

BENZODIAZEPINE RECEPTOR LIGAND ACTIONS ON GABA RESPONSES. β -CARBOLINES, PURINES

JOHN H. SKERRITT and ROBERT L. MACDONALD *

Department of Neurology, University of Michigan, Ann Arbor, MI 48109, U.S.A.

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The effects of several β -carboline and purine ligands for benzodiazepine receptors were studied upon GABA (4-aminobutyric acid) responses and upon diazepam enhancement of GABA responses, using mouse spinal cord neurons in dissociated cell culture. While the potent convulsant β -carboline DMCM (methyl-6,7-dimethoxy-4-ethyl-carboline-3-carboxylate), reduced GABA responses, methyl-carboline-3-carboxylate (β CCMe) and the corresponding ethyl ester (β CCeEt) did not alter GABA responses. The propyl ester (β CCPr) enhanced GABA responses in a concentration-dependent fashion, while both β CCMe and β CCPr blocked diazepam enhancement of GABA responses. β CCPr may thus have partial agonist activity. Two purines with moderate benzodiazepine receptor affinity, 1-methylisoguanosine (MeIG) and 6-dimethylaminopurine (DMAP), weakly enhanced GABA responses. MeIG also significantly antagonized diazepam enhancement of GABA responses. Inosine and hypoxanthine had no apparent actions upon GABA responses or upon diazepam enhancement of such responses. The results with β -carbolines are consistent with their behavioural profile in vivo and with neurochemical studies of their effects upon GABA-benzodiazepine receptor complexes. Furthermore, certain purines are also able to interact with these complexes.

Benzodiazepine-GABA receptor complex Spinal cord neuronal cultures β -Carboline-3-carboxylates
Amino acid electrophysiology Purines

1. Introduction

The identification of specific binding sites in the central nervous system for benzodiazepines (Braestrup and Squires, 1977; Möhler and Okada, 1977) has prompted searches for endogenous ligands for these sites. In addition to various peptides (Chiu and Rosenberg, 1981; Guidotti et al., 1983), β -carboline-3-carboxylate ethyl ester (β CCeEt) (Braestrup et al., 1980) and certain purines including inosine and hypoxanthine (Skolnick et al., 1978; Asano and Spector, 1979; Möhler et al., 1979) have been suggested to serve such a role. While some β -carbolines and purines

also have benzodiazepine agonist or antagonist-like activity, thorough evidence that similar endogenous compounds modulate benzodiazepine receptors in vivo is lacking. β CCeEt has been shown more recently to be an extraction artifact and unlikely to occur in the central nervous system (Braestrup and Nielsen, 1980; Möhler, 1981).

However, the alkyl esters of β -carboline-3-carboxylic acid are in general potent ligands for benzodiazepine binding sites and as a class have a range of pharmacological profiles. While the compounds act as benzodiazepine antagonists, some are anxiogenic and potent convulsants (e.g. methyl-6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate, DMCM), others proconvulsant (e.g. β CCeEt) and compounds with larger alkyl substitutions such as β -carboline-3-carboxylate propylester (β CCPr) have anticonvulsant activity (Cepeda

* To whom all correspondence should be addressed: Neuroscience Laboratory Building, 1103 East Huron, Ann Arbor, MI 48109, U.S.A.

et al., 1981; Braestrup et al., 1982; Oakley and Jones, 1982; Valin et al., 1982; Jensen et al., 1983). As such, they have proven valuable in the study of benzodiazepine receptor multiplicity and function.

Inosine and hypoxanthine are relatively weak inhibitors of benzodiazepine binding (Huang et al., 1980; Skerritt et al., 1982b), although it is possible that brain levels sufficiently high to interact with benzodiazepine receptors could be achieved *in vivo* under certain physiological conditions. Further, these purines do have benzodiazepine-like activity in some behavioural assays (Skolnick et al., 1979), and like clonazepam bind selectively to central rather than peripheral benzodiazepine receptors (Skerritt et al., 1982a).

In the previous paper (Skerritt and Macdonald, 1983) we demonstrated that certain benzodiazepines and nonbenzodiazepine anticonvulsant/anxiolytics enhanced GABA responses in mouse spinal cord (SC) neurons in cell culture at concentrations likely to be found in cerebrospinal fluid during therapy. Further, such enhancement was antagonized by Ro 15-1788, a benzodiazepine antagonist. In the present study we have investigated the effects of β -carboline and purine ligands for benzodiazepine receptors and have demonstrated a correlation between the *in vivo* actions of these substances and their effects upon GABA responses of mouse SC neurons in cell culture.

2. Materials and methods

Methods used in the present study are outlined in the accompanying paper (Skerritt and Mac-

donald, 1984). The β -carboline esters used were a generous gift of Dr. C. Braestrup, A/S Ferrosan, Denmark. They were dissolved in vehicle (0.1% or less dimethylsulfoxide) and applied to neurons in the same manner as the benzodiazepines. Purines were dissolved directly in recording medium. 1-Methylisoguanosine was a gift of Roche Products, Australia, and inosine, hypoxanthine and 6,6-dimethylaminopurine were purchased from Sigma.

3. Results

The convulsant DMCM at 100 nM reduced GABA responses in all SC neurons tested (fig. 1A, table 1). While diazepam reversibly enhanced responses of SC neurons to iontophoretically applied GABA in a concentration-dependent fashion (see previous paper), neither β -carboline-3-carboxylic acid methyl ester (β CCMe) (fig. 1B) nor β CCEt (fig. 1C) altered GABA responses at 100 nM or 10 μ M (table 1). The corresponding propyl ester (β CCPr) weakly but significantly enhanced GABA responses at 100 nM (table 1). β CCPr enhanced GABA responses in a concentration-dependent fashion (fig. 2, table 1); 10 nM was ineffective while enhancement at 10 μ M ($84 \pm 17\%$, $n = 10$ cells) (fig. 1D) was not statistically significantly different from maximal enhancement by diazepam ($81 \pm 10\%$ at 10 nM, $n = 13$ cells). β CCMe (100 nM) markedly decreased diazepam enhancement of GABA responses on all 6 cells tested (fig. 3A, table 1) while β CCEt (100 nM)

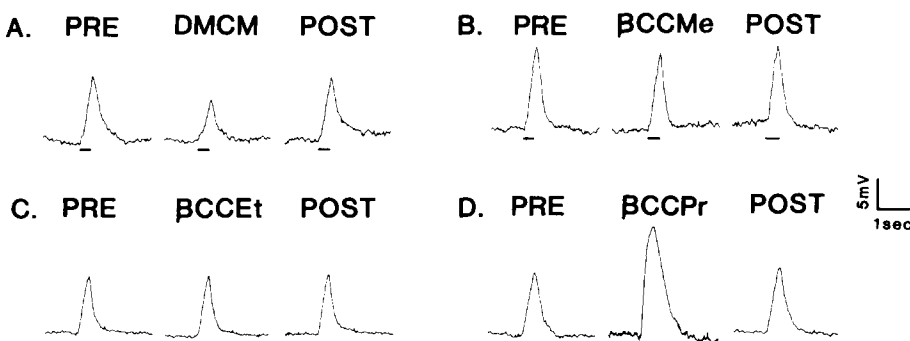


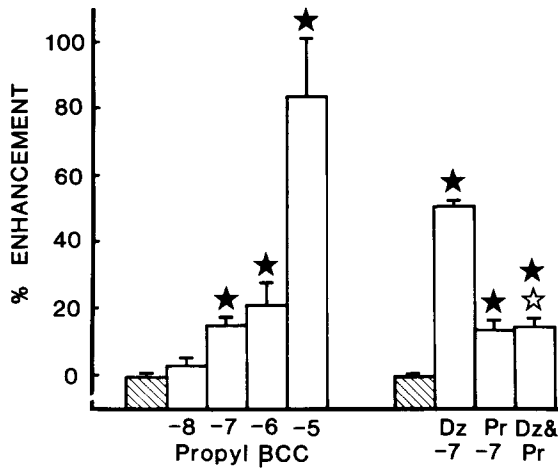
Fig. 1. Reversible modulation of GABA responses by β -carboline esters: (A) DMCM 100 nM (MP = -75 mV). (B) β CCMe 100 nM (MP = -72 mV). (C) β CCEt 100 nM (MP = -78 mV) (D) β CCPr 10 μ M (MP = -82 mV). A-D represent separate cells.

TABLE 1

Effects of β -carboline esters upon GABA responses of cultured spinal cord neurons and their enhancement by diazepam (100 nM).

	(n)	% Control GABA response	(n)	% Inhibition of diazepam (100 nM) enhancement of GABA response	% cells with > 20% inhibition of DZ enhancement of GABA response
DMCM 100 nM	9	53.3 \pm 5.4 ^a		n.t.	n.t.
β CCMe 100 nM	6	98.0 \pm 3.9	6	76.5 \pm 2.6 ^b	100%
10 μ M	6	106.7 \pm 3.4		n.t.	n.t.
β CCEt 100 nM	13	95.2 \pm 2.9	9	24.0 \pm 9.7	44%
10 μ M	8	101.9 \pm 3.9		n.t.	n.t.
β CCPr 100 nM	18	114.5 \pm 2.9 ^a	14	86.5 \pm 6.5 ^b	85%
10 μ M	10	183.7 \pm 17.3 ^a		n.t.	n.t.

^a P < 0.001 from control (responses to buffer containing vehicle only), Student's 2-tailed t-test. ^b P < 0.001 from mean enhancement by diazepam. (n) = number of cells studied. n.t. = not tested.



antagonized diazepam (by more than 20%) in 4 of 9 cells studied (table 1). β CCPr (100 nM) reduced further enhancement of responses by diazepam (fig. 3B, table 1).

The purine, 1-methylisoguanosine (MeIG), enhanced GABA responses weakly (fig. 4A) but in a

Fig. 2. Concentration-dependence of β CCPr enhancement of GABA responses. Drug concentrations shown are logarithm molar. Control bars are hatched. β CCPr (Pr, 100 nM) significantly antagonized enhancement of GABA responses by diazepam (DZ, 100 nM). Filled stars: P < 0.05 from control (vehicle) GABA responses, Student's t-test, 2-tailed. Empty stars: P < 0.05 from diazepam (DZ, 100 nM).

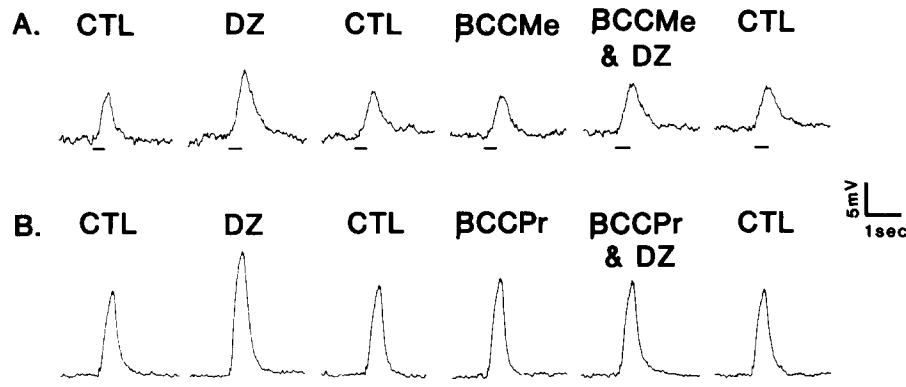


Fig. 3. Blockade of diazepam enhancement of GABA responses by β -carboline esters. (A) Diazepam (100 nM) reversibly enhanced GABA responses, and β CCMe (100 nM), which itself did not alter GABA responses, blocked enhancement by diazepam. (B) Enhancement of GABA responses by 100 nM diazepam was blocked by β CCPr (100 nM) which itself produced a weak enhancement of GABA responses in SC neurons. GABA responses returned to control values within 6 min following removal of the β -carboline esters. Cell A, MP = -72 mV; cell B, MP = -75 mV.

concentration-dependent fashion (table 2). Significant enhancement (8.6%) was produced at 10 μM MeIG, with 22% enhancement being produced at 500 μM . The purine, 6-dimethylaminopurine (DMAP), at 500 μM also enhanced GABA responses (by more than 10%) on 4 of 6 cells studied (fig. 4B, table 2). Neither inosine nor hypoxanthine enhanced GABA responses at 100 μM or 1 mM; at 1 mM GABA responses were weakly inhibited (table 2). At 100 μM , MeIG enhanced GABA responses and significantly reduced the further enhancement of GABA responses by diazepam (fig. 4C, table 2). Further, inosine (1 mM) failed to alter enhancement of GABA responses by diazepam (100 nM) (fig. 4D, table 2).

In no cells studied did any of the β -carbolines or purines alter resting membrane potential or conductance. Furthermore, at a concentration which enhanced GABA responses (10 μM), βCCPr , like diazepam, failed to affect glutamate responses of SC neurons (responses $97.4 \pm 2.4\%$ control, $n = 4$ cells).

4. Discussion

The results obtained on modulation of GABA responses by β -carboline esters are in keeping with their *in vivo* actions and their effects on GABA-benzodiazepine receptor complexes as studied by neurochemical methods. DMCM had potent convulsive activity in mice (Braestrup et al., 1982) and in the present study was found to potently antagonize GABA responses. βCCMe had weak convulsant activity and was anxiogenic in conflict tests in mice, while βCCEt was also anxiogenic and facilitated the convulsant activity of certain compounds, while not itself producing seizures (Braestrup et al., 1982; Oakley and Jones, 1982; Corda et al., 1983; De Carvalho et al., 1983). Neither βCCMe nor βCCEt reduced GABA responses in cultured SC neurons, although in cat spinal cord *in situ* some antagonism has been observed (Polc et al., 1981, 1982). βCCMe reduced diazepam enhancement of GABA responses while βCCEt had a lesser (29% reduction) effect, which

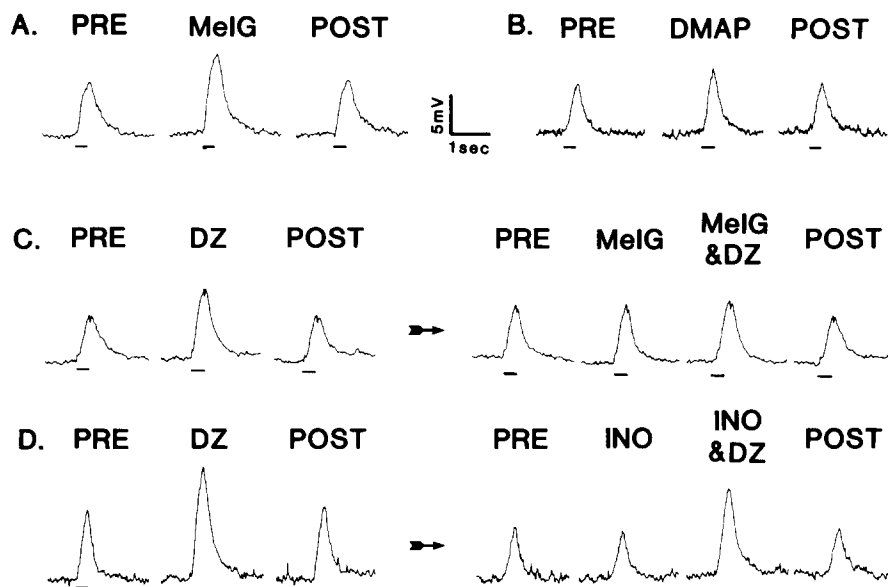


Fig. 4. Effects of certain purines upon GABA responses and their enhancement by diazepam (DZ). (A) 1-Methylisoguanosine (MeIG, 500 μM ; MP = -80 mV) and (B) 6-dimethylaminopurine (DMAP, 500 μM ; MP = -62 mV) reversibly enhanced GABA responses. (C) Diazepam (100 nM) enhancement was reduced by 100 μM MeIG, which itself weakly enhanced GABA responses (MP = -80 mV). (D) Inosine (INO, 100 μM) weakly depressed GABA responses but did not alter their (percentage) enhancement over control levels (PRE), by 100 nM diazepam (MP = -75 mV).

TABLE 2

Effects of purines and diazepam upon GABA responses of cultured spinal cord neurons.

		Number of cells studied (n)	Mean % enhancement	% cells with responses enhanced by more than 10%
(1) l-Methylisoguanosine	10 μ M	10	+8.6 \pm 3.6 ^a	40
	100 μ M	29	+12.8 \pm 2.9 ^b	48
	500 μ M	12	+22.0 \pm 4.2 ^b	83
6-Dimethylaminopurine	500 μ M	6	+25.3 \pm 6.8 ^b	67
	100 μ M	6	+5.2 \pm 2.5	0
Inosine	1 mM	16	-8.6 \pm 3.0 ^a	0
	100 μ M	9	-0.8 \pm 2.5	0
Hypoxanthine	1 mM	8	-15.3 \pm 3.4 ^a	0
	100 μ M	18	+11.8 \pm 2.9 ^a	50
(2) l-Methylisoguanosine	100 μ M	18	+66.5 \pm 5.7 ^b	100
Diazepam (DZ)	100 nM	18	+46.1 \pm 4.6 ^c	88
l-MeIG + DZ		18	+46.1 \pm 4.6 ^c	88
Inosine	1 mM	8	-11.9 \pm 4.9 ^a	0
Diazepam (DZ)	100 nM	8	+70.1 \pm 12.7 ^b	0
Inosine + DZ		8	+68.4 \pm 9.3 ^c	0

^a $P < 0.05$, ^b $P < 0.005$ from cells miniperfused with drug-free control medium, ^c $P < 0.05$ from diazepam (100 nM) alone (Student's t-test, 2-tailed).

failed to achieve statistical significance. Both β CCMe and β CCeT blocked the anticonvulsant activity of diazepam in mice (Oakley and Jones, 1982).

Other neurochemical and behavioural studies have revealed results corresponding to those of this study. GABA markedly inhibited the binding of [³H]DMCM (Braestrup et al., 1982) while β CCMe and β CCeT did not alter basal low affinity GABA binding, but inhibited its enhancement by diazepam (Skerritt et al., 1983). In keeping with neurochemical results, β CCPr and diazepam enhanced GABA responses over a similar concentration range (Skerritt et al., 1983). Physiological investigations reveal it actually is a partial agonist since low concentrations of β CCPr (100 nM), which produced only moderate enhancement of GABA responses, prevented further effects of diazepam (fig. 3). β CCPr inhibited β CCMe seizures (Valin et al., 1982) and antagonized audiogenic seizures in mice (Jensen et al., 1983). The latter study found that proportionately greater occupancy of benzodiazepine receptors by β CCPr than diazepam was needed for its anticonvulsant activity. This could arise from the similar potency but lower efficacy of β CCPr relative to diazepam as an enhancer of GABA actions.

The purines, inosine and hypoxanthine, were the first described putative endogenous benzodiazepine receptor ligands. Inosine has been demonstrated to have benzodiazepine-like activity against pentylentetrazole seizures, yet in certain behavioural models it was able to antagonize diazepam (Skolnick et al., 1979; Crawley et al., 1981). However, at no concentration tested did inosine or hypoxanthine markedly alter GABA responses, and 1 mM inosine failed to alter response enhancement by diazepam. Inosine was a weak inhibitor of benzodiazepine binding in cultured SC neurons (K_i 2.8 mM, Huang et al., 1980) so it cannot be ruled out that higher inosine concentrations (e.g. 10 mM) may have some activity. However, the relevance of such concentrations to the in vivo actions of the purine is questionable. The finding that inosine failed to alter GABA responses is in contrast to a report of the ability of inosine to reverse diazepam inhibition of rat substantia zona reticulata neurons and to produce a diazepam-like inhibition of firing rate at higher dose (Skolnick et al., 1983). However, these effects may not necessarily be mediated by GABA-coupled benzodiazepine receptors. Inosine has also been reported to have direct effects upon membrane potential and conductance of cultured spi-

nal neurons (MacDonald et al., 1980), although in the present experiments no direct effects of inosine or any other purine were observed.

Neurochemical studies have indicated that while the affinities of inosine and hypoxanthine for benzodiazepine antagonist binding were unaltered by 10 μ M GABA, the potencies of certain purines with higher receptor affinities (eg. MeIG, DMAP) could be altered by GABA, albeit in a different manner from that of diazepam (Skerritt et al., 1982b). Micromolar concentrations of these purines produced a moderate but statistically significant concentration-dependent enhancement of GABA responses in cultured SC neurons, while 100 μ M MeIG partially blocked enhancement of GABA responses by diazepam. Certain purines may therefore act as partial agonists at benzodiazepine receptors. Taken together with the apparent selectivity of purines for central rather than peripheral benzodiazepine receptors (Skerritt et al., 1982a), it is possible that purines could be developed with clinically useful selective actions on benzodiazepine receptors.

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