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# Tolerance to Anticonvulsant Effects of Some Benzodiazepines in Genetically Epilepsy Prone Rats

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DE SARRO G., E. DONATO DI PAOLA, U. AGUGLIA AND A. DE SARRO. *Tolerance to anticonvulsant effects of some benzodiazepines in genetically epilepsy prone rats.* PHARMACOL BIOCHEM BEHAV **55**(1) 39–48, 1996.—The development of tolerance to the anticonvulsant effects of clonazepam, clobazam, and diazepam were studied in genetically epilepsy-prone rats following intraperitoneal (IP) or oral administration. The anticonvulsant effects were evaluated on seizures evoked by means of auditory stimulation (109 dB, 12–16 kHz). All compounds showed 60 min after IP injection antiseizure activity with $ED_{50}$ against clonus of 0.24 μmol $kg^{-1}$ for clonazepam, 0.72 μmol $kg^{-1}$ for diazepam, and 3.9 μmol $kg^{-1}$ for clobazam. After 120 min of oral administration the $ED_{50}$ against clonus of 2.37 μmol $kg^{-1}$ for clonazepam, 15.8 μmol $kg^{-1}$ for diazepam, and 30 μmol $kg^{-1}$ for clobazam. The dose chosen for the chronic treatment were 2.5 μmol $kg^{-1}$ for clonazepam, 15 μmol $kg^{-1}$ for diazepam, and 30 μmol $kg^{-1}$ for clobazam. The animals were treated three times daily for 4 or 6 weeks. Auditory stimulation was administered 60 min after drug IP injection on various days. During treatment, tolerance was observed as a loss of drug anticonvulsant effects. No changes of occurrence of audiogenic seizures was observed in rats treated with vehicle. Tolerance to the anticonvulsant activity developed most rapidly during clobazam treatment, less rapidly following diazepam treatment, and most slowly during clonazepam treatment. Sixty minutes after IP injection on various days of chronic treatment the motor impairment induced by these benzodiazepines was also studied by means of a rotarod apparatus. The tolerance to the motor impairment developed more rapidly than the anticonvulsant effects. The response to auditory stimulation to benzodiazepines was stopped 24 and 48 h after chronic treatment with these compounds, showing no residual drug effects and that rats were still tolerant. The genetically epilepsy-prone rats is a reliable and sensitive model for studying long-term effects of anticonvulsant drugs.

Epilepsy Clobazam Diazepam Clonazepam Tolerance Benzodiazepines Anticonvulsants Audiogenic seizures Genetically epilepsy-prone rats

MOST commonly, tolerance to the anticonvulsant action of benzodiazepines has been studied using suppression of pentylenetetrazol seizures in animals. A report of tolerance to the actions of diazepam against strychnine and picrotoxin, but not pentylenetetrazol (22), suggested that tolerance might not occur to the antipentylenetetrazol action. Subsequently, File (7) showed tolerance to the antipentylenetetrazol action of diazepam in mice. Tolerance to the antipentylenetetrazol effect of diazepam was also reported in rats treated with flurazepam (29) and with diazepam (14). Several studies have shown that the rate of benzodiazepine tolerance development is not uniform. For example, tolerance developed more rapidly in some strains of mice than others (8). Gent and Haigh (13), studying the effects of clobazam to raise the threshold for pentylenetetrazol seizures in mice, showed that the tolerance to clobazam developed very rapidly, whereas tolerance during clonazepam treatment progressed more slowly (12). In another study, tolerance was studied in amygdala-kindled rats treated with diazepam (23). In these experiments tolerance was observed for the effect of diazepam on seizure latency

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and duration although the degree of tolerance was not marked. In rats treated three times daily with clonazepam (22) a slight degree of tolerance was demonstrated by the decreasing clonazepam effect on seizure duration and amygdala afterdischarges duration, with no loss of drug effect as measured by seizure latency or seizure stage. In addition, Young et al. (38,39) described tolerance in amygdala-kindled rats during treatment with clonazepam or clobazam. Clinical reports suggest a similar situation, for instance, an especially rapid development of tolerance to clobazam (11,25,31,32). Because no data of tolerance to anticonvulsant effects of benzodiazepines were reported in genetically epilepsy-prone rats, in the present report we intend to study the possible development of tolerance to diazepam, clonazepam, and clobazam in this particular strain of rodents. This experimental genetic model of epilepsy would lend itself to following development of tolerance over time. In this strain of rats, seizures are relatively stables, so that drug effects on seizure activity can be measured repeatedly and the rate of tolerance development can be monitored. Unlike experiments in which chemical convulsants are used, there is no possibility of drug interaction and no concern that reduced anticonvulsant effectiveness might result from the treated animal becoming more sensitive to the convulsant drug during chronic benzodiazepine treatment. Benzodiazepines are among the most potent drugs effective against audiogenic seizure in genetically epilepsy prone rat (4,27).

To allow better comparisons among the different benzodiazepines, we initially attempted to choose anticonvulsant doses that would be approximately equieffective, based on preliminary dose–response activity of these benzodiazepines and on the results of some recent studies (29,35). A three-times-daily dosing frequency was used for all drugs. In addition, the effects on motor function of benzodiazepines were assessed by rotarod test. The resulting effects were examined to determine whether these benzodiazepines had similar profiles of tolerance.

## METHOD

### *Animals*

Sprague–Dawley rats were purchased from Charles River (Calco, Como, Italy). Genetically epilepsy prone rats (GEPRs), a strain derived from Sprague–Dawley rats, were kindly supplied by our breeding stock (Institute of Pharmacology, Messina) from a colony originally instituted at the Lousiana State University at Shreveport, LA, by Dr. P. C. Jobe. Progenitors of this latter were raised at the University of Arizona and named [UAZ:AGS (SD)]. The rats were housed three or four per cage in stable conditions of humidity (60 ± 5%) and temperature (21 ± 2°C) and allowed free access to food and water until the time of the experiments. Animals were maintained on a 12 L:12 D cycle (lights on 0700–1900, off 1900–0700 h). GEPRs were tested three times at weekly intervals between 6 and 8 weeks of their life, and only animals that showed an audiogenic seizure in all three exposures to sound stimulation were used for these experiments.

The experimental protocol and all the procedures involving animals and their care were conducted in conformity with the istitutional guidelines and the European Council Directive of laws and policies.

### *Anticonvulsant Activity*

Seizures were induced in GEPRs, 180–260 g, 12–18 weeks old, male or female ($n$ = 240) by exposing them to a mixed frequency sound of 12–16 kHz, 109 dB intensity under a hemispheric Plexigas dome (58 cm diameter). Individual animals were initially tested 10 min before sound stimulation for assessment of locomotor activity and then placed into the dome box for habituation and assesment of anticonvulsant activity. Auditory stimulation was applied for 60 s or until the onset of convulsions occurred. A full seizure response (S.R.) consisted of one or two running phases, followed by a convulsion (clonus of forelimbs, hindlimbs, head, pinnae, vibrissae, and tail) and tonic extension to give a score of 9 (18). In particular, the audiogenic seizure response was assessed on the following scale previously reported (5): 0 = no response; 1 = running only; 2 = two running phases, followed by a clonic convulsion (clonus of forelimbs, hindlimbs, head, pinnae, vibrissae, and tail); 3 = one running phase, followed by a clonic convulsion (clonus of forelimbs, hindlimbs, head, pinnae, vibrissae, and tail); 4 = two running phases followed by tonus of neck, trunk, and forelimb and hindlimb clonus; 5 = one running phase followed by tonus of neck, trunk, and forelimb and hindlimb clonus; 6 = two running phases followed by nearly complete tonic extension except hindfeet; 7 = one running phase followed by nearly complete tonic extension except hindfeet; 8 = two running phases followed by complete tonic extension; and 9 = one running phase followed by complete tonic extension. The maximum response was recorded for each animal. Behavioural changes were observed during the period between drug administration and auditory testing.

### *Chronic Treatment*

The chronic treatment dose of each benzodiazepine was administered three times daily, at 0700, 1500, and 2100 h. Diazepam, clonazepam, and clobazam were administered by gastric intubation. The doses used were: diazepam 15 μmol $kg^{-1}$, clonazepam 2.5 μmol $kg^{-1}$, and clobazam 30 μmol $kg^{-1}$. Control rats received orally vehicle as appropriate. Diazepam and clonazepam treatments lasted 6 weeks, while clobazam treatment lasted 4 weeks only. One and 2 days (i.e., 24 and 48 h) after the end of chronic treatment, the benzodiazepine was again administered IP 60 min before auditory stimulation to test for residual drug effect and to determine if tolerance would persist at least this long. Another group of Sprague–Dawley rats were tested 60 min after drug injection for motor impairment using rotarod apparatus.

### *Effect of Diazepam, Clonazepam, and Clobazam*

On the morning of days 1, 3, 5, and 7, and then twice weekly, genetically epilepsy prone rats received an IP anticonvulsant test dose instead of the usual chronic treatment dose. The doses were: diazepam 2.5 and 10 μmol $kg^{-1}$; clobazam 15 and 30 μmol $kg^{-1}$, and clonazepam 0.5 and 1 μmol $kg^{-1}$; these doses had decreased the incidence of tonic or clonic component of audiogenic seizures by 80–90%. Genetically epilepsy prone rats received an auditory stimulation 60 min after the IP administration of a benzodiazepine and the occurrence of the seizure phases were recorded. After seizure activity was recorded, the remainder of the daily chronic treatment dose was administered. Control rats received appropiate vehicles in place of these doses.

### *Effects on Motor Movements*

Sprague–Dawley rats ($n$ = 196) were trained to do coordinated motor movements continuously for 5 min on a rotarod apparatus 4 cm in diameter 4.5 rpm (U. Basile, Comerio,

Varese, Italy). Impairment of coordinated motor movements was definited as inhability of the animals to retain on the rotarod for a 5-min test period according to Dunham and Miya (6). In particular, 60 min after IP drug injection on days 1, 3, and 5 of chronic treatment and then every 5 days the motor impairment induced by these benzodiazepines was studied. After that the effects on motor movements were recorded, the remainder of the daily chronic treatment dose was administered.

*GABA and Benzodiazepine Receptor Binding Analysis*

At the end of the experiments, rats were decapitated, the brain removed, and stored at −80°C until used. To study the binding of benzodiazepine receptors, fresh unfrozen cortex samples were homogenized with a Teflon pestle homogenizer in 15 vol (wt/vol) of 0.32 M sucrose, processed with a Soniprep sonicator, and centrifuged for 10 min (12,000 × *g* at +4°C). Supernatant was centrifuged for 20 min (40,000 × *g*). The resulting pellet was resuspended in 15 vol Tris-HCl buffer (50 mM, pH 7.4, +4°C), and the homogenate was again centrifuged for 20 min (40,000 × *g*). This washing of the homogenate was repeated twice, and the final pellet was suspended in 10 vol Tris-HCl; 100 μl aliquots of this suspension were taken for incubation in a 1-ml vol on crushed ice for 30 min.

Tritiated flunitrazepam (specific activity 77.4 Ci/mmol, NEN, Boston, MA) was added to the incubation tube to yield a final concentration of 0.11 nM for the study of benzodiazepine binding; for Scatchard analysis, six concentrations ranging between 0.06 and 4.3 nM were used. Nonspecific binding was determined after a 10 μM concentration of nonradioactive flunitrazepam was added.

After incubation, the contents were sucked through Whatman GF/B filters (W &R Balston Ltd, England), which were rinsed twice with 4 ml ice-cold Tris-HCl buffer. The filters were allowed to solubilize in ACS scintillation liquid (Radiochemical Centre, Amersham, England) for 24 h, after which the radioactivity was measured with a Wallac-LKB liquid scintillation counter (LKB-Wallac, Turku, Finland). All measurements were made in duplicate test tubes. Specific binding was calculated by subtracting nonspecific binding from total binding. Protein content of the homogenate was measured using the method described by Lowry et al. (24).

To measure the binding of GABA receptors, fresh samples of rat brain cortex were homogenized in 32 vol 0.32 M sucrose and the homogenate was centrifuged for 20 min (40,000 × *g*, at +4°C). The resulting pellet was suspended in 32 vol ice-cold Tris-HCl buffer (50 mM, pH 7.4), and the resulting homogenate was again centrifuged. This washing was repeated once more, and the final homogenate was used in 300 μl aliquots for incubation in a 1-ml volume on crushed ice for 30 min. Filtration of the homogenate was performed as described for benzodiazepine binding. Tritiated muscimol (specific activity 12.2 Ci/mol, from the Radiochemical Centre, Amersham, England) was added to the incubation tube to yield a concentration of 6.4 nM. Non specific binding was determined after adding a 10 μM concentration of nonradioactive GABA to another set of test tubes. Measurement of radioactivity was performed as above.

The ability of GABA receptors to increase their ligand binding in the presence of benzodiazepine agonists was determined by comparing the binding of tritiated muscimol in the presence of a 10 μM concentration of diazepam and in the absence of diazepam. The ability of benzodiazepine receptors to increase their binding in the presence of GABA-agonists was also measured in a similar manner. GABA (10 μM) was added to the incubation mixture of homogenate with tritiated flunitrazepam. In both cases, the results from rats administered benzodiazepines were compared with those from control rats.

*Statistical Analysis*

For each rat, maximum response to auditory stimuli was recorded. Specific comparisons, using Student's *t*-test, were made between the results on the first treatment day and the final treatment day between baseline values and results recorded 24 and 48 h after the final chronic dose.

The effects of treatment were also statistically analyzed using nonparametric methods. A Kruskall–Wallis analysis of variance was first carried out and if this was significant a Mann–Whithey *U*-test was used to compare control and drug-treated animals. The percentage of animals exhibiting tonic extension (S.R. = 6–7) or clonic phase (S.R. = 4–5) of the audiogenic seizure was determined for each dose of compounds administered, and these values were plotted against corresponding doses for calculation of $ED_{50}$ (with 95% confidence limits). Median neurotoxic dose ($TD_{50}$ with 95% confidence limits), the dose that made 50% of animals fall from the rotarod, was calculated.

The $ED_{50}$ and $TD_{50}$ values for each compound were determined using the method of Litchfield and Wilcoxon (20). At least 32 animals were used to calculate each $ED_{50}$ and $TD_{50}$ values.

*Drug*

Diazepam, clobazam, and clonazepam were purchased from Sigma St. Louis, MO). For IP injections, diazepam was administered in a standard vehicle consisting of 40% propylene glycol, 10% benzyl alcohol, 0.2 M sodium benzoate, and 0.02 M benzoic acid in distilled water. Clonazepam and clobazam solution for IP injection were made in 60% rather than 40% propylene glycol. For chronic oral administration, each drug was suspended in 0.5% Tween 80 in distilled water sonicated and vigorously shaken before administration. For systemic administrations, all compounds were administered IP or orally by gastric gavage (0.4 ml/100 g of body weight of the rat). At least six animals were used for each dose level studied.

## RESULTS

Two hundred forty genetically epilepsy prone rats were used. Neither injections of the vehicle containing 60% propylene glycol (10 rats) nor chronic oral treatment with vehicle containing 0.5% tween 80 in distilled water (15 rats) had effects on any audiogenic seizure phases ($p > 0.13$) (Table 1). To allow better comparisons among the different benzodiazepines, we attempted to choose anticonvulsant doses that would be approximately equieffective, based on preliminary dose–response activity of these benzodiazepines (Table 1). After 120 min of oral adminstration the $ED_{50}$ against clonus were 2.37 (1.49–3.77) μmol $kg^{-1}$ for clonazepam, 15.8 (9.61–25.78) μmol $kg^{-1}$ for diazepam, and 30 (19.1–47.17) μmol $kg^{-1}$ for clobazam. After 120 min of oral adminstration the $ED_{50}$ against tonus were 1.2 (0.58–2.49) μmol $kg^{-1}$ for clonazepam, 9.82 (7.72–12.52) μmol $kg^{-1}$ for diazepam, and 20 (15.07–26.57) μmol $kg^{-1}$ for clobazam. No residual drug effects was observed 8 h after the single oral treatment with the doses of benzodiazepines used in the chronic treatment. A similar approach was done to study the motor impairment induced by these benzodi-

TABLE 1

THE EFFECT OF CLOBAZAM, CLONAZEPAM AND DIAZEPAM ON AUDIOGENIC SEIZURES IN GENETICALLY EPILEPSY-PRONE RATS-9S

| Drug Dose (umol kg$^{-1}$) | Median Seizure Score | | | |
|---|---|---|---|---|
| | IP Administration | | Oral Administration | |
| | Vehicle | Drug | Vehicle | Drug |
| Clobazam | | | | |
| 2.5 | 9 ± 0 (8) | 8.0 ± 0.4 (8) | | |
| 5 | 9 ± 0 (8) | 6.0 ± 0.4 (8) | | |
| 10 | 9 ± 0 (8) | 5.0 ± 0.4 (8)** | 9 ± 0 (8) | 8.0 ± 0.4 (8) |
| 15 | 9 ± 0 (8) | 4.0 ± 0.3 (6)** | 9 ± 0 (8) | 7.0 ± 0.4 (8) |
| 20 | 9 ± 0 (8) | 3.0 ± 0.3 (6)** | 9 ± 0 (8) | 5.0 ± 0.5 (6) |
| 30 | 9 ± 0 (8) | 1.0 ± 0.4 (6)** | | |
| 40 | 9 ± 0 (8) | 0.5 ± 0.4 (6)** | 9 ± 0 (8) | 4.0 ± 0.5 (6)** |
| 60 | 9 ± 0 (8) | 0 ± 0 (6)** | 9 ± 0 (8) | 1.0 ± 0.4 (8)** |
| Clonazepam | | | | |
| 0.1 | 9 ± 0 (8) | 8.0 ± 0.3 (8)* | | |
| 0.25 | 9 ± 0 (8) | 5.0 ± 0.4 (8)** | 9 ± 0 (8) | 9 ± 0 (8) |
| 0.5 | 9 ± 0 (8) | 4.0 ± 0.4 (8)** | 9 ± 0 (8) | 8.0 ± 0.4 (8) |
| 0.75 | 9 ± 0 (8) | 3.0 ± 0.4 (8)** | | |
| 1 | 9 ± 0 (8) | 1.0 ± 0.4 (6)** | 9 ± 0 (8) | 7.0 ± 0.4 (6) |
| 2.5 | 9 ± 0 (8) | 0 ± 0 (6)** | 9 ± 0 (8) | 5.0 ± 0.3 (6)* |
| 5 | | | 9 ± 0 (8) | 3.0 ± 0.3 (8)** |
| 10 | 9 ± 0 (8) | 0 ± 0 (6) | 9 ± 0 (8) | 2.0 ± 0.4 (8)** |
| Diazepam | | | | |
| 0.5 | 9 ± 0 (8) | 9.0 ± 0 (8) | | |
| 0.75 | 9 ± 0 (8) | 7.0 ± 0.3 (8) | | |
| 1 | 9 ± 0 (8) | 6.0 ± 0.4 (8) | | |
| 2.5 | 9 ± 0 (8) | 4.0 ± 0.3 (8)** | 9 ± 0 (8) | 9 ± 0 (8) |
| 5 | 9 ± 0 (8) | 3.0 ± 0.4 (6)** | 9 ± 0 (8) | 8.0 ± 0.4 (8) |
| 7.5 | 9 ± 0 (8) | 1.0 ± 0.4 (6)** | 9 ± 0 (8) | 6.0 ± 0.4 (8) |
| 10 | | | 9 ± 0 (8) | 4.0 ± 0.4 (6)** |
| 15 | 9 ± 0 (8) | 0.5 ± 0.3 (6)** | | |
| 20 | 9 ± 0 (8) | 0 ± 0 (6)** | 9 ± 0 (8) | 3.0 ± 0.5 (8)** |
| 40 | 9 ± 0 (8) | 0 ± 0 (6)** | 9 ± 0 (8) | 2.0 ± 0.4 (8)** |

Groups of genetically epilepsy-prone rats were injected IP or orally with the stated doses of the drugs or vehicle and exposed to auditory stimulation 60 min after drug IP injection or 120 min after oral administration. Incidence of each seizure phase was recorded and median seizure score ± interquartile range for each dose level studied is expressed. Number of rats used for each dose is reported in parentheses. Significant differences in the incidence of seizure phases between concurrent control and drug-treated group are denoted by $*p < 0.05$; $**p < 0.01$ using Mann–Whitney $U$-test.

azepines in Sprague–Dawley rats. The relative $TD_{50}$ values and protective index ($TD_{50}/ED_{50}$) are reported in Table 2.

*Chronic Treatment With Diazepam in Genetically Epilepsy-Prone Rats*

There was a statistically significant loss of diazepam effects over time as measured by the occurrence of various phases of the audiogenic seizures in oral treatment groups (Fig. 1, Table 3). The effect of the 2.5 μmol kg$^{-1}$ and 10 μmol kg$^{-1}$ IP of diazepam on incidence of seizure phases decreased in parallel as tolerance developed. On day 21, after injection of 2.5 μmol kg$^{-1}$ and 10 μmol kg$^{-1}$ IP of diazepam, the occurrence of various phases of the audiogenic seizure was significantly different to that observed on the first treatment day. The development of tolerance to the anticonvulsant effects of diazepam was more evident throughout the treatment (Fig. 1). On days 43 and 44 (24 and 48 h after the end of chronic

TABLE 2

EFFECTS OF CLOBAZAM, CLONAZEPAM AND DIAZEPAM ON AUDIOGENIC SEIZURES AND ROTAROD PERFORMANCE IN GENETICALLY EPILEPSY-PRONE RATS-9S AND SPRAGUE–DAWLEY RATS

| Compound | $ED_{50}$ | $TD_{50}$ | $TD_{50}/ED_{50}$ |
|---|---|---|---|
| Clobazam | 3.9 (3.0–5.0) | 104.0 (56–171) | 24 |
| Clonazepam | 0.24 (0.17–0.34) | 3.26 (2.09–5.42) | 14 |
| Diazepam | 0.72 (0.49–1.1) | 11.5 (8.6–15.3) | 12 |

Methods have been described in the text. Values represent the amount of compound protecting 50% of the tested animals from the clonic phase of sound-induced seizures (ED50) or impairing performance in 50% of the tested animals in the rotarod procedure (TD50). Numbers in parentheses are 95% confidence limits for each value. The data are expresssed as umol kg$^{-1}$, IP TD50/ED50 = Protective index.

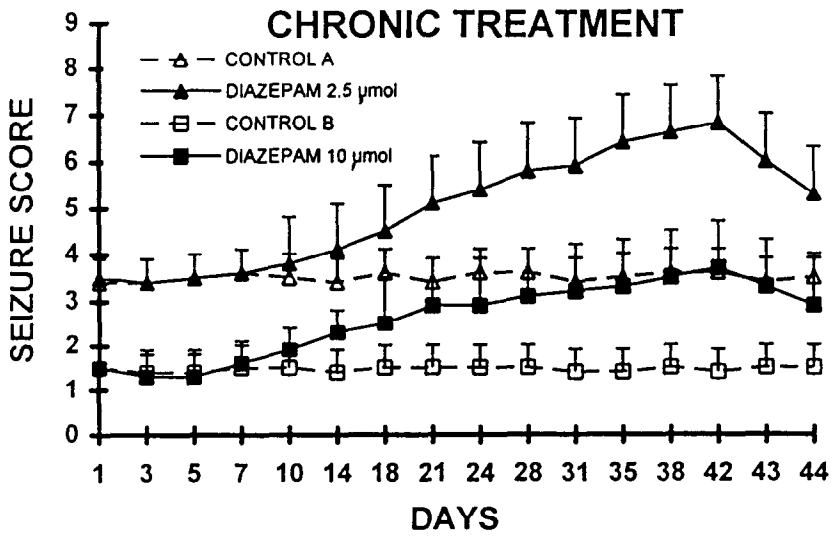


FIG. 1. Anticonvulsant effects of diazepam during chronic treatment with 15 µmol $kg^{-1}$ PO, three times daily. The seizure response per group ($n$ = 6–8) of drug treated was determined 60 min after IP administration of the indicated doses of drug. Chronic treatment ended on day 42.

treatment), following the IP administration of diazepam, we observed the presence of some audiogenic seizure phases, suggesting that tolerance was still present. Two out of eight genetically epilepsy-prone rats receiving diazepam chronically showed a stage 3 at 48 h after the end of chronic treatment when the animals were put into the Plexiglas dome before the test.

*Tolerance to the Effects of Diazepam on Motor Movements*

Tolerance to the effects of diazepam on motor function was observed as a significant reduction in the ataxia and as minor impairment of rotarod test in Sprague–Dawley rats (Table 4). Diazepam-induced impairment of performance on the rotarod test was first significantly reduced on day 10 of treatment. Tolerance to anticonvulsant and motor activity persisted throughout the treatment period and was still present 48 h after the end of chronic treatment (Tables 3 and 4).

*Chronic Treatment With Clobazam in Genetically Epilepsy-Prone Rats*

In rats chronically treated with clobazam there was a statistically significant reduction in the anticonvulsant effect of this benzodiazepine as assessed by incidence of all phases of the audiogenic seizures (Fig. 2). Seven days after the doses of clobazam (15 and 30 µmol $kg^{-1}$ IP) the incidence of various phases of audiogenic seizures was significantly different to that observed on the first treatment day as baseline values (Table 3). In addition, doubling the dose of clobazam to 30 µmol $kg^{-1}$ IP we observed after 2 weeks of treatment an anticonvulsant effect similar to that showed on the first day by 15 µmol $kg^{-1}$. When 15 and 30 µmol $kg^{-1}$ of clobazam was again tested 24 and 48 h after the end of chronic treatment (on days 29 and 30), the anticonvulsant effects of this benzodiazepine were still significantly reduced, showing that animals were still tolerant (Table 3). Three out of eight genetically epilepsy-prone rats receiving clobazam chronically showed a stage 3 at 48 h after the end of chronic treatment when the animals were put into the Plexiglas dome before the test.

*Tolerance to the Effects of Clobazam on Motor Movements*

Initially, 100 µmol $kg^{-1}$ IP of clobazam impaired rotarod test in four out of eight animals. During chronic treatment, there was a significant decrease of side effects of clobazam (Table 4). Tolerance to clobazam-induced motor impairment was first evident on day 5 and was still present 48 h after chronic treatment has been stopped (Table 4). Tolerance to motor activity persisted throughout the treatment period and was still present 48 h after the end of chronic treatment (Table 4).

*Chronic Treatment With Clonazepam in Genetically Epilepsy-Prone Rats*

There was a statistically significant reduction of clonazepam effect over time as measured by the occurrence of various phases of the audiogenic seizures in chronic treatment groups (Fig. 3, Table 3). The effect of the 0.5 and 1 µmol $kg^{-1}$ dose of clonazepam on the incidence of seizure phases decreased in parallel as tolerance developed. On day 24, after IP injection of 0.5 and 1 µmol $kg^{-1}$ of clonazepam, the occurrence of various phases of the audiogenic seizure were almost similar to that observed on the first treatment day, while a significant decrease of the anticonvulsant effects of clonazepam was first evident on day 38 (Table 3 and Fig. 3). On days 43 and 44 (24 and 48 h after the end of chronic treatment), we observed that some effects due to tolerance were still present at 24 h but they were not evident 48 h after stopping chronic treatment (Fig. 3).

*Tolerance to the Effects of Clonazepam on Motor Movements*

Tolerance to the effects of clonazepam on motor function was observed as a minor impairment of rotarod test in normal

TABLE 3

THE EFFECT OF CHRONIC TREATMENT WITH CLOBAZAM, DIAZEPAM AND CLONAZEPAM ON AUDIOGENIC SEIZURES IN GENETICALLY EPILEPSY-PRONE RATS-9S

| Days of Chronic Treatment | Median Seizure Score | | | |
|---|---|---|---|---|
| | Vehicle | Drug | Vehicle | Drug |
| | | clobazam 15 | | clobazam 30 |
| 1 | 3.5 (8) | 3.5 (8) | 1.4 (8) | 1.4 (8) |
| 3 | 3.4 (8) | 3.2 (8) | 1.4 (8) | 1.4 (8) |
| 5 | 3.6 (8) | 3.9 (8) | 1.3 (8) | 1.8 (8) |
| 7 | 3.3 (8) | 5.0 (8)* | 1.4 (8) | 2.7 (8)* |
| 10 | 3.5 (8) | 5.3 (8)** | 1.4 (8) | 2.9 (8)** |
| 14 | 3.4 (8) | 5.7 (8)** | 1.4 (8) | 3.1 (8)** |
| 18 | 3.6 (8) | 6.4 (8)** | 1.3 (8) | 3.1 (8)** |
| 21 | 3.5 (8) | 6.8 (8)** | 1.5 (8) | 3.2 (8)** |
| 24 | 3.4 (8) | 7.1 (8)** | 1.4 (8) | 3.3 (8)** |
| 28 | 3.4 (8) | 7.4 (8)** | 1.5 (8) | 3.4 (8)** |
| 29 | 3.6 (8) | 6.5 (8)** | 1.3 (8) | 2.6 (8)** |
| 30 | 3.5 (8) | 5.8 (6)** | 1.4 (8) | 2.1 (8)* |
| | | diazepam 2.5 | | diazepam 10 |
| 1 | 3.4 (8) | 3.5 (8) | 1.5 (8) | 1.5 (8) |
| 3 | 3.4 (8) | 3.4 (8) | 1.4 (8) | 1.3 (8) |
| 5 | 3.5 (8) | 3.5 (8) | 1.4 (8) | 1.3 (8) |
| 7 | 3.6 (8) | 3.6 (8) | 1.5 (8) | 1.6 (8) |
| 10 | 3.5 (8) | 3.8 (8) | 1.5 (8) | 1.9 (8) |
| 14 | 3.4 (8) | 4.1 (8) | 1.4 (8) | 2.3 (8) |
| 18 | 3.6 (8) | 4.5 (8) | 1.5 (8) | 2.5 (8) |
| 21 | 3.4 (8) | 5.1 (8)* | 1.5 (8) | 2.9 (8)* |
| 24 | 3.6 (8) | 5.4 (8)* | 1.4 (8) | 2.9 (8)** |
| 28 | 3.6 (8) | 5.8 (8)** | 1.5 (8) | 3.1 (8)** |
| 31 | 3.4 (8) | 5.9 (8)** | 1.4 (8) | 3.2 (8)** |
| 35 | 3.5 (8) | 6.4 (8)** | 1.4 (8) | 3.3 (8)** |
| 38 | 3.6 (8) | 6.6 (8)** | 1.5 (8) | 3.5 (8)** |
| 42 | 3.6 (8) | 6.8 (8)** | 1.4 (8) | 3.7 (8)** |
| 43 | 3.4 (8) | 6.0 (8)** | 1.5 (8) | 3.3 (8)** |
| 44 | 3.5 (8) | 5.3 (8)* | 1.5 (8) | 2.9 (8)* |
| | | clonazepam 0.5 | | clonazepam 1 |
| 1 | 3.4 (8) | 3.5 (8) | 1.4 (8) | 1.4 (8) |
| 3 | 3.4 (8) | 3.4 (8) | 1.4 (8) | 1.5 (8) |
| 5 | 3.5 (8) | 3.6 (8) | 1.5 (8) | 1.4 (8) |
| 7 | 3.6 (8) | 3.5 (8) | 1.5 (8) | 1.5 (8) |
| 10 | 3.5 (8) | 3.6 (8) | 1.4 (8) | 1.5 (8) |
| 14 | 3.4 (8) | 3.6 (8) | 1.5 (8) | 1.6 (8) |
| 18 | 3.6 (8) | 3.8 (8) | 1.4 (8) | 1.7 (8) |
| 21 | 3.5 (8) | 3.9 (8) | 1.5 (8) | 1.9 (8) |
| 24 | 3.6 (8) | 4.2 (8) | 1.4 (8) | 2.2 (8) |
| 28 | 3.6 (8) | 4.4 (8) | 1.4 (8) | 2.6 (8) |
| 31 | 3.4 (8) | 4.7 (8) | 1.5 (8) | 2.8 (8) |
| 35 | 3.5 (8) | 4.9 (8) | 1.5 (8) | 2.9 (8)* |
| 38 | 3.4 (8) | 5.2 (8)** | 1.4 (8) | 3.2 (8)** |
| 42 | 3.6 (8) | 5.4 (8)** | 1.5 (8) | 3.4 (8)** |
| 43 | 3.4 (8) | 3.8 (8) | 1.4 (8) | 2.8 (8) |
| 44 | 3.5 (8) | 3.6 (6) | 1.5 (8) | 2.2 (8) |

Groups of GEPR were injected IP with the stated doses of the drugs or saline (or vehicle) and exposed to auditory stimulation 60 min after drug IP injection. Incidence of each seizure phase was recorded and median seizure score for each dose level studied is expressed. Number of rats used for each dose is reported in parentheses. Significant differences in the incidence of seizure phases between concurrent control and drug-treated group are denoted by $*p < 0.05$; $**p < 0.01$ using Mann–Whitney $U$-test. The doses of benzodiazepines are expressed in μmol/kg.

TABLE 4

INFLUENCE OF CHRONIC TREATMENT WITH CLONAZEPAM, CLOBAZAM AND DIAZEPAM ON THE MOTOR IMPAIRMENT ASSESSED BY THE ROTAROD TEST

| Treatment (days) | Clobazam | Clonazepam | Diazepam |
|---|---|---|---|
| 1 | 104 (56–171) | 3.4 (2.1–5.4) | 11.5 (8.6–15.3) |
| 3 | 122 (78–192) | 3.4 (2.2–5.3) | 13.6 (9.0–20.6) |
| 5 | 158 (95–262)* | 3.7 (2.4–5.7) | 16.2 (9.6–27.3) |
| 10 | 164 (97–277)* | 4.2 (2.7–6.5) | 18.4 (10.1–33.5)* |
| 15 | 173 (101–296)* | 4.6 (3.0–7.1) | 18.8 (10.3–34.3)* |
| 20 | 180 (105–309)* | 5.0 (3.4–7.4)* | 19.1 (10.4–35.1)* |
| 25 | 187 (110–318)* | 5.2 (3.5–7.7)* | 19.3 (10.6–35.1)* |
| 30 | 165 (99–275)* | 5.4 (3.6–8.1)* | 19.9 (11.1–35.7)* |
| 35 | | 5.5 (3.6–8.4)* | 20.5 (11.4–36.9)* |
| 40 | | 5.6 (3.5–9.0)* | 21.5 (11.6–39.8)* |
| 44 | | 5.3 (3.6–7.8)* | 19.3 (10.5–35.5)* |

Values are expressed as umol $kg^{-1}$ of at least 32 animals according to the methods of Lichtfield and Wilcoxon (1949).

Significant differences among TD50 values are denoted: $*p < 0.05$.

rats (Table 4). Clonazepam-induced impairment of performance on the rotarod test was first significantly reduced on day 20 in the rats receiving orally clonazepam. This tolerance persisted throughout the treatment period and was still present 48 h after the end of chronic treatment (Table 4).

*Receptor Studies*

Administration of benzodiazepine clobazam, diazepam, or clonazepam for 4 or 6 weeks did not change the maximal binding capacity ($B_{max}$) of ($^3$H)flunitrazepam or the affinity of binding ($K_d$) in the cortex (Table 5). Neither did administration of these benzodiazepines have any effect on diazepam-activated ($^3$H)muscimol binding or GABA-activated ($^3$H)flunitrazepam binding (Table 6).

## DISCUSSION

The present study examines the anticonvulsant profiles of clobazam, clonazepam, and diazepam in genetically epilepsy-prone rats. The relative anticonvulsant activity of these benzodiazepines appears similar in this strain of rats following oral and IP administration. In addition, after oral administration clonazepam is from 5- to 10-fold more potent than diazepam and much more potent than clobazam. The different potencies of these benzodiazepines may reflect their different efficacies for binding to the benzodiazepine receptors involved in the generation or propagation of seizures (36). Previous data have demonstrated the greater affinity for binding to benzodiazepine sites of clonazepam in comparison to diazepam and clobazam (33,36,37). The ranking order of anticonvulsant potency of the benzodiazepines studied in this strain of rats is in general agreement with results previously reported in various rodent seizure models (4,15,16,21,29,30). In addition, the present experiments demonstrate that tolerance to anticonvulsant effects of diazepam, clonazepam, and clobazam occurs also in genetically epilepsy-prone rats. Tolerance to the anticonvulsant effects develops most rapidly following clobazam treatment, less rapidly following diazepam treatment, and most slowly following clonazepam treatment.

The rate of development of tolerance observed in the present study is in agreement with previous animal and clinical reports (13,30,39). Oxley (25) showed that tolerance to the anticonvulsant effects of clobazam appeared within 1–8 months in 77% of patients receiving this benzodiazepine. Similar results were described by Schmidt et al. (32). Tolerance to clonazepam appeared to be less prominent. Browne (2) and Frosche and Engels (9) reported that the development of tolerance to anticonvulsant effects of clonazepam was observed in 33 and 32% of the patients, respectively.

The rate of development of tolerance also varied according

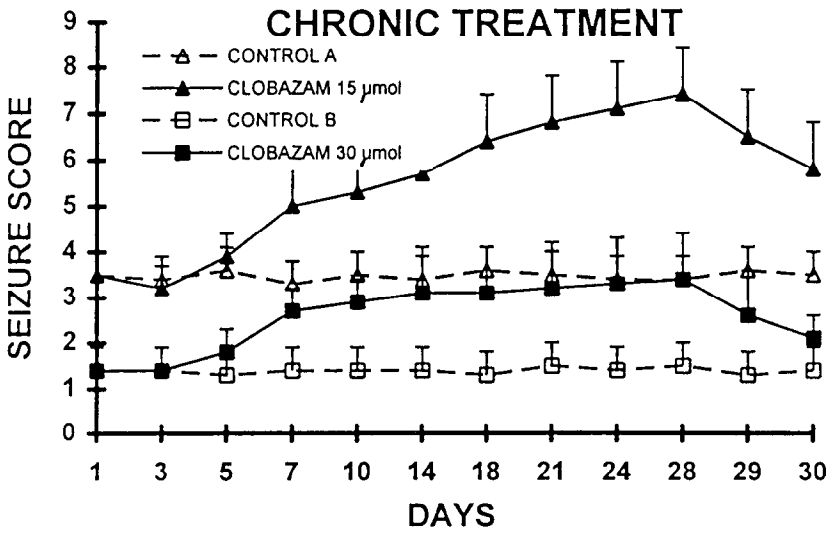


FIG. 2. Anticonvulsant effects of clobazam during chronic treatment with 30 µmol $kg^{-1}$ PO, three times daily. The seizure response per group ($n$ = 6–8) of drug treated was determined 60 min after IP administration of the indicated doses of drug. Chronic treatment ended on day 28.

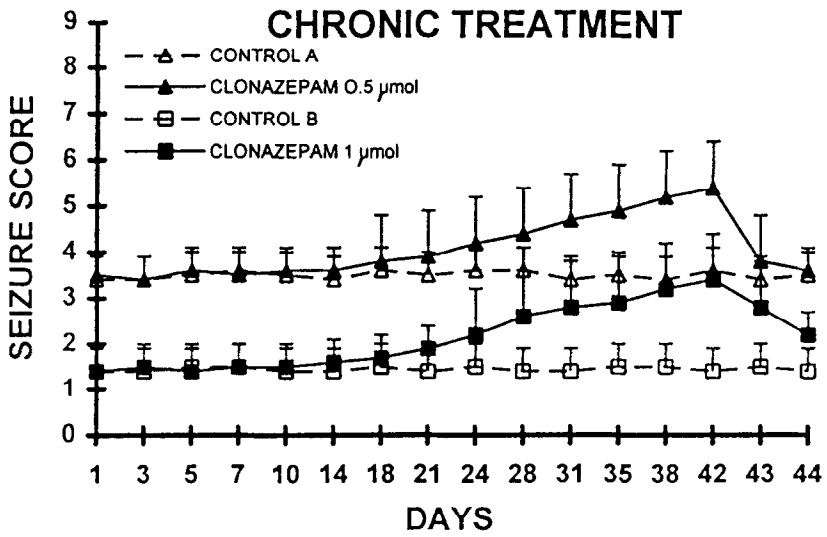


FIG. 3. Anticonvulsant effects of clonazepam during chronic treatment with 2.5 μmol $kg^{-1}$ PO, three times daily. The seizure response per group ($n$ = 6–8) of drug treated was determined 60 min after IP administration of the indicated doses of drug. Chronic treatment ended on day 42.

to the drug effects. In fact, tolerance to motor impairment developed more quickly than to the anticonvulsant activity for all three benzodiazepines. Overall, these data are similar to the usual clinical situation, in which tolerance to the side effects of benzodiazepines develops more quickly than tolerance to the anticonvulsant effects (2). The different profile of the benzodiazepines studied may be due to the number and the type of benzodiazepine receptors affected (16,17,36). This hypothesis is also supported by biochemical studies, which show that clonazepam may not be a full agonist while diazepam and clobazam are considered full agonists (26,36). These studies have demonstrated that rodents are less likely to became tolerant to the anticonvulsant effects of clonazepam and other partial benzodiazepine receptor agonists (3) than to the anticonvulsant effects of diazepam and clobazam. Indeed, these studies showed little evidence of a dependence syndrome on withdrawal from the chronic treatment (19,30,35,36). The reliability of these results is dependent on the stability of the audiogenic seizures in this strain of rats (4,27,34). The lower incidence of tolerance to clonazepam in comparison to clobazam and diazepam requires an explanation. Previous studies demonstrated that tolerance may be partly masked by the dose of benzodiazepine used (29,30,38,39). To avoid this problem in our study we have used two ranges of equieffective doses for each compound examined. A comparison of benzodiazepine anticonvulsant action is difficult because pharmacokinetic factors (e.g., metabolites) contribute to the differences in anticonvulsant potency and tolerance phenomena among the benzodiazepines. Active metabolites of diazepam and clobazam such as desmethyl-diazepam and desmethyl-clobazam contribute to the anticonvulsant effects of these compounds, whereas the major metabolite of clonazepam, for instance, 7-aminoclonazepam, does not contribute to the antiseizure activity of clonazepam (35). The different pharmacological profiles of these benzodiazepines suggest alternative explanations: the different behavioural, anticonvulsant, and tolerance effects of the compounds studied may reflect different degrees of efficacy at more than one benzodiazepine receptor site. The results of the radioreceptor assay show no significant effects of chronic treatment on brain levels of benzodiazepine receptors in the cortex. In addition, the affinity of ($^3$H)flunitrazepam binding and the maximal number of benzodiazepine binding sites does not change, and GABA-activated flunitrazepam and diazepam-activated muscimol binding do not change. These observations suggest that the development of tolerance to the anticonvulsant effects of these benzodiazepines is not principally due to a down regulation of GABA–benzodiazepine receptors. In fact, the number of receptors was not modified in all groups of animals. The fact that tolerance was still evident 48 h after the end of chronic treatment with diazepam and clobazam, when it was unlikely that residual drug would be present (22), and the ability to restore the anticonvulsant effec-

TABLE 5

EFFECTS OF CHRONIC ADMINISTRATION OF BENZODIAZEPINES ON ($^3$H) FLUNITRAZEPAM BINDING IN RAT CORTEX

| | Displacement of ($^3$H) Flunitrazepam Binding | |
|---|---|---|
| | $B_{max}$ (pmol/mg protein) | $K_d$ (nM) |
| Subjects | | |
| Controls ($n$ = 6) | 4.58 ± 0.33 | 2.22 ± 0.12 |
| Clobazam 15 μmol ($n$ = 6) | 4.47 ± 0.35 | 2.29 ± 0.18 |
| Clobazam 30 μmol ($n$ = 5) | 4.53 ± 0.29 | 2.33 ± 0.15 |
| Controls ($n$ = 8) | 4.79 ± 0.32 | 2.19 ± 0.18 |
| Diazepam 2.5 μmol ($n$ = 6) | 4.64 ± 0.36 | 2.27 ± 0.22 |
| Diazepam 10 μmol ($n$ = 6) | 4.50 ± 0.45 | 2.36 ± 0.21 |
| Controls ($n$ = 6) | 4.83 ± 0.43 | 2.21 ± 0.16 |
| Clonazepam 1 μmol ($n$ = 6) | 4.61 ± 0.30 | 2.34 ± 0.20 |

Values are mean ± SEM of the maximal binding capacity ($B_{max}$) or the affinity ($K_d$) of ($^3$H) flunitrazepam binding to cortical homogenate of rat brain.

TABLE 6

EFFECTS OF CHRONIC ADMINISTRATION OF CLOBAZAM, DIAZEPAM OR CLONAZEPAM ON EHNANCEMENT OF GABA AND BENZODIAZEPINE RECEPTOR BINDING

| Subjects | Increase in GABA Receptor Binding (%) | Increase in BDZ Receptor Binding (%) |
|---|---|---|
| Controls | 23.7 ± 13.4 (8) | 18.9 ± 3.5 (10) |
| Clobazam 30 μmol | 23.4 ± 12.8 (6) | 19.7 ± 3.1 (6) |
| Diazepam 10 μmol | 22.6 ± 14.5 (6) | 18.2 ± 2.8 (6) |
| Clonazepam 1 μmol | 21.9 ± 13.7 (5) | 16.8 ± 3.9 (5) |

Values are expressed as percentage of increase (means ± SEM) in binding in the presence of an enhancer.

($^3$H) Muscimol (6.4 nM) was used as ligand and diazepam (10 μM) was used as ehnancer for GABA receptor binding assay. ($^3$H) Flunitrazepam (0.11 nM) was used as ligand, and GABA (10 μM) as enhancer for benzodiazepine binding assay. The number of experiments is shown in parentheses.

tiveness when the dose of clobazam was doubled, suggests that tolerance is a functional phenomena (35).

Spontaneous seizures were not observed when rats were handled during chronic drug or vehicle treatment while they sometimes occurred 48 h after the final dose of clobazam and diazepam. This rebound hyperexcitability may be related to rebound phenomena observed clinically (1,10,28). It shows that clonazepam differs from the other two benzodiazepines and that the lack of rebound phenomena may be related to the slower development of tolerance with this compound.

The present observations suggest that epilepsy seizure-prone rats can be useful for studying the long-term efficacy of novel anticonvulsant drugs.

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