Epilepsia, 30(1):17–25, 1989
Raven Press, Ltd., New York

Effects of Antiepileptic Drugs on GABA Responses and on Reduction of GABA Responses by PTZ and DMCM on Mouse Neurons in Cell Culture

*†Peter P. De Deyn and †Robert L. Macdonald

**Department of Neurology, University of Michigan Medical Center, Ann Arbor, Michigan, U.S.A., and †Research Institute, Born Bunge Foundation, University of Antwerp, Laboratory of Neurochemistry and Neuropharmacology, Antwerp, Belgium*

Summary: The mechanisms of action of antiepileptic drugs effective against generalized absence seizures (antiabsence AEDs) remain uncertain. Antiabsence AEDs are generally effective against seizures induced in experimental animals by pentylenetetrazol (PTZ) and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), drugs which reduce GABAergic inhibition. Thus, antiabsence AEDs have been suggested to enhance GABAergic inhibition. We studied the effects of several AEDs on GABA responses recorded from mouse spinal cord neurons grown in primary dissociated cell culture. Four antiabsence AEDs were included: ethosuximide (ESM), dimethadione (DMO), sodium valproate (VPA), and diazepam (DZP). Two experimental AEDs, CGS 9896 and ZK 91296, with anticonvulsant action against PTZ- or DMCM-induced seizures were also included. Possible effects of the antiabsence and experimental AEDS on PTZ- and DMCM-induced inhibition of GABA responses were also evaluated. PTZ and DMCM reversibly reduced GABA responses in a concentration-dependent manner. PTZ completely inhibited GABA responses at 10 m*M* (IC_{50} of 1.1 m*M*), whereas DMCM-induced inhibition of GABA responses reached a plateau level of 39% of control values at 1 μ*M* (IC_{50} of 33 n*M*). ESM (1,200 μ*M*), DMO (6 m*M*), VPA (200 μ*M*), CGS 9896 (2 μ*M*), and ZK 9896 (2 μ*M*) did not alter GABA responses. DZP enhanced GABA responses in a concentration-dependent manner. The inhibition of GABA responses produced by PTZ 1 m*M* was unaltered by ESM (600 μ*M*), DMO (6 m*M*), CGS 9896 (1 μ*M*), or ZK 9896 (1 μ*M*). Coapplication of VPA (200 μ*M*) and PTZ (1 m*M*) slightly enhanced the PTZ effect. DZP (>10 n*M*), however, reversed the PTZ-induced reduction of GABA responses. The DMCM (250 nM) inhibition of GABA-responses was unaltered by ESM (600 μ*M*), DMO (2 m*M*), or VPA (200 μ*M*). CGS 9896 (2 μ*M*) and ZK 91296 (2 μ*M*), however, antagonized the DMCM effect. DZP (>10 n*M*) significantly reversed the DMCM-induced inhibition of GABA responses. The lack of effect of VPA, ESM, and DMO on postsynaptic GABA responses suggests that direct enhancement of postsynaptic GABA action is not a common mechanism of action of antiabsence AEDs. The AEDs DZP, CGS 9896, and ZK 91296 all reversed DMCM, but not PTZ, reduction of GABA responses, suggesting that these AEDs blocked DMCM seizures by acting at benzodiazepine receptors. However, since only DZP enhanced GABA responses, it is unclear how CGS 9896 and ZK 91296 blocked PTZ seizures. **Key Words:** Anticonvulsants—GABA—Neuron culture—Cell culture—Spinal cord neurons—Convulsants.

A reduction in central nervous system (CNS) GABAergic inhibition has been suggested to be a cause of epilepsy (Meldrum, 1979; Roberts, 1980; Lloyd et al., 1981; Olsen, 1981), and it has been suggested that the mechanism of action of antiepileptic drugs (AEDs) used in the treatment of generalized absence seizures (antiabsence AEDs) may involve enhancement of GABAergic inhibition (Macdonald and McLean, 1986).

The $GABA_A$ receptor is an oligomeric complex containing binding sites for GABA, barbiturates, benzodiazepines, β-carbolines and picrotoxinlike drugs (Olsen, 1981; Olsen and Leeb-Lundberg, 1981). The structure of the $GABA_A$ receptor was recently deduced from cDNA clones and is composed of two α and two β peptide subunits which span the neuronal membrane to form a chloride channel (Schofield et al., 1987). GABA binds to the

Received February 1988; revision accepted June 1988.
Address correspondence and reprint requests to Dr. R. L. Macdonald at University of Michigan, Neuroscience Laboratory Building, 1103 East Huron St., Ann Arbor, MI 48104-1687, U.S.A.

receptor to open the chloride channel, resulting in membrane hyperpolarization and inhibition.

Several convulsants, including bicuculline, picrotoxin, penicillin, D-tubocurarine, pentylenetetrazol (PTZ) and α-keto-δ-guanidino-valeric acid have been shown to be GABA antagonists, blocking the inhibitory action of GABA by interacting with either GABA or picrotoxin binding sites (Curtis et al., 1971; Curtis et al., 1972; Hill et al., 1972; Hill and Simmonds, 1973; Macdonald and Barker, 1978*a*; Johnston and Willow 1981; Olsen and Leeb-Lundberg, 1981; De Deyn et al., 1988). Benzodiazepine receptor ligands which were convulsant or proconvulsant, such as the β-carbolines βCCM, βCCE, and DMCM and the pyrazoloquinoline CGS 8216 reduced GABAergic inhibition through an allosteric interaction with the $GABA_A$ receptor (Braestrup et al., 1983; Peterson, 1983).

AED reduction of seizures induced in experimental animals by either intravenous (i.v.) or subcutaneous (s.c.) administration of PTZ has been used to predict antiabsence efficacy of drugs such as diazepam (DZP), trimethadione (TMO), sodium valproate (VPA), and ethosuximide (ESM) (Swinyard, 1969). The antiabsence AEDs were also effective against DMCM-induced seizures (Petersen, 1983). Experimental drugs also have an anticonvulsant action in experimental animals against PTZ or DMCM seizures. The pyrazoloquinoline, CGS 9896, was anticonvulsant in the PTZ test (Petrack et al., 1983), and the β-carboline, ZK 91296, was protective against PTZ- and DMCM-induced seizures (Petersen et al., 1984; Klockgether et al., 1985).

Enhancement of GABA-mediated inhibition has been proposed as the mechanism of action of several AEDs used extensively in human therapy (Eccles et al., 1963; Nicoll, 1972; Macdonald and Barker, 1978*b*, 1979; Gent and Phillips, 1980; Shultz and Macdonald, 1981). Anticonvulsant benzodiazepines such as DZP directly enhance GABA responses (Choi et al., 1977; Macdonald and Barker, 1978*b*), whereas VPA increases brain and synaptosomal GABA concentration (Godin et al., 1969; Simler et al., 1973; Iadarola and Gale, 1979). However, although DZP has been shown to enhance GABA responses, no clear action of TMO [or its active metabolite, dimethadione (DMO)] VPA, or ESM on GABAergic inhibition at clinically relevant concentrations has been reported.

In this study, we determined whether or not the antiabsence (ESM, DMO, VPA, and DZP) and two experimental (CGS 9896 and ZK 91296) AEDs had an action on postsynaptic GABA responses on mouse spinal cord neurons grown in primary dissociated cell culture at clinically relevant concentrations. We also determined the concentration-dependent effects of the convulsant drugs PTZ and DMCM on GABA responses and the effects of the AEDS on PTZ- and DMCM-induced reduction of GABA responses.

MATERIALS AND METHODS

Primary dissociated cell culture

Cultures of spinal cord neurons were prepared from dissected spinal cords and attached dorsal root ganglia from 12–14-day-old fetal mice as described previously (Ransom et al., 1977). Experiments conformed to the policy of the American Physiological Society. 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 miminal essential medium (MEM) supplemented with 5.5 g glucose and 1.5 g $NaHCO_3$/L, 5% heat-inactivated horse serum, and 5% Nu-Serum II (Collaborative Research) 325 mOsm] and then plated on sterile collagen-coated 35-mm dishes. 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 4–6 to suppress the growth of rapidly dividing non-neuronal cells. Medium was changed twice weekly. Cultures were maintained for 4–9 weeks before electrophysiologic experiments were made.

Experimental procedures

Solutions

All recordings were made in a high magnesium ion concentration of Dulbecco's phosphate-buffered saline (PBS) after removal of growth medium. The elevated magnesium ion concentration in the recording solution suppressed spontaneous synaptic and action potentials. The recording solution contained (in m*M*): NaCl 137, Na_2HPO_4, 8.06, KCl 2.68, KH_2PO_4 1.47, $CaCl_2$ 1, $MgCl_2$ 10, and glucose 5.6 (pH 7.4). Heavy paraffin oil was applied to the surface of the bathing solution to retard evaporation.

Solutions of drugs were always prepared on the day of the experiment in the following manner: DMCM, DZP, and VPA were dissolved in dimethylsulfoxide (DMSO) to form 10-, 1-, and 60-m*M* stocks, respectively. DMO, ESM, and PTZ were dissolved in recording solution to form 12-, 6-, and 10-m*M* stocks, respectively. Aliquots were removed and diluted in bathing medium to obtain the

applied concentrations. The final solutions contained ≤0.1% DMSO.

DMCM was applied at concentrations between 1 n*M* and 10 μ*M*. PTZ concentrations ranged between 62.5 μ*M* and 10 m*M*. In the experiments testing the effects of AEDs on DMCM- and PTZ-induced reduction of GABA responses, DMCM was used at a concentration of 250 n*M* and PTZ was used at a concentration of 1 m*M*. The ranges of AED concentrations tested were determined from human clinical pharmacologic data when available. Concentrations equivalent to the clinically useful therapeutic range in CSF or comparable to the free levels in serum were used (Gugler et al., 1977; Eadie and Tyrer, 1980; Sherwin, 1982; Wilder and Karas, 1982).

Experimental apparatus

For experiments, the culture dish containing bathing solution was placed on a microscope 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.

Electrophysiologic recordings

Intracellular recordings were made from the somata of spinal cord (>20 μm) neurons using glass micropipettes (25–50 MΩ) filled with 3M KCl. Use of an active bridge circuit (Model 8100, Dagan, Minneapolis, MN, U.S.A.) allowed simultaneous recording of membrane potential and injection of current (for steady-state polarization or periodic stimulation) using a single micropipette. The preamplifier output signal was led to a six-channel polygraph (Model 2600, Gould Instruments, Cleveland, OH, U.S.A.) for continuous recording.

GABA responses

GABA (0.5 *M*, pH 3.4) was applied iontophoretically using 500-ms duration rectangular current pulses at 5-s intervals. Tips of iontophoretic pipettes were positioned to within 2 μm of neuronal somata. The use of 3M KCl-filled micropipettes resulted in elevation of intracellular chloride ion concentration and a shift in the chloride equilibrium potential from about −65 mV to about −20 mV. Under these conditions, an increase in chloride conductance resulted in an outward chloride current giving depolarizing GABA responses (Nowak et al., 1982). Responses of ~10–15 mV in amplitude were evoked following membrane hyperpolarization (to between −70 and −90 mV) to avoid saturation at or near the chloride equilibrium potential.

Direct effects of convulsant drugs and AEDs on GABA responses were accepted only if the GABA responses returned to control amplitude within 5–10 min of removal of the drug-containing micropipette. In the studies evaluating effects of coapplication of the AEDs and the convulsant drugs on GABA responses, data were accepted only when the original direct effect of the convulsant drug could again be evoked after recovery of the GABA response from the combination effect.

Drug application

For evaluation of drug effects on GABA responses, all drugs were applied by a perfusion micropipette. A blunt-tipped (10–15 μm) micropipette, filled with the recording solution containing convulsant or convulsant and AED, was positioned 15–30 μm from the soma of the cell under study. The open end of the perfusion micropipette was connected to a pressure regulator, set between 0.4 and 0.8 psi, by tight-fitting polyethylene tubing. Pressure-pulse duration, regulated by a voltage-activated three-way valve, was 10 s. Under these conditions, local perfusion produced no artifacts, and application of recording solution (with or without vehicle) was virtually free of effects. When the effect of coapplication of convulsant drugs and AEDS was studied, the drugs were applied through one perfusion micropipette to avoid flow artifacts. The concentrations reported in this study are those contained in the perfusion micropipettes. Although some reduction in the concentration of drug at the neuron may have occurred due to dilution in the surrounding medium, increasing durations of application or altering the position of the perfusion micropipette did not increase the effects of the drugs. Thus, although there may be some small inaccuracy in the drug concentrations, the reported concentrations are probably close to those at the surface of the neuron.

The perfusion micropipettes and recording micropipettes were held by Leitz micromanipulators. To decrease leakage of drugs into the bathing medium, the tips of the perfusion micropipettes were kept in the oil phase between drug application trials. They were lowered in the aqueous phase only when drug application was desired.

Drugs

CGS 9896 [2-(*p*-chlorophenyl)-2,5-dihydropyrazolo)-4,3-C)quinolin-3(5H)-1] was obtained from Ciba-Geigy (Summit, NJ, U.S.A.). DZP was obtained from Hoffman-LaRoche (Nutley, NJ, U.S.A.). ZK 91296 (ethyl 5-benzyloxy-4-methoxymethyl-β-carboline-3-β-carboxylate) and DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) were obtained from A/S Ferrosan (Denmark).

DMO (5,5-dimethyl-2,4-oxazolidine-dione), PTZ (6,7,8,9-tetrahydro-5H-tetrazolo-[1,5-a]azepine), and GABA were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). VPA and ESM were obtained from Parke-Davis/Warner-Lambert Research Laboratories (Ann Arbor, MI, U.S.A.).

Algebraic and statistical methods

Mean values and SD were calculated for GABA response amplitudes before, during, and after drug application. Mean values and SD for the effects of the coapplied convulsant drugs and AEDs on GABA responses were also calculated.

All effects were expressed as percentage of change in GABA response from control GABA response. The statistical significance of differences between GABA responses with and without drug application was calculated using the two-tailed Student's *t* test; $p < 0.05$ was considered statistically significant.

RESULTS

Direct effects of convulsant drugs PTZ and DMCM on GABA responses

Application of PTZ or DMCM to spinal cord neurons did not alter resting membrane potential or conductance. Application of recording solution (with or without vehicle) (n = 10) did not significantly alter GABA responses (100.8 ± 1.9%).

PTZ and DMCM rapidly and reversibly reduced GABA responses. The PTZ effect was concentration-dependent (Fig. 1). A significant 6.2 ± 2.1% decrease of GABA responses ($p < 0.001$) was obtained at 125 μ*M*, and complete inhibition was obtained at 10 m*M*. The DMCM effect was also concentration dependent (Fig. 2). A significant 9.9 ± 2.3% decrease of GABA responses ($p < 0.001$) was found at 10 n*M*. The inhibition of GABA responses reached a maximum of 39% at 1 μ*M*. Although both PTZ and DMCM reduced GABA responses, DMCM was more potent than PTZ. The estimated IC_{50} values for the inhibition of GABA responses were 1.1 m*M* for PTZ and 33 n*M* for DMCM.

Direct effect of the AEDs on GABA responses

ESM (600 and 1,200 μ*M*), DMO (2 and 6 m*M*), CGS 9896 (2 μ*M*), and ZK 91296 (2 μ*M*) did not alter GABA responses significantly (Table 1). VPA (200 μ*M*) produced a small (2.1%) but significant reduction of GABA responses. DZP applied at concentrations between 1 and 500 n*M* enhanced GABA responses in a concentration-dependent manner. For DZP, a significant 12.6 ± 3.4% enhancement of GABA responses ($p < 0.001$) was obtained at 10 n*M*, and a peak enhancement of 82.4 ± 5.7% was obtained at 500 n*M* (Table 1).

Effects of AEDs on PTZ-induced antagonism of GABA responses

The reduction of GABA responses produced by PTZ (1 m*M*) was unaltered by ESM (600 μ*M*) and DMO (2 and 6 m*M*) (Table 2). Similarly, the benzodiazepine receptor antagonists, CGS 9896 (1 μ*M*) and ZK 91296 (1 μ*M*), did not alter PTZ (1 m*M*) reduction of GABA responses (Table 2 and Fig. 4A and B). VPA (200 μ*M*), however, coapplied with PTZ (1 m*M*), slightly enhanced ($p < 0.05$) the inhibition of GABA responses induced by PTZ (Table 2).

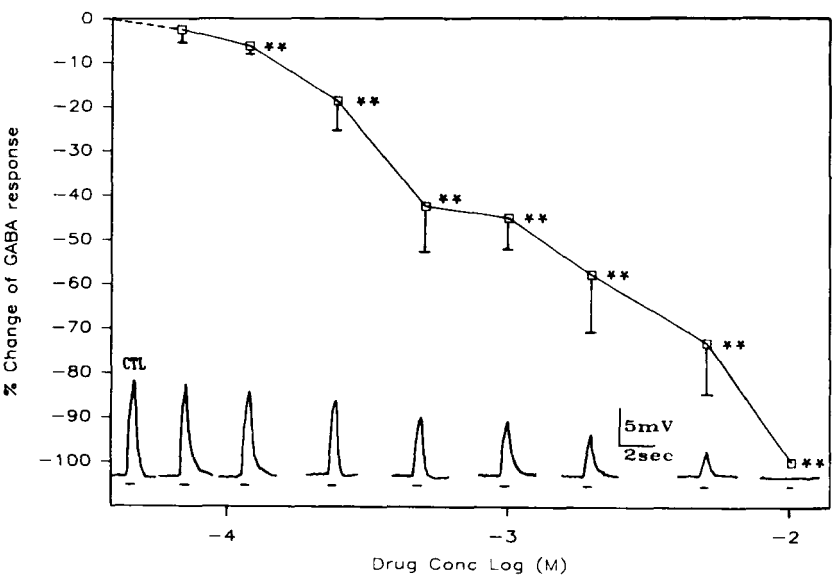

FIG. 1. Concentration-dependent effects of pentylenetetrazol (PTZ) on GABA responses on spinal cord neurons. GABA responses (bottom) show the effect on one cell of the respective concentrations of applied PTZ. Initial control (CTL) GABA response shows a stable response before drug application. GABA responses returned to control values within 2 min after removal of PTZ. Iontophoretic GABA application is indicated by a dash. Effects are expressed as percentage of change of the original GABA response. Data are means plus SD of responses from 3 to 13 neurons. SD shown in one direction only. **p < 0.001 from control GABA responses. Drug concentrations shown on abscissa are logarithm molar.

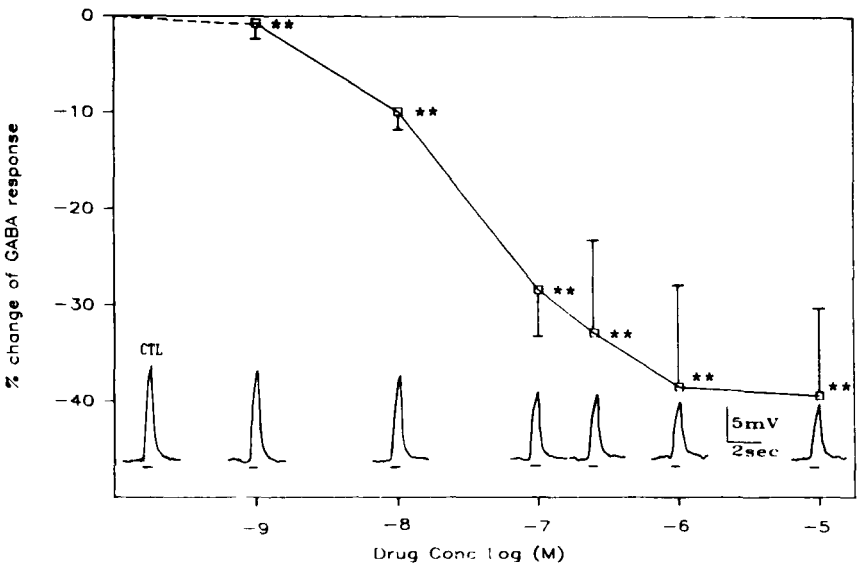

FIG. 2. Concentration-dependent effects of methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) on GABA responses on spinal cord neurons. GABA responses (bottom) show the effect on one cell of the respective concentrations of applied DMCM. Initial control (CTL) shows a stable GABA response before drug application. GABA responses returned to control values within 10 min after removal of the DMCM-containing micropipette. Iontophoretic GABA application is indicated by a dash. Effects are expressed as percentage of change of the original GABA response. Data are means plus SD of responses from 5 to 15 neurons. SD shown in one direction only. **p < 0.001 from control GABA responses. Drug concentrations shown on abscissa are logarithm molar.

In contrast, DZP with a threshold concentration of 10 n*M* ($p < 0.05$) significantly reversed the PTZ-induced reduction of GABA responses. Coapplication of PTZ (1 m*M*) and DZP (100 n*M*) resulted in a significant 7.4 ± 5.3% enhancement of the GABA response ($p < 0.001$) (Table 2 and Fig. 3A).

Effects of AEDs on DMCM-induced decreases of GABA responses

DMCM (250 n*M*) reduction of GABA-responses was unaltered by ESM (600 μ*M*), DMO (2 m*M*), or VPA (200 μ*M*) (Table 3). Coapplication of DZP, CGS 9896 or ZK 91296, however, influenced the DMCM-induced inhibition of GABA-responses (Table 3). CGS 9896 (2 μ*M*) and ZK 91296 (2 μ*M*) significantly antagonized the DMCM-induced inhibition of GABA responses (Table 3 and Fig. 4C and D). DZP, with a threshold concentration of 10 n*M* ($p < 0.05$), significantly reversed the DMCM-induced inhibition of GABA responses (Fig. 3B). Simultaneous application of DMCM 250 n*M* and DZP 500 n*M* resulted in a small increase (5.5%) in GABA responses compared to control responses (Table 3).

TABLE 1. *Direct effects of AEDS on GABA responses*

AED	No. of cells studied (n)	GABA response (% control ± SEM)
ESM 600 μ*M*	8	102.3 ± 3.3
ESM 1,200 μ*M*	5	100.4 ± 2.1
DMO		
2 m*M*	3	99.3 ± 1.7
6 m*M*	4	99.3 ± 1.4
VPA 200 μ*M*	8	97.9 ± 1.7[a]
CGS 9896 2 μ*M*	10	100.2 ± 4.6
ZK 91296 2 μ*M*	6	100.3 ± 2.7
DZP		
1 n*M*	5	100.1 ± 1.2
10 n*M*	5	112.6 ± 3.4[b]
100 n*M*	38	143.7 ± 17.2[b]
500 n*M*	5	182.4 ± 5.7[b]

[a] $p < 0.01$, [b] $p < 0.001$, from control, Student's two-tailed *t* test.

AED, antiepileptic drug; ESM, ethosuximide; DMO, dimethadione; VPA, sodium valproate; DZP, diazepam.

TABLE 2. *Effects of AEDS on PTZ-induced antagonism of GABA responses*

AED	No. of cells studied (n)	GABA response (% control ± SEM)
PTZ 1 m*M*	7	60.2 ± 7.4
PTZ 1 m*M* + ESM 600 μ*M*		61.3 ± 5.8
PTZ 1 m*M*	4	61.9 ± 3.8
PTZ 1 m*M* + DM 2 m*M*		62.4 ± 5.4
PTZ 1 m*M*	3	58.0 ± 5.6
PTZ 1 m*M* + DMO 6 m*M*		59.1 ± 11.1
PTZ 1 m*M*	4	66.3 ± 8.9
PTZ 1 m*M* + CGS 9896 1 μ*M*		67.3 ± 8.4
PTZ 1 m*M*	4	62.3 ± 7.2
PTZ 1 m*M* + ZK 91296 1 μ*M*		61.0 ± 11.6
PTZ 1 m*M*	7	60.0 ± 6.2
PTZ 1 m*M* + VPA 200 μ*M*		57.8 ± 5.4[a]
PTZ 1 m*M*	5	63.2 ± 7.2
PTZ 1 m*M* + DZP 1 n*M*		61.5 ± 8.5
PTZ 1 m*M*	6	67.6 ± 6.2
PTZ 1 m*M* + DZP 10 n*M*		70.6 ± 4.7[a]
PTZ 1 m*M*	8	64.0 ± 6.3
PTZ 1 m*M* + DZP 100 n*M*		107.4 ± 5.3[b]
PTZ 1 m*M*	2	57.6 ± 9.0
PTZ 1 m*M* + DZP 500 n*M*		126.8 ± 8.1[b]

Abbreviations as in Table 1.

[a] $p < 0.05$, [b] $p < 0.001$ from PTZ 1 m*M*, Student's 2-tailed *t* test, paired samples.

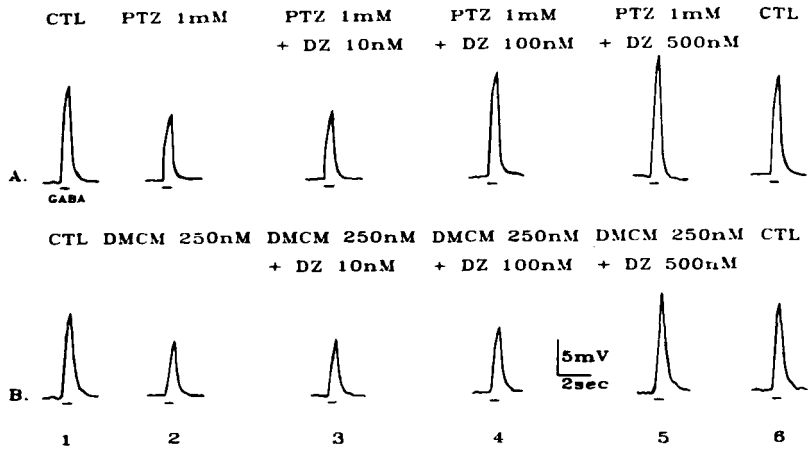

FIG. 3. Blockade of pentylenetetrazol (PTZ 1 m*M*) (panel A) and DMCM (250 n*M*) (panel B) -induced reduction of GABA responses on spinal cord neurons by DZP (10, 100, and 500 n*M*). Column 2 shows PTZ and methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) effects. Columns 3–5 show effects of simultaneous convulsant and diazepam (DZP) applications. Columns 1 and 6 show control (CTL) GABA responses before and after drug applications, respectively. Control responses between drug applications are not shown. Iontophoretic GABA application is indicated by a dash.

DISCUSSION

To understand the mechanism(s) of action of antiabsence AEDs better, we investigated the effects of some clinically and/or experimentally proven AEDs on GABA responses recorded from mouse spinal cord neurons in cell culture. The effects of two convulsant drugs used in chemical animal models of epilepsy, PTZ and DMCM, were studied as well. Furthermore, the possible antagonistic effect of the AEDS on PTZ- and DMCM-induced reduction of GABA responses was determined.

The convulsant drugs PTZ and DMCM reduced GABA responses in a concentration-dependent manner, consistent with previous observations (Pellmar and Wilson, 1977; Macdonald and Barker, 1978*a*; Jensen and Lambert, 1983; Skerritt and Macdonald, 1984). This reduction of postsynaptic GABAergic inhibition might underlie the convulsant activity of these drugs. Our results also confirm that DMCM exerts its effect on GABA responses through an interaction with the benzodiazepine receptor. Indeed, the benzodiazepine receptor antagonists CGS 9896 and ZK 91296 (De Deyn and Macdonald, 1987) completely antagonized the DMCM effect. DMCM is an inverse agonist at the benzodiazepine receptor (Jensen and Lambert, 1983; Skerritt and Macdonald, 1984), which probably explains the protective activity of ZK 91296 against DMCM-induced seizures (Klockgether et al., 1985). PTZ, however, was previously demonstrated to inhibit GABA responses through an interaction with a different site on the GABA receptor, possibly the picrotoxin receptor (Macdonald and Barker, 1977, 1978*a*).

The anticonvulsants ESM, DMO, and VPA ap-

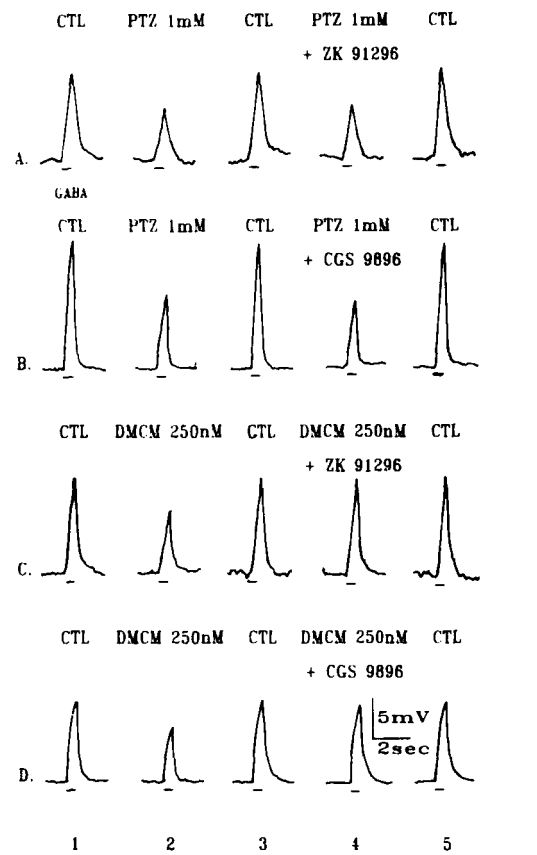

FIG. 4. Lack of antagonism of pentylenetetrazol (PTZ 1 m*M*) and antagonism of methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM 250 n*M*)-induced reduction of GABA-responses on spinal cord neurons by ZK 91296 (2 μ*M*, panels A and C) and CGS 9896 (2 μ*M*, panels B and D). Column 2 shows the PTZ and DMCM effect. Column 4 shows the effect of simultaneous convulsant and experimental antiepileptic drug (AED) applications. Columns 1, 3, and 5 show control (CTL) responses, respectively, before, between, and after drug applications. Iontophoretic GABA application is indicated by a dash.

TABLE 3. *Effects of AEDS on DMCM-induced antagonism of GABA responses*

AED	No. of cells studied (n)	GABA response (% control ± SEM)
DMCM 250 n*M*	6	62.4 ± 5.8
DMCM 250 n*M* + ESM 600 μ*M*		64.5 ± 7.0
DMCM 250 n*M*	5	60.6 ± 3.6
DMCM 250 n*M* + DMO 2 m*M*		57.8 ± 3.3
DMCM 250 n*M*	5	62.3 ± 4.4
DMCM 250 n*M* + VPA 200 μ*M*		61.7 ± 6.4
DMCM 250 n*M*	4	58.7 ± 4.0
DMCM 250 n*M* + DZP 10 n*M*		63.3 ± 4.1[a]
DMCM 250 n*M*	5	60.5 ± 5.5
DMCM 250 n*M* + DZP 100 n*M*		71.7 ± 13.4[a]
DMCM 250 n*M*	6	58.7 ± 2.6
DMCM 250 n*M* + DZP 500 n*M*		105.5 ± 9.9[b]
DMCM 250 n*M*	6	63.0 ± 6.5
DMCM 250 n*M* + CGS 9896 2 μ*M*		99.0 ± 1.3[b]
DMCM 250 n*M*	5	59.2 ± 5.5
DMCM 250 n*M* + ZK 91296 2 μ*M*		99.2 ± 3.6[b]

Abbreviations as in Table 1.
[a] $p < 0.05$, [b] $p < 0.001$ from DMCM 250 n*M*, Student's two-tailed *t* test, paired samples.

plied at concentrations equivalent to the clinically useful therapeutic range in cerebrospinal fluid (CSF), did not alter GABA responses. Moreover, these drugs did not alter neuronal membrane potential or input resistance. These results confirm previous findings for ESM and VPA (Barnes and Dichter, 1984; Macdonald et al., 1984; Macdonald et al., 1985; McLean and Macdonald, 1986; Buchalter and Dichter, 1986). At higher concentrations, however, previous studies demonstrated an enhancement of GABA responses by VPA (Macdonald and Bergey, 1979; Harrison and Simmonds, 1982). The observed concentration-dependent effect of DZP on GABA responses with a maximal direct effect of 82.4% is consistent with previous findings (Choi et al., 1977; Macdonald and Barker, 1978*b*; Skerritt et al., 1984). The two experimental AEDs CGS 9896 and ZK 91296, however, applied at a concentration of 2 μ*M* remained inactive in this paradigm. This finding is consistent with previous work describing CGS 9896 and ZK 91296 to be pure antagonists at the benzodiazepine receptor (De Deyn and Macdonald, 1987).

With the exception of DZP, none of the tested AEDs antagonized or reversed PTZ-induced inhibition of GABA responses. The applied PTZ concentration (1 m*M*) was comparable to the critical brain PTZ level for onset of seizures in mice (Yonekawa et al., 1980). Behavioral studies, however, demonstrated the protective action of VPA, DMO, ESM, CGS 9896, and ZK 91296 against PTZ-induced seizures (Chen et al., 1963; Krall et al., 1978; Petrack et al., 1983; Petersen et al., 1984). DZP (100 n*M*) coapplied with PTZ (1 m*M*) reversed the inhibition of GABA responses. This is consistent with behavioral findings for this benzodiazepine receptor agonist (Haefely et al., 1981).

Neither VPA, DMO, nor ESM antagonized DMCM-induced reduction of GABA responses. DZP, however, antagonized the DMCM-induced inhibition of GABA responses. This is probably due to its high-affinity binding at the benzodiazepine receptor in competition with DMCM (Haefely et al., 1981). CGS 9896 and ZK 91296, although devoid of any intrinsic effect, completely antagonized the DMCM-induced reduction of GABA responses. This is explained by the previously demonstrated antagonistic effect of these compounds at the benzodiazepine receptor (De Deyn and Macdonald, 1987). The demonstrated antagonism of the DMCM effect might be the underlying mechanism for the protective action against DMCM-induced seizures for ZK 91296 (Klockgether et al., 1985).

The lack of effects of VPA, ESM, and DMO on postsynaptic GABA action contrasts with their clinical efficacy against generalized absence seizures, and suggests that direct postsynaptic enhancement of GABAergic inhibition is not a common mechanism of action of antiabsence AEDs. These results also suggest that the disturbance responsible for generalized absence seizures may not be localized to the GABA receptor. Alternatively, the therapeutic efficacy of these AEDs may be due to the stimulation of GABA synthesis and/or the inhibition of GABA metabolism as has been shown for VPA (Völzke and Doose, 1973; Hammond and Wilder, 1985). Finally, there may be alterations in pathologic neurons, and their interactions may not have been present in our neurons in culture.

Our results suggest that DZP most probably exerts its antiabsence effect by enhancing GABA-mediated inhibition through an interaction with the benzodiazepine receptor. CGS 9896 and ZK 91296, two experimentally proved AEDs, were shown to be pure antagonists at the benzodiazepine receptor. Their anticonvulsant effect against DMCM-induced seizures can be explained by their benzodiazepine receptor antagonistic effect. Their mechanism against PTZ-induced seizures is less clear. The existence of an endogenous benzodiazepine receptor inverse agonist has been proposed (Guidotti et al., 1982). CGS 9896 and ZK 91296 could enhance GABAergic inhibition by displacing this endogenous compound. Regardless of mechanism of action, CGS 9896 and ZK 91296 may be clinically useful AEDs.

Acknowledgment: We thank Nancy Fox for technical

assistance and Liliane Van den Eynde and Marjorie Mills for secretarial assistance. Dr. Peter De Deyn is the recipient of a Rotary International Foundation Award.

REFERENCES

Barnes DM, Dichter MA. Effects of ethosuximide and tetramethyl-succinimide on cultured cortical neurons. *Neurology* 1984;25:620–5.

Braestrup C, Nielsen M, Honore T, Jensen LH, Petersen EN. Benzodiazepine receptor ligands with positive and negative efficacy. *Neuropharmacology* 1983;22:1451–7.

Buchhalter JR, Dichter MA. Effects of valproic acid in cultured mammalian neurons. *Neurology* 1986;36:259–62.

Chen G, Weston JK, Bratton AC Jr. Anticonvulsant activity and toxicity of phensuximide, methsuximide and ethosuximide. *Epilepsia* 1963;4:66–76.

Choi DW, Farb DH, Fischbach GD. Chlordiazepoxide selectively augments GABA action in spinal cord cell cultures. *Nature* 1977;269:342–4.

Curtis DR, Duggan AW, Felix D, Johnston GAR. Bicuculline, an antagonist of GABA and synaptic inhibition in the spinal cord of the cat. *Brain Res* 1971;32:69–96.

Curtis DR, Game CJA, Johnston GAR, McCulloch RM, MacLachlan RM. Convulsive action of penicillin. *Brain Res* 1972;43:242–5.

De Deyn PP, Macdonald RL. CGS 9896 and ZK 91296, but not CGS 8216 and Ro 15-1788, are pure benzodiazepine receptor antagonists on mouse neurons in cell culture. *J Pharmacol Exp Ther* 1987;242:48–55.

De Deyn PP, Marescau B, Macdonald RL. Effects of α-keto-δ-guanidinovaleric acid on inhibitory amino acid responses in mouse neurons in cell culture. *Brain Res* 1988; 449:54–60.

Eadie MJ, Tyrer JH. *Anticonvulsant therapy*. Edinburgh: Churchill-Livingstone, 1980.

Eccles JC, Schmidt R, Willis WD. Pharmacological studies on presynaptic inhibition. *J Physiol* 1973;168:500–30.

Gent JP, Phillips NI. Sodium di-*n*-propylacetate (valproate) potentiates responses to GABA and muscimol on single central neurons. *Brain Res* 1980;197:275–8.

Godin Y, Heiner L, Mark J, Mandel P. Effects of di-*n*-propylacetate, an anticonvulsant compound, on GABA metabolism. J Neurochem 1969;19:869–73.

Gugler R, Schell A, Eichelbaum M, Fröscher W, Shulz HV. Disposition of valproic acid in man. *Eur J Clin Pharmacol* 1977;12:125–32.

Guidotti A, Forchetti CM, Ebstein B, Costa E. Purification and characterization of an endogenous peptide putative effector for the benzodiazepine recognition site. In: Usdin E, Skolnick P, Tallman Jr JF, et al., eds. *Pharmacology of benzodiazepines*. London: Macmillan, 1982:529–35.

Haefely W, Pieri L, Polc P, Schasffner R. General pharmacology and neuropharmacology of benzodiazepine derivatives. In: Hoffmeister F, Stille G, eds. *Handbook of experimental pharmacology*, Vol 55. Berlin: Springer-Verlag, 1981:13–262.

Hammond EJ, Wilder BJ. Gamma-vinyl GABA: a new antiepileptic drug. *Clin Neuropharmacol* 1985;8:1–12.

Harrison NL, Simmonds MA. Sodium valproate enhances responses to GABA receptor activation only at high concentrations. *Brain Res* 1982;250:201–4.

Hill RG, Simmonds MA. A method for comparing the potencies of γ-aminobutyric acid antagonists on single cortical neurons using microiontophoretic techniques. *Br J Pharmacol* 1973;48:1–11.

Hill RG, Simmonds MA, Straughan DW. Antagonism of GABA by picrotoxin in the feline cerebral cortex. *Br J Pharmacol* 1972;49:37–51.

Iadarola MJ, Gale K. Dissociation between drug-induced increase in nerve terminal and non-nerve terminal pools of GABA in vivo. *Eur J Pharmacol* 1979;59:125–9.

Jensen MS, Lambert JDC. The interaction of the β-carboline derivative DMCM with inhibitory amino acid responses on cultured mouse neurons. *Neurosci Lett* 1983;40:175–9.

Johnston GAR, Willow M. Barbiturates and GABA receptors. Costa E, Di Chiara G, Gessa GL, eds. *GABA and benzodiazepine receptors*. New York: Raven Press, 1981:191–8.

Klockgether T, Schwarz M, Turski L, Sontag K-H. ZK 91296, an anticonvulsant β-carboline which lacks muscle relaxant properties. *Eur J Pharmacol* 1985;110:309–15.

Krall RL, Penry JK, White BG, et al. Antiepileptic drug development: II. Anticonvulsant drug screening. *Epilepsia* 1978;19:409–28.

Lloyd KG, Munari C, Bossi L, et al. Biochemical evidence for the alterations of GABA-mediated synaptic transmission in pathological brain tissue (stereo EEG or morphological definition) from epileptic patients. Morselli PL, Lloyd KG, Loscher W, eds. *Neurotransmitters, seizures and epilepsy*. New York: Raven Press, 1981:325–38.

Macdonald RL, Barker JL. Pentylenetetrazol and penicillin are selective antagonists of GABA-mediated post-synaptic inhibition in cultured mammalian neurons. *Nature* 1977;267: 720–1.

Macdonald RL, Barker JL. Specific antagonism of GABA-mediated postsynaptic inhibition in cultured mammalian spinal cord neurons: a common mode of convulsant action. *Neurology* 1978*a*;28:325–30.

Macdonald RL, Barker JL. Benzodiazepines specifically modulate GABA-mediated postsynaptic inhibition in cultured mammalian neurons. *Nature* 1978*b*;271:563–4.

Macdonald RL, Barker JL. Enhancement of GABA-mediated postsynaptic inhibition in cultured mammalian neurons. *Brain Res* 1979;167:323–36.

Macdonald RL, Bergey GK. Valproic acid augments GABA-mediated post-synaptic inhibition in cultured mammalian neurons. *Brain Res* 1979;170:558–62.

Macdonald RL, McLean MJ. Anticonvulsant drugs: mechanisms of action. In: *Basic mechanisms of the epilepsies*. Delgado-Escueta AV, Ward Jr AA, Woodbury DM, Porter RJ, eds. New York: Raven Press, 1986:713–36 (Advances in neurology; vol. 44).

Macdonald RL, Skerritt JH, McLean MJ. Anticonvulsant drug actions on GABA responses and sustained repetitive firing of neurons in cell culture. *Neuropharmacology* 1984;23:843–4.

Macdonald RL, McLean MJ, Skerritt JH. Anticonvulsant drug mechanisms of action. *Fed Proc* 1985;44:2634–9.

McLean MJ, Macdonald RL. Sodium valproate, but not ethosuximide, produces use- and voltage-dependent limitation of high frequency repetitive firing of action potentials of mouse central neurons in cell culture. *J. Pharmacol Exp Ther* 1986;237:1001–11.

Meldrum B. Convulsant drugs, anticonvulsants and GABA-mediated neuronal inhibition. In: Krogsgaard-Larsen P, Scheel-Kruger J, Kofod H, eds. *GABA-neurotransmitters*. Copenhagen: Munksgaard, 1979:390–405.

Nicoll RA. The effects of anesthetics on synaptic excitation and inhibition in the olfactory bulb. *J Physiol* 1972;223:803–14.

Nowak LM, Young AB, Macdonald RL. GABA and bicuculline actions on mouse spinal cord and cortical neurons in cell culture. *Brain Res* 1982;244:155–64.

Olsen RW. The GABA postsynaptic membrane receptor-ionophore complex: site of action of convulsant and anticonvulsant drugs. *Mol Cell Biochem* 1981;39:261–79.

Olsen RW, Leeb-Lundberg F. Convulsant and anticonvulsant drug binding sites related to GABA-regulated chloride ion channels. In: Costa E. Di Chiara G and Gessa GL, eds. *GABA and benzodiazepine receptors*. New York: Raven Press, 1981:93–102.

Pellmar TC and Wilson WA. Synaptic mechanism of pentylenetetrazole: selectivity for chloride conductance. *Science* 1977;197:912–4.

Petersen EN. DMCM: Potent convulsive benzodiazepine receptor ligand. *Eur J Pharmacol* 1983;94:117–24.

Petersen EN, Jensen LH, Honore T, et al. ZK 91296, a partial agonist at the benzodiazepine receptors. *Psychopharmacology* 1984;83:240–8.

Petrack B, Czernik AJ, Cassidy JP, et al. Benzodiazepine recep-

tor ligands with opposing pharmacologic actions. *Psychopharmacology* 1983;38:129–37.
Ransom BR, Neale E, Henkart M, et al. Mouse spinal cord in cell culture. I. Morphology and intrinsic neuronal electrophysiologic properties. *J Neurophysiol* 1977;40:1132–50.
Roberts E. Epilepsy and antiepileptic drugs: a speculative synthesis. In: Glaser GH, Penry JK, Woodbury DM, eds. *Antiepileptic drugs: mechanisms of action.* New York: Raven Press, 1980:667–713.
Schofield PR, Darlison MG, Fujita N, et al. Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature* 1987;328:221–7.
Schulz DW, Macdonald RL. Barbiturate enhancement of GABA-mediated inhibition and activation of chloride ion conductance: correlation with anticonvulsant and anesthetic actions. *Brain Res* 1981;109:177–88.
Sherwin AL. Ethosuximide. Relation of plasma concentration to seizure control. In: Woodbury DM, Penry JK, Pippenger CE, eds. *Antiepileptic drugs.* 2nd Ed. New York: Raven Press, 1982:637–45.
Simler S, Ziesielski L, Maitre M, et al. Effect of sodium di-*n*-propylacetate on audiogenic seizures and brain gamma-aminobutyric acid level. *Biochem Pharmacol* 1973;22: 1701–8.
Skerritt JH, Macdonald RL. Benzodiazepine receptor ligand actions on GABA-responses. β-carbolines, purines. *Eur J Pharmacol* 1984;101:135–41.
Skerritt JH, Werz MA, McLean MJ, Macdonald RL. Diazepam and its anomalous *p*-chloro-derivative Ro 5-4864: Comparative effects on mouse neurons in cell culture. *Brain Res* 1984;310:99–105.
Swinyard EA. Laboratory evaluation of antiepileptic drugs. *Epilepsia* 1969;10:107–19.
Völzke E, Doose H. Dipropylacetate (Depakine, Ergenyl) in the treatment of epilepsy. *Epilepsia* 1973;14:185–93.
Wilder BJ and Karas BJ. Valproate-relation of plasma concentration to seizure control. In: Woodbury DM, Penry JK, Pippenger CE, eds. *Antiepileptic drugs, 2nd Ed.,* New York: Raven Press, 1982:591–8.
Yonekawa WD, Kupferberg HJ, Woodbury DM. Relationship between pentylenetetrazol-induced seizures and brain pentylenetetrazol levels in mice. *J Pharmacol Exp Ther* 1980; 214:589–93.

RESUMEN

Los mecanismos de acción de las medicaciones antiepilépticas eficaces contra los ataques generalizados de ausencia (AEDs antiausencia) permanecen inciertos. Los AEDs antiausencia son, generalmente, eficaces contra ataques experimentales inducidos por el pentilentetrazol (PTZ) y el metil-6,7-dimetoxy-4-etil-βcarbolina-3-carboxilato (DMCM) en animales, medicaciones que reducen la inhibición GABAérgica. Hemos estudiado los efectos de varios AEDs sobre respuestas-GABA registradas en las neuronas de la médula espinal de ratones que habian crecido en cultivos de células primarieas disociadas. Cuatro AEDs antiausencia fueron incluidos: etoxusimida (ESM), dimetadiona (DMO), valproato sódico (VPA) y diazepan (DZP). También se incluyeron dos AEDs experimentales, CGS 9896 y ZK 91296, con acción anticonvulsiva contra los ataques inducidos por PTZ o DMCM. También se valoraron los posibles efectos de los AEDs antiausencia y experimentales sobre el PTZ y la inhibición de las respuestas-GABA inducidas por el DMCM. El PTZ y el DMCM redujeron las respuestas-GABA de modo reversible y dependiendo de sus concentraciones. El PTZ inhibió cmpletamente las respuestas-GABA a 10 mM (IC_{50} de 1.1 m*M*) mientras que la inhibición de las respuestas GABA inducida por el DMCM alcanzó un nivel estable del 39% de los valores control con 1 μ*M* (IC_{50} de 33 m*M*). La ESM (1200 μ*M*), la DMO (6 mM), el VPA (200 μ*M*), el CGS 9896 (2 μ*M*) y el ZK 9896 (2 μ*M*) no alteraron las respuestas-GABA. El DZP aumentó las respuestas GABA de una manera concentración-dependiente. La inhibición de las respuestas-GABA producidas por el PTZ (1 m*M*), no se alteró con las ESM (600 μ*M*), la DMO (6 m*M*), el CGS 9896 (1 μ*M*) o el ZK 9896 (1 μ*M*). La co-aplicacion de VPA (200 μ*M*) y el PTZ (1 mM) aumentó ligeramente los efectos del PTZ. Sin embargo el DZP (>10 n*M*) revirtió la reducción de las respuestas GABA inducidas por el PTZ. La reducción de las respuestas GABA producida por el DMCM (250 n*M*) no fue alterada por la ESM (600 μM), la DMO (2 m*M*) o el VPA (200 μ*M*). Sin embargo el CGS 9896 (2 μ*M*), y el ZK 91296 (2 μ*M*), antagonizaron el efecto del DMCM. El DZP (>10 n*M*) revirtió significativamente la inhibición de las respuestas GABA inducidas por el DMCM. La falta de efectos de CPA, ESM y DMO sobre las respuestas GABA post-sinápticas sugiere que el incremento de la acción GABA post-sináptica no es un mecanismo común de actuación de las AEDs antiausencia. Todas las AEDs DZP, CGS 9896 y ZK 91296 revirtieron la reducción de las respuestas GABA producidas por el DMCM pero no las inducidas por el PTZ lo que sugiere que estos AEDs bloquean los ataques DMCM actuando sobre los receptores de la benzodiazepina. Sin embargo, puesto que el incremento de las respuestas GABA sólo se produce por el DZP, permanece todavía sin aclarar el por qué el CGS 9896 y el ZK 91296 bloquean los ataques producidos por el PTZ.

(A. Portera-Sánchez, *Madrid*)

ZUSAMMENFASSUNG

Der Wirkmechansimus von Antiepileptika gegen generalisierte Absencen ist unklar. Antiabsencemittel sind generell wirkungsvoll gegen PTZ- und Methyl-6,7-Dimethoxy-4-Äthyl-β-Carbolin-3-Carboxylat (DMCM) induzierte tierexperimentelle Anfälle, also von Medikamenten, die die GABA-erge Inhibition reduzieren. Es wurde vermutet, daß Antiabsencemittel die GABA-erge Inhibition verstärken. Wir untersuchten die Wirkung von verschiedenen Antiepileptika auf GABA-Antworten in spinalen Mäuseneuronen, die in Zellkulturen gewachsen waren. Es wurden 4 Absencemittel untersucht: Ethosuximid (ESM), Dimethadion (DMD), Sodium Valproat (VPA) und Diazepam (DZP). Zusätzlich wurden 2 experimentelle Antiepileptika, CGS 9896 und ZK 91296, die gegen PTZ0 oder DMCM-induzierte Anfälle wirkungsvoll sind, eingeschlossen. Mögliche Wirkungen der Antiabsence- und experimentellen Antiepileptika auf PTZ- und DMCM-induzierte Hemmung der GABA-Antworten wurden ebenfalls ausgewertet. PTZ und DMCM zeigten eine konzentrationsabhängige reversible Reduktion der GABA-Antworten. PTZ zeigte eine komplette Hemmung der GABA-Antworten bei 10 mM (IC_{50} 1,1 m*M*), DMCM-Hemmung der GABA-Antworten zeigte ein Plateau von 39% der Kontrollwerte bei 1 μ*M* (IC_{50} von 33 m*M*). ESM (1200 μ*M*), DMD (6 m*M*), VPA (200 μ*M*), CGS 9896 (2 μ*M*) und ZK 9896 (2 μ*M*) änderten nicht die GABA-Antworten. DZP verstärkte die GABA-Antworten konzentrationsabhängig. Die durch PTZ (1 m*M*) hervorgerufene Hemmung der GABA-Antworten war bei ESM (600 μ*M*), DMD (6 m*M*), CGS 9896 (1 m*M*) und ZK 3836 (1 m*M*) unverändert. Zusätliche Anwendung von VPA (200 m*M*) und PTZ (1 m*M*) verstärkten geringfügig den PTZ-Effekt. DZP (10 nM) kehrte die durch PTZ hervorgerufene Reduktion der GABA-Antworten um. Die durch DMCM (250 n*M*) hervorgerufene Hemmung der GABA-Antworten war durch ESM (600 μ*M*), DMD (2 m*M*) und VPA (200 μ*M*) unbeeinflusst. CGS 9896 (2 μ*M*) und ZK 91296 (2 μ*M*) antagonisierten die DMCM-Wirkung. DZP (>10 n*M*) kehrte die durch DMCM-induzierte Hemmung der GABA-Antworten um. Das Fehlen einer Wirkung von VPA. ESM und DMD auf die postsynaptischen GABA-Antworten legen nahe, daß eine direkte Verstärkung der postsynaptischen GABA-Aktion kein gemeinsamer Mechanismus der Antiabsencemittel darstellt. Die Antiepileptika DZP, CGS 9896 und ZK 91296 kehrten die DMCM-Wirkung auf die GABA-Antworten um, jedoch nicht die von PTZ, was vermuten läßt, daß diese Antiepileptika die DMCM-Anfälle über die Wirkung an den Benzodiazipin-Rezeptoren verhinderte. Da jedoch nur DZP GABA-Antworten verstärkte, ist unklar, in welcher Weise CGS 9896 und ZK 91296 die PTZ-Anfälle verhinderten.

(C. Benninger, *Heidelberg*)