultaneous applications of diazepam and the putative antagonists. Columns 1, 3 and 6 show control responses (Con), before, between and after drug applications. Iontophoretic GABA-application is indicated by a horizontal bar. CGS 9896 1 μM and ZK 91296 1 μM almost completely blocked diazepam (100 nM)-enhancement of responses to GABA. PK 9084 10 μM and buspirone 10 μM did not antagonize responses to GABA.

Discussion

Diazepam, zopiclone and CL 218,872 enhanced responses to GABA in a concentration-dependent manner. Ro 15-1788, which is considered a classical

Table 3 Direct effect and antagonism of diazepam-induced enhancement of responses to GABA by CGS 9896 and ZK 91296 on mouse cerebral hemisphere neurones

Drugs and concentrations		*Number of neurones studied*	*Response to GABA* (% control)
CGS 9896	2 μM	4	100.1 ± 1.0
ZK 91296	2 μM	4	100.3 ± 0.8
Diazepam	100 nM	10	137.3 ± 11.2*
Diazepam	100 nM + CGS 9896 2 μM	3	100.7 ± 3.8
Diazepam	100 nM + ZK 91296 2 μM	5	99.5 ± 2.4

Data shown are mean ± s.d. The asterisk indicates a significant drug-induced change in the GABA response ($P < 0.05$).

antagonist or weak partial agonist at the benzodiazepine receptor (Hunckeler *et al.*, 1981; Skerritt & Macdonald, 1983), antagonized these effects, suggesting that the benzodiazepine receptor may be the site of action for these anxiolytics. These findings are consistent with previous results for diazepam (Squires & Braestrup, 1977; Möhler & Okada, 1977), zopiclone (Blanchard *et al.*, 1979; 1983; Trifiletti & Snyder, 1984) and CL 218,872 (Squires *et al.*, 1979; Lippa *et al.*, 1979a,b). Diazepam, with a maximal direct effect inducing 82% enhancement of responses to GABA, may be considered a full agonist at the benzodiazepine receptor. This result confirms previous findings (Skerritt *et al.*, 1984) obtained with this methodological approach. Zopiclone, with a maximal enhancement of 64%, also appeared to be a full agonist at the benzodiazepine receptor. CL 218,872, with a maximal enhancement of only 20%, was a partial agonist at the benzodiazepine receptor. The concentration-dependent effects presented here for zopiclone and CL 218,872 are consistent with earlier studies using only single drug concentrations (Skerritt & Macdonald, 1984).

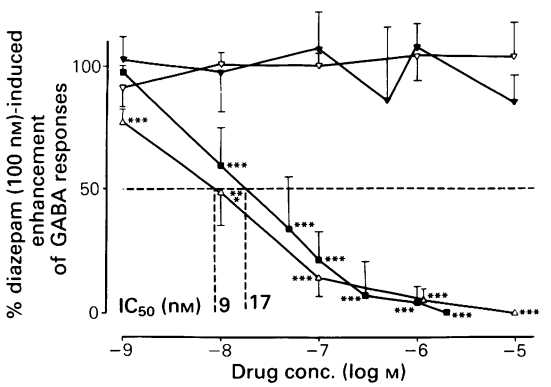

Figure 4 Effects of diazepam (100 nM)-induced enhancement of responses to γ-aminobutyric acid (GABA) on spinal cord neurones produced by CGS 9896 (■, $n = 5$–8), ZK 91296 (△, $n = 5$–6), PK 9084 (▼, $n = 4$–8) and buspirone (△, $n = 5$) applied in concentrations ranging from 1 nM to 10 μM. Effects are expressed as remaining % of the initial enhancement of responses to GABA induced by diazepam 100 nM. Data shown are means and vertical lines indicate s.d. Standard deviations smaller than the symbols are not shown and they are shown only in one direction. n = numbers of cells for all concentrations tested. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ from initial 100 nM diazepam-induced enhancement. CGS 9896 (IC_{50} = 17 nM) and ZK 91296 (IC_{50} = 9 nM) concentration-dependently antagonized the diazepam-enhancement of responses to GABA. However, PK 9084 and buspirone did not antagonize significantly diazepam-enhancement of responses to GABA.

PK 8165 had a mixed effect on responses to GABA: inducing an enhancement at nM concentrations and a reduction at μM concentrations. However, pressure ejection of nM concentrations of PK 8165, after diffusion of Ro 15-1788, was without significant effect on responses to GABA. In contrast, CGS 8216 (Yokoyama *et al.*, 1982) did not antagonize the decrease of responses to GABA induced by μM concentrations of PK 8165. This suggests that PK 8165 could have another site of action in addition to the benzodiazepine receptor. While PK 8165 would act as a partial agonist at the benzodiazepine-receptor, the inhibitory effect on responses to GABA at μM concentrations could be mediated through another receptor.

CGS 9896, ZK 91296, buspirone and PK 9084 in concentrations ranging from 1 nM to 10 μM, were devoid of any significant direct effect on responses to GABA in spinal cord neurones. CGS 9896 and ZK 91296 were free also of significant effects on responses to GABA in cerebral hemisphere neurones. In contrast, in an experiment testing the antagonist effect on diazepam-induced enhancement of responses to GABA, CGS 9896 and ZK 91296 acted concentration-dependently on spinal cord and hemisphere neurones. CGS 9896 and ZK 91296, therefore, could be classified in this system as pure benzodiazepine receptor antagonists (DeDeyn &

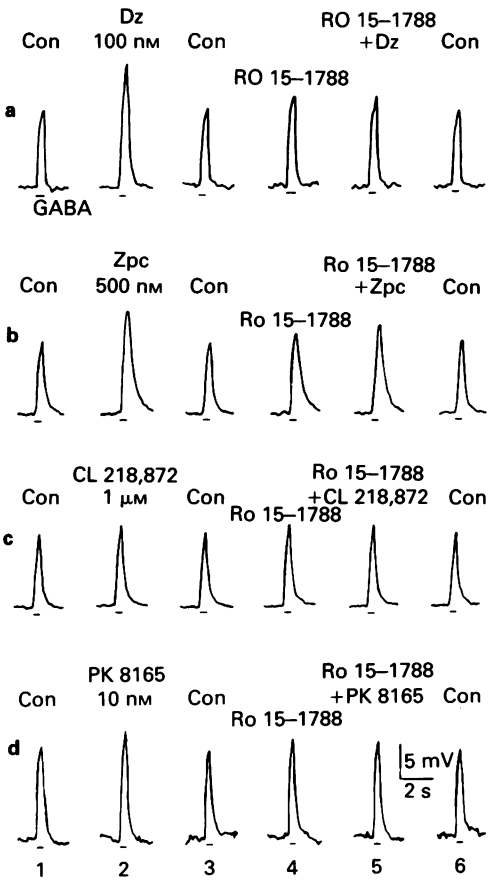

Figure 5 Blockade by Ro 15-1788 of the direct enhancing effect of diazepam (Dz) 100 nM (a), zopiclone (Zpc) 500 nM (b), CL 218,872 1 μM (c) and PK 8165 10 nM (d) on responses to γ-aminobutyric acid (GABA) in spinal cord neurones. The Ro 15-1788 concentration was 500 nM (a, c and d) or 100 nM (b). Each row represents responses obtained in one neurone. Column 2 shows the direct effect of the different drugs on responses to GABA. Column 4 shows the direct effect of Ro 15-1788. Column 5 shows the effect of simultaneous application of the tested drug and Ro 15-1788. Columns 1, 3 and 6 show control responses (Con) before, between and after drug applications. Iontophoretic GABA-application is indicated by a horizontal bar. GABA was applied every 5 s. Ro 15-1788 antagonized the enhancement of responses to GABA produced by diazepam, CL 218,872 and PK 8165, suggesting that the benzodiazepine receptor might be the site of action for these drugs.

Macdonald, 1987). However, buspirone and PK 9084, in concentrations from 1 nM to 10 μM, remained inactive. These findings suggest that buspirone and PK 9084 are not benzodiazepine-receptor ligands. This is consistent with previous results for buspirone (Riblet *et al.*, 1982). However, for the phenylquinolines PK 9084 and PK 8165, the binding studies were less conclusive. While shown to be benzodiazepine receptor ligands *in vitro* (Gee *et al.*, 1983; Benavides *et al.*, 1984 ; Keane *et al.*, 1984), one *in vivo* study (Keane *et al.*, 1984) failed to demonstrate an affinity for the benzodiazepine receptor.

The data presented in this study are not entirely consistent with a unitary hypothesis that these drugs are anxiolytic and anticonvulsant by directly enhancing postsynaptic GABAergic inhibition. The classification of diazepam and zopiclone (Haefely *et al.*, 1979; Duriez *et al.*, 1979; Julou *et al.*, 1983) as full agonists and CL 218,872 (Squires *et al.*, 1979; Lippa *et al.*, 1979b) and PK 8165 (Le Fur *et al.*, 1981) as partial agonists at the benzodiazepine receptor is consistent with their anxiolytic action. Administered at relatively high doses, PK 8165 was proconvulsant (File & Simmonds, 1984). This is consistent, as suggested by our findings, with an action of this compound mediated via a receptor independent of the benzodiazepine receptor. Several neurochemical parameters, such as GABA-ratio, photo shift, chloride shift and TBPS shift (Ehlert *et al.*, 1982; Karobath *et al.*, 1983; Wood *et al.*, 1984) are consistent with our findings that diazepam, zopiclone, CL 218,872 and PK 8165 have agonist or partial agonist actions on benzodiazepine receptors. A discrepancy, however, was found between the lack of direct effects on responses to GABA on mouse spinal cord and hemisphere neurones for the benzodiazepine-receptor ligands CGS 9896 and ZK 91296 and their anxiolytic and anticonvulsant actions (Yokoyama *et al.*, 1982; Petrack *et al.*, 1983; Petersen *et al.*, 1984). Neurochemical studies predicted a partial agonistic profile for CGS 9896 (Gee & Yamamura, 1982) and ZK 91296 (Stephens *et al.*, 1984; Petersen *et al.*, 1984). However, the benzodiazepine receptor antagonist effects of CGS 9896 and ZK 91296 obtained in this study are consistent with the behavioural findings (Brown *et al.*, 1984; Bernard *et al.*, 1985; Klockgether *et al.*, 1985). The existence of an endogenous ligand for the benzodiazepine receptor could explain the lack of an intrinsic effect of CGS 9896 and ZK 91296 in our experimental system, as opposed to behavioural and neurochemical findings. The interaction of an endogenous ligand with anxiogenic and proconvulsant activity could explain the absence of intrinsic activity *in vitro* and the observed selective agonist activity *in vivo* for CGS 9896 and ZK 91296. The existence of an endogenous anxiogenic benzodiazepine receptor ligand has indeed been suggested (Guidotti *et al.*, 1982; Costa & Guidotti, 1985).

Another hypothesis for the non-sedative anxiolytic profile of benzodiazepine receptor ligands could be the mediation of their pharmacological effects via

specific benzodiazepine receptor subtypes. Only for CL 218,872 (Klepner *et al.*, 1979; Squires *et al.*, 1979) and CGS 9896 (Boast *et al.*, 1985) have different affinities for benzodiazepine receptors in different brain regions been proposed. For CL 218,872, its specific interaction with a cerebellar subtype of benzodiazepine receptor (Type I) has been suggested as the basis for its selective anxiolytic effect (Klepner *et al.*, 1979). However, the pharmacological significance of this receptor subtype has been challenged because the discriminitive properties of CL 218,872 disappear at 37°C (Gee *et al.*, 1982). Moreover, this triazolopyridazine concentration-dependently enhanced responses to GABA on our non-cerebellar neurones. A recent study, using autoradiographic techniques, showed a significantly different affinity for CGS 9896, less than 2 times higher, for cerebellar cortex than for dentate gyrus (Boast *et al.*, 1985). Practical implications of such a small difference in affinity seem unlikely, and moreover, the cerebellum is a brain region not believed to be related to anxiety but is rather involved in motor function.

Our findings failed to indicate any interaction of PK 9084 or buspirone with the benzodiazepine receptor complex. While PK 9084 is poorly established as an anxioselective agent, buspirone has been shown to be efficaceous in several clinical trials (Goldberg & Finnerty, 1979; Goldberg & Finnerty, 1982; Rickels *et al.*, 1982). These results are consistent with the conclusion that anxiety can be treated by modification of non-GABAergic neurotransmission, possibly including 5-hydroxytryptaminergic, noradrenergic and dopaminergic systems (for review, see: Hoehn-Saric, 1982; Braestrup, 1982).

In conclusion, in a system testing postsynaptic responses to GABA, two clearly sedative anxiolytics, diazepam and zopiclone, were found to be full benzodiazepine receptor agonists. CL 218,872 and PK 8165, initially thought to be non-sedative anxiolytics but later shown to be sedative anxiolytics, were found to be partial benzodiazepine receptor agonists. These results are consistent with neurochemical and behavioural findings for these compounds and suggest that enhancement of GABAergic inhibition by benzodiazepine receptor agonists is anxiolytic and sedative. However, the non-sedative anxiolytics CGS 9896 and ZK 91296 were shown to be benzodiazepine receptor antagonists. PK 9084 and buspirone were found to be free of any interaction with the benzodiazepine receptor complex. Our electrophysiological findings together with other neurochemical and behavioural data suggest that facilitation of GABA-ergic neurotransmission may be sufficient, but not necessary for anxiolysis, that benzodiazepine receptor agonists produce sedative anxiolysis and that benzodiazepine receptor antagonists or weak partial agonists may produce non-sedative anxiolysis.

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