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BENZODIAZEPINE RECEPTOR LIGAND ACTIONS ON GABA RESPONSES. BENZODIAZEPINES, CL 218872, ZOPICLONE

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The effects on GABA (4-aminobutyric acid) responses of several benzodiazepine and nonbenzodiazepine benzodiazepine receptor ligands were examined using mouse spinal cord neurons in dissociated cell culture. Diazepam, clonazepam and nitrazepam enhanced GABA responses potently at low nanomolar concentrations. Diazepam and clonazepam were most potent with significant enhancement at 1nM and peak enhancement of 80.7 and 50.2% at 10 nM respectively. Nitrazepam was least potent with no significant enhancement at 1 nM and enhancement of only 20.7% at 10 nM. The benzodiazepine antagonist, Ro 15-1788, blocked enhancement by diazepam but also weakly enhanced GABA responses at low micromolar concentrations, suggesting partial agonist activity. The convulsant benzodiazepine, Ro 5-4864, did not enhance GABA responses at any concentration tested but antagonized GABA responses at 1 μM and above. Diazepam shifted GABA dose-response curves to the left by decreasing the apparent K_D but without altering the apparent Vmax (Lineweaver-Burk analysis). Two nonbenzodiazepine anxiolytic/anticonvulsants, CL 218872 and zopiclone, were weak enhancers of GABA responses at high nanomolar concentrations. These results with benzodiazepines, CL 218872 and zoplicone are consistent with their anxiolytic and anticonvulsant profile in vivo and with studies of their effects upon low affinity GABA binding in vitro.

Spinal cord neuronal cultures Benzodiazepine GABA receptor complex Anticonvulsants Anxiolytics Amino acid electrophysiology

1. Introduction

A diversity of actions have been found for benzodiazepines in central nervous system, including modulation of presynaptic calcium uptake (Leslie et al., 1980; Paul et al., 1983), inhibition of adenosine uptake (Phillis et al., 1980), blockade of responses of cortical neurons to excitant amino acids (Assumpcao et al., 1979), inhibition of glycine antagonist binding (Young et al., 1974), alteration of sodium permeabilities (Schwarz and Spielmann, 1983), as well as interactions with numerous enzymatic activities in the brain (Haefely et al., 1981). Recently however, the most studied effects of benzodiazepines have been on synaptic inhibition mediated by GABA (see Haefely and Polc, 1983). Benzodiazepines have been demonstrated to enhance GABA neurotransmission (Schmidt et al., 1967; Haefely et al., 1975; Costa et al., 1975; Choi et al., 1977; Macdonald and Barker, 1978), and specific binding sites for radiolabelled benzodiazepines in brain membrane preparations have been described (Braestrup and Squires, 1977; Möhler and Okada, 1977). Numerous quantitative studies have been published describing interactions of a variety of benzodiazepines with these binding sites (see Haefely et al., 1981; Möhler and Richards, 1983; Skerritt et al., 1982a; Skerritt and Johnston, 1983). Furthermore, certain nonbenzodiazepine compounds including CL 218872 and zopiclone also bind with high affinity to benzodi-

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azepine receptors and have anxiolytic and anticonvulsant actions in common with benzodiazepines (Blanchard et al., 1979; Klepner et al., 1979). The triazolopyridazine, CL 218872, has been considered a selective ligand for a subclass of benzodiazepine receptor (Lippa et al., 1979). While there have been several studies of the binding properties and in vivo activities of CL 218872 and the piperazine carboxylate zopiclone (Blanchard et al., 1979; Klepner et al., 1979; Lippa et al., 1979), little is known of their physiological actions.

Many physiological studies of the effects of benzodiazepine receptor ligands upon GABA responses have involved the application of benzodiazepines by iontophoresis (Macdonald and Barker, 1978; Nestoros, 1980), and thus, the actual drug concentrations applied were unknown. Since iontophoresis requires the use of concentrated drug solutions (and thus water soluble compounds), only a few of the compounds of experimental or clinical interest have been examined. To surmount many of these difficulties, mouse spinal cord (SC) neurons in cell culture have been used to study anticonvulsant drug actions (see Macdonald, 1983a). Intracellular recordings from single neurons can be made and known concentrations of drugs can be applied directly to the cell surface by local pressure ejection (miniperfusion). Benzodiazepines have been shown previously to selectively enhance GABA responses of chick and mouse SC neurons in cell culture (Choi et al., 1977; Macdonald and Barker, 1978). Furthermore, in addition to benzodiazepines, the actions of barbiturates, hydantoins, iminostilbenes and valproic acid have been studied using this preparation (see Macdonald, 1983b).

In the present paper, we report studies on alterations of GABA responses by known concentrations of benzodiazepines, CL 218872 and zopiclone. In the following paper (Skerritt and Macdonald, 1984a), the effects of certain β-carboline esters and purines, benzodiazepine receptor ligands with a wide spectrum of behavioral activities, upon GABA responses of SC neurons in cell culture are presented. The results with benzodiazepines, CL 218872, zopiclone, β-carbolines and purines correlate well both qualitatively and quantitatively with the known neurochemical and behavioral effects of these compounds.

2. Materials and methods

Cell cultures were prepared from spinal cords with attached dorsal root ganglia from 12 to 14 day old mouse embryos, as described earlier (Ransom et al., 1977; Nowak et al., 1982) and maintained for 5-8 weeks prior to electrophysiological experiments. Spinal cord neurons were penetrated under visual guidance on the modified stage of an inverted phase-contrast microscope, using high resistance (25-40 MΩ) glass micropipettes filled with either 4 M potassium acetate (KAc) or 3 M potassium chloride (KCl). Cultures were bathed in a phosphate buffered saline containing an elevated magnesium ion concentration in order to suppress spontaneous activity (composition in mM: NaCl 143.4, KCl 4.2, $CaCl_2$ 0.9, $MgCl_2$ 10.0 and glucose 5.6 in 9.5 mM sodium phosphate buffer at pH 7.35-7.40), and maintained at 33-34°C for electrophysiological investigations. Using a conventional bridge circuit, simultaneous current injection and membrane potential measurement were possible with a single recording micropipette; data were recorded on a 6-channel Gould polygraph.

GABA (0.5 M, pH 3.2) or S-glutamate (0.5 M, pH 10.0) were applied iontophoretically using 400 ms duration rectangular current pulses (+0.5 to +20 nA for GABA, −5 to −80 nA for S-glutamate) at 4 s intervals. Benzodiazepines, CL 218872, zopiclone or vehicle (0.1% or less dimethylsulfoxide) were applied by miniperfusion (0.2 psi ejection pressure, 30 s duration) from pipettes positioned 15-100 μm from the soma of the cell under study. Previous studies using miniperfusion have demonstrated that drug concentrations achieved at the neuronal membrane are similar to those in the miniperfusion pipette (Heyer et al., 1982). Nonetheless, a small dilutional reduction in the local concentration of drug cannot be ruled out. Application of medium containing vehicle only did not alter responses to iontophoretically applied GABA or S-glutamate. During assessment of drug action upon GABA responses, cells were impaled with 3 M KCl-containing micropipettes, and for glutamate iontophoresis or membrane conductance studies, 4 M KAc-containing micropipettes were used. GABA responses of 6-9 mV amplitude were

evoked following membrane hyperpolarization (to −70 to −90 mV), and glutamate responses of similar amplitude obtained near resting membrane potential (−60 to −70 mV) were used for assessment of drug actions. Data were accepted only if the amino acid responses returned to control levels within five min following removal of the drug-containing pipette from the vicinity of the cell under study. For assessment of possible antagonism of diazepam effects, the putative antagonist was applied for 30 s by ejection before application of diazepam followed by diffusion during diazepam application.

When KAc recording micropipettes were used, application of GABA of SC neurons rapidly and reversibly produced membrane hyperpolarization and an increase in membrane conductance. Use of KCl-containing recording micropipettes allows chloride ions to enter the cell, changing the equilibrium potential for chloride ions from about −65 mV to −20 mV, and GABA responses become depolarizing (Nowak et al., 1982).

Diazepam, nitrazepam, clonazepam, Ro 5-4864 and Ro 15-1788 (flumazepil) were obtained from Hoffman-LaRoche, Nutley, NJ, USA. CL 218872 [3-methyl-6-[3-trifluromethylphenyl]1,2,4-triazolo[4,3-b]pyridazine] was provided by Lederle Laboratories, NY, USA, and zopiclone [6-(5-chloro-2-pyridyl)-6,7-dihydro-7-oxo-5H-pyrrolo[3,4-b]pyrazin5-yl]-4-methyl-1-piperazine carboxylate was a gift of Rhone-Pulenc Recherches, France. GABA and S-glutamic acid were purchased from Sigma.

3. Results

Nitrazepam, clonazepam and diazepam reversibly enhanced responses of SC neurons to iontophoretically applied GABA in a concentration-dependent fashion (fig. 1). Diazepam (fig. 1, filled circles) produced significant enhancement of GABA responses at 1 nM. At 100 nM, diazepam enhanced GABA responses by at least 15% in all 113 cells studied at this concentration with a mean enhancement 65 ± 3%. While there was some cell to cell variation in the extent of enhancement by diazepam, little variation in the mean enhancement produced by diazepam between cultures (6-12 cells per culture) occured. Maximal enhancement (80.7 ± 9.7%) for diazepam occurred at 10 nM,

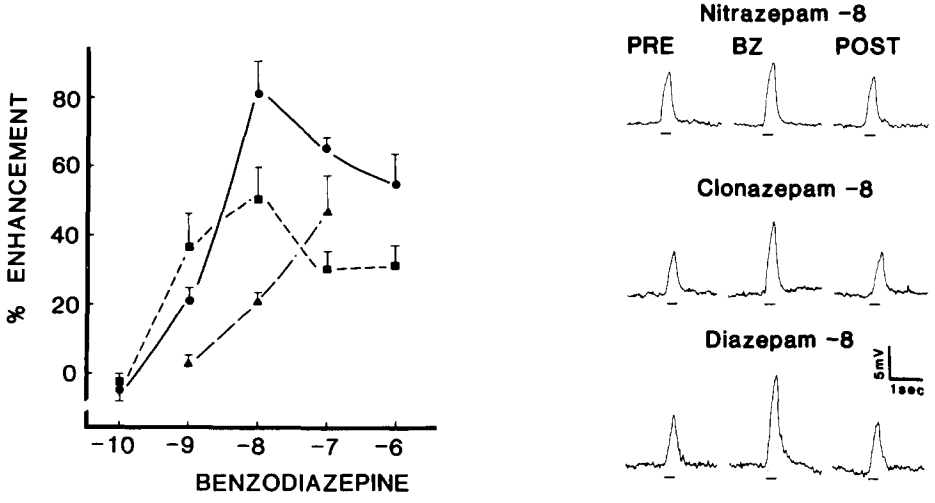

Fig. 1. Concentration-dependent enhancement of GABA responses in SC neurons by diazepam (circles), clonazepam (squares) and nitrazepam (triangles). Drug concentrations shown are logarithm molar. Data shown are means (±S.E.M.) for effects in 7 or more cells at each drug and concentration shown, with at least 3 cultures used for the study of each drug. Specimen records on right show diazepam (membrane potential, MP = −79 mV), clonazepam (MP = −80 mV) and nitrazepam (MP = −76 MV) enhancement of GABA responses at 10 nM. Post responses were obtained 2 min following removal of benzodiazepine-containing pipette. KCl recordings were used.

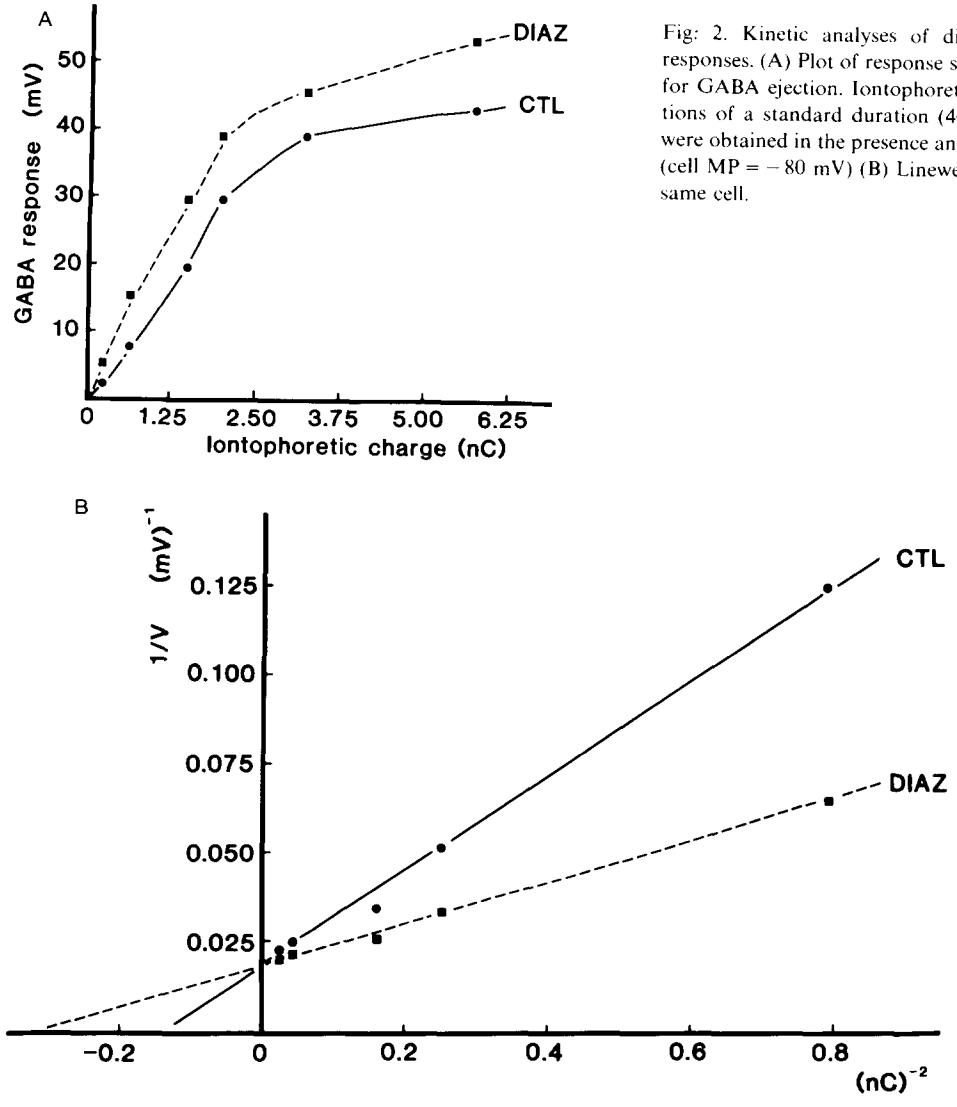

Fig: 2. Kinetic analyses of diazepam effects upon GABA responses. (A) Plot of response size versus iontophoretic charge for GABA ejection. Iontophoretic current was varied for ejections of a standard duration (400 ms), and GABA responses were obtained in the presence and absence of 100 nM diazepam (cell MP = − 80 mV) (B) Lineweaver-Burk plot of data for the same cell.

while enhancement at higher diazepam concentrations decreased slightly. Clonazepam enhanced GABA responses over a similar concentration range (fig. 1, filled squares); however, the maximal enhancement by clonazepam (at 10 nM, 50.2 ± 10.0%) was somewhat lower. Nitrazepam was less potent than either diazepam or clonazepam with significant enhancement (20.7 ± 2.2%) occurring at 10 nM but not at 1 nM (fig. 1, filled triangles).

GABA dose-response curves were obtained by applying increasing current through a GABA iontophoretic pipette at a constant pulse duration (400 ms). GABA response amplitude (mV) was then plotted against iontophoretic charge (current times duration; nanocoulombs, nC). Diazepam (100 nM) enhanced the GABA responses at all GABA charges applied (fig. 2A), thus shifting the GABA dose-response curve to the left (4 cells). For kinetic analysis, Lineweaver-Burk plots were made (fig. 2B). Since GABA dose-response curves on mouse spinal cord neurons in cell culture are sigmoidal and have log-log slopes of about 2.0 (Nowak et al., 1982), the reciprocal of GABA response amplitude was plotted against the re-

TABLE 1

Effects of Ro 15-1788 upon basal and diazepam-enhanced GABA responses on mouse spinal cord neurons in cell culture.

	Number of cells studied (n)	GABA response % control
(1)Ro 15-1788 100 nM	5	92.4 ± 2.4
Diazepam (DZ) 100 nM	5	194.4 ± 7.8 [a]
DZ 100 μM + Ro 15-1788 100 nM	4	165.0 ± 7.1 [a,b]
(2)Ro 15-1788 1 μM	15	121.9 ± 2.9 [a]
Diazepam (DZ) 100 nM	6	162.0 ± 11.0 [a]
DZ 100 nM + Ro 15-1788 1 μM	6	124.4 ± 6.0 [a,b]
(3)Ro 15-1788 10 μM	9	120.4 ± 4.2 [a]
Diazepam 100 nM	9	172.2 ± 13.3 [a]
DZ 100 nM + Ro 15-1788 10 μM	6	129.5 ± 4.6 [a,b]

[a] $P < 0.01$ from vehicle, [b] $P < 0.02$ from 100 nM diazepam, Student's 2-tailed t-test.

ciprocal of the square of the GABA iontophoretic charge. The Lineweaver-Burk plots were linear with and without diazepam and intersected the ordinate at similar values (no change in apparent V_{max}). However, diazepam reduced the abcissa intercept (reduction of apparent K_D).

Ro 15-1788 (100 nM), a benzodiazepine antagonist (Hunkeler et al., 1981), produced partial blockade of enhancement of GABA responses by 100 nM diazepam. Higher concentrations (1-10 μM) of Ro 15-1788 weakly enhanced GABA responses but blocked their further enhancement by diazepam (table 1). The anomalous benzodiazepine, Ro 5-4864, which lacks the usual behavioural and clinical profile of diazepam, clonazepam or nitrazepam (Zbinden and Randall, 1967), did not enhance GABA responses. At 1 μM Ro 5-4864 inhibited GABA responses by 23.8 ± 2.9% (9 cells), and at 10 μM, the compound produced 58.1 ± 2.0% inhibition (15 cells).

CL 218872 failed to alter GABA responses at 100 nM (99.2 ± 1.6% control, n = 11 cells) but weakly enhanced responses at 1μM (13.8 ± 2.7% enhancement, n = 9 cells, $P < 0.001$ compared to vehicle alone) (fig. 3). CL 218872 (100 nM) did not alter the enhancement of GABA responses by nitrazepam (100 nM) in 5 cells studied (nitrazepam enhanced GABA responses 156 ± 16% control; nitrazepam enhanced GABA responses with CL 218872 151 ± 14% control). Zopiclone (100 nM) enhanced GABA responses (fig. 3) by 20.4 ± 2.3% (n = 14 cells).

In no cell studied did any of the benzodiazepines, CL 218872, or zopiclone alter resting membrane potential or conductance. Several benzodiazepines were applied at high concentrations for their possible effects on responses of SC neurons to the excitatory neurotransmitter S-gluta-

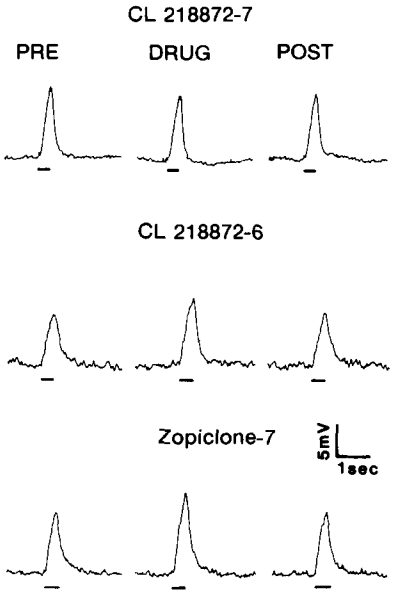

Fig. 3. Effects of CL 218872 and zopiclone upon GABA responses of SC neurons. CL 218872 at 100 nM (MP = −70 mV) failed to alter GABA responses in any cell tested, while 1 μM (MP = −72 mV) weakly enhanced such responses. Zopiclone 100 nM (MP = −75 mV) also weakly enhanced GABA responses.

mate. However, neither diazepam (1 M: 98.8 ± 1.0% control, n = 10 cells), clonazepam (1 μM: 101.7 ± 2.0% control, 6 cells), Ro 15-1788 (1 M: 101.6 ± 0.6% control, 6 cells), nor Ro 5-4846 (10 M: 97.4 ± 1.2% control, 5 cells) altered glutamate responses.

4. Discussion

For an identified effect of a benzodiazepine to have relevance to its therapeutic mechanism, such an effect must occur at concentrations found in the central nervous system during therapy. While serum levels of diazepam are generally in the low micromolar range, diazepam is highly (94-99%) bound to plasma proteins, and the free plasma and cerebrospinal fluid (CSF) concentrations of diazepam are likely between 5-120 nM (see Eadie and Tyrer, 1980). Although daily doses and usual effective serum levels of clonazepam are lower than those for diazepam, the degree of plasma protein binding is lower (55-88%), and so the likely range of clonazepam CSF concentration is similar to that of diazepam. Similar reasoning would suggest that effective free serum and CSF nitrazepam concentrations are about 2-fold higher than those for clonazepam or diazepam (Rieder, 1973). Only some of the actions of benzodiazepines occur at these nanomolar levels. At these concentrations diazepam stimulated synaptosomal calcium uptake (Paul et al., 1983). One benzodiazepine, midazolam, augmented a calcium-dependent potassium conductance in CA_1 hippocampal pyramidal neurons at 1-10 nM (Carlen et al., 1983), and at similar concentrations, flurazepam and diazepam have been shown to enhance GABA effects in cultured mouse spinal and cortical neurons respectively (MacDonald and Barker, 1982; White et al., 1981). Indeed, several studies have clearly demonstrated either (or both) enhancement or depression of GABA responses by benzodiazepines (Macdonald and Barker, 1978; MacDonald and Barker, 1982).

The present results indicate that three benzodiazepines used widely in the therapy of certain types of epilepsy enhanced GABA responses over concentration ranges coincident with their expected CSF levels during treatment. While no enhancement of GABA responses by diazepam occurred at subtherapeutic concentrations (0.1 nM), significant enhancement occurred at low therapeutic concentrations with a plateau effect at higher levels (10 nM-1 μM). Similar results were obtained with clonazepam. While the affinity of clonazepam for benzodiazepine receptors is severalfold higher than for diazepam (Braestrup and Squires, 1977), clonazepam is not a more potent enhancer of GABA binding than diazepam (Skerritt and Johnston, 1983). Nitrazepam was a less potent enhancer of GABA responses, in agreement with the greater CSF concentrations calculated for anticonvulsant activity and neurochemical results. It should be noted, however, that benzodiazepines of vastly differing receptor affinities have been found to differ correspondingly in their GABA potentiation in the cerebral cortex in vivo (Nestoros, 1980).

Importantly, enhancement of GABA responses by diazepam was blocked by the benzodiazepine antagonist, Ro 15-1788, which blocks the anticonvulsant action of diazepam (Hunkeler et al., 1981). At higher concentrations, Ro 15-1788 enhanced GABA responses, confirming partial agonist activity (Skerritt and Macdonald, 1984b). In addition, the 4′-choro derivative of diazepam, Ro 5-4864, which does not have the usual anticonvulsant spectrum of diazepam and instead has some convulsant activity, antagonized rather than enhanced GABA responses.

Based on Lineweaver-Burk analysis, the enhancement of GABA responses by diazepam appeared to be due to an enhancement of the affinity of GABA receptors, consistent with results obtained using binding techniques (Skerritt et al., 1982b). These results taken with neurochemical data (Skerritt and Johnston, 1983; Skerritt et al., 1982a,b) would support the notion that enhancement of GABA responses by benzodiazepines arises from an interaction with a specific benzodiazepine receptor.

Zopiclone, a nonbenzodiazepine benzodiazepine receptor ligand with anxiolytic and anticonvulsant activity (Blanchard et al., 1979), weakly, but significantly, enhanced GABA responses of SC neurons. In binding studies, 100 nM zopiclone

produced less enhancement of GABA binding than similar diazepam, clonazepam or nitrazepam concentrations (Skerritt and Johnston, 1983). CL 218872 was a very weak enhancer of GABA responses on SC neurons. Others (Owen et al., 1982), however, found that CL 218872 (10 μM) failed to influence GABA responses in cultured SC neurons. Although it is a moderately potent antagonist of pentylenetetrazole-induced seizures, CL 218872, unlike diazepam, has little activity against bicuculline seizures and little sedative activity in the rotorod test in mice (Klepner et al., 1979). It has been suggested that CL 218872 may distinguish between benzodiazepine receptor subclasses with differing brain regional distribution and affinities (Klepner et al., 1979; Lippa et al., 1979; Regan et al., 1981). CL 218872 enhanced GABA binding to membranes from whole rat brains with similar potency and efficacy to diazepam (Skerritt and Johnston, 1983). However, in the spinal cord the binding properties of [^{3}H]CL 218872 and the interactions of this triazolopyridazine with GABA receptors have not yet been investigated (H.I. Yamamura, personal communication).

CL 218872 has also been reported to antagonize diazepam-induced loss of righting reflex in mice, suggesting that CL 218872 may have some partial agonist activity (Gee et al., 1983). However, no antagonism of nitrazepam enhancement of GABA responses was observed using 100 nM CL 218872. In view of the high benzodiazepine doses required to produce loss of righting reflex, it may be possible that a non-GABA related mechanism may be involved in this case.

Several classes of compound known to interact with benzodiazepine binding sites modified GABA responses of SC neurons in primary dissociated cell culture. Further, such interaction seemed to be selective in that none of the compounds affected membrane potential or conductance and none of the drugs tested on S-glutamate responses (diazepam, clonazepam, Ro 5-4864 and Ro 15-1788) altered the effects of the excitatory neurotransmitter. While benzodiazepines have been reported to antagonize excitatory amino acid responses in the rat cerebral cortex, the molar concentrations of benzodiazepine required are unknown (Assumpacao et al., 1979). Diazepam, clonazepam and nitrazepam, however, enhanced GABA at low nanomolar concentrations, coincident with their expected free serum therapeutic ranges, and diazepam enhancement was blocked by the benzodiazepine antagonist Ro 15-1788. These electrophysiological results together with other neurochemical and behavioral data suggest that regulation of GABA receptor function is an important mechanism in the therapeutic actions of the benzodiazepines. SC neurons in cell culture appear useful in the assessment of novel benzodiazepine receptor ligands and of molecular influences upon GABA-benzodiazepine receptor complexes.

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