Pharmacology Biochemistry & Behavior, Vol. 39, pp. 711–716. © Pergamon Press plc, 1991. Printed in the U.S.A. 0091-3057/91 $3.00 + .00

Differential Tolerance to the Antipentylenetetrazol Activity of Benzodiazepines in Flurazepam-Treated Rats

HOWARD C. ROSENBERG, ELIZABETH I. TIETZ AND TED H. CHIU

Department of Pharmacology, Medical College of Ohio, P.O. Box 10008, Toledo, OH 43699

Received 1 February 1991

ROSENBERG, H. C., E. I. TIETZ AND T. H. CHIU. *Differential tolerance to the antipentylenetetrazol activity of benzodiazepines in flurazepam-treated rats.* PHARMACOL BIOCHEM BEHAV **39**(3) 711–716, 1991.—Rats were treated for one week with flurazepam (FZP). After an additional two days with no treatment, each rat was injected with one of seven benzodiazepines (BZs). Several different doses of each BZ were evaluated. Ten min later, 100 mg/kg pentylenetetrazol (PTZ) was injected, IP, and convulsive activity was recorded. Rats treated for a week with FZP were tolerant to ataxia induced by each of the seven BZs tested. There was a dose-dependent anti-PTZ effect for each BZ. Whether or not tolerance to the anti-PTZ effect was found depended on the particular BZ used. Tolerance was found for four of the drugs: diazepam, clobazam, flurazepam and desalkylflurazepam. However, no tolerance was found to the anti-PTZ actions of midazolam, triazolam or clonazepam. Brain BZ levels were measured by the ability of brain extracts to displace specifically bound [^{3}H]flunitrazepam in vitro. There was no significant effect of one week of flurazepam treatment. It was proposed that differences among BZs in their interactions with receptors allowed some to circumvent the mechanism responsible for tolerance to the anti-PTZ effect.

Benzodiazepines Tolerance Cross-tolerance Pentylenetetrazol Anticonvulsant Chronic treatment

IT is well established that chronic benzodiazepine (BZ) administration can produce tolerance (12,27). Tolerance does not develop uniformly, but rather depends on several factors. One factor is the measure of drug action under study. For example, tolerance to sedation and motor impairment can develop with little or no tolerance to behavioral disinhibition (3,19). Similarly, tolerance to BZ locomotor impairment was shown to develop more rapidly than tolerance to the anticonvulsant effect (30).

Another factor that may influence tolerance development is the particular drug used for chronic treatment. Thus tolerance to anticonvulsant actions developed more quickly during clobazam than during clonazepam treatment of amygdala kindled rats (36,42). The same dissimilarity between the rate of tolerance development during treatment with clobazam or clonazepam was observed in mice in which pentylenetetrazol (PTZ) was infused to determine drug effects on seizure threshold (11). Further study revealed a rapid development of tolerance during treatment with nitrazepam, and a more slowly developing tolerance during treatment with chlordiazepoxide or midazolam (9). Other details of the chronic treatment are also probably important in the development of tolerance. For example, variations in the development of tolerance to BZs have also been shown to occur between different strains of the same species (5).

Another indication of the nonuniformity of BZ tolerance is the regional variation in CNS response to chronic treatment. When BZ receptor downregulation is produced, it varies according to brain region (23, 26, 35). Other experiments showing regional differences in the effects of chronic BZ treatment have used electrophysiological recording (41), measurement of GABA-mediated $^{36}Cl^-$ flux (20), and measurement of the ability of GABA to stimulate [^{3}H]flunitrazepam binding in BZ-tolerant rats (34). Such findings show that BZ tolerance is not uniform, and suggest multiple sites and mechanisms for tolerance.

One possibility that has received little attention is that the ability to demonstrate tolerance following chronic BZ treatment may depend on the particular drug used for testing. In an earlier study, Gent et al. (10) measured BZ action by elevation of PTZ seizure threshold, and demonstrated cross-tolerance to the anticonvulsant action of several BZs. Though the data suggested some differences between certain pairs of chronic treatment and test drugs, there was no clear pattern, and significant tolerance was demonstrated in all but one case out of many. In the present study, the possibility that choice of test drug might determine whether or not tolerance could be shown was explored in groups of rats that had all received a standard one week BZ treatment. These rats were then tested with one of seven different BZ agonists. For each of these BZs, several doses were chosen to be sure that tolerance could be detected if it were indeed present [cf. (29)].

METHOD

Chronic Treatment

Male Sprague-Dawley rats (225–275 g) were housed in a climate-controlled room with a 12 h light/dark cycle, with free ac-

cess to standard rat food. They were given flurazepam (FZP) in a 0.02% saccharin solution as drinking water for one week, according to the procedure described previously (25, 29, 34, 35, 37). Supplying FZP in the drinking water is an efficient way to produce tolerant rats without the problems sometimes associated with repeated parenteral injections. The FZP concentration was adjusted daily, based on the volume consumed over the previous day and the weight of the rat, to provide up to 100 mg/kg daily for 3 days, and 150 mg/kg for the next 4 days (but subject to a maximum concentration of 1.0, then 1.5 mg/ml). Control rats were handled identically, but received undrugged saccharin solution. The FZP dose actually consumed during the treatment did not differ from that recently reported in rats treated in the same way (37). Only rats that had consumed an average of at least 100 mg/kg FZP daily were included in the study. As in previous work (25), rats given this treatment did not show any ataxia or sedation.

After the week of treatment, FZP was replaced with undrugged saccharin solution. All testing was done 48 h later, at which time approximately 99% of active BZ had been eliminated from brain (29). This is in keeping with the very short elimination half-lives of FZP and its active metabolites in the rat [$t_{1/2}$'s of about two hours; (17)]. Each rat was tested only once. Equal numbers of treated and control rats were tested on a given day. As in previous studies using this dosing technique (25,37), no spontaneous abstinence signs were produced.

Behavioral Testing

Seven different drugs were used to test for tolerance. These drugs were: FZP; desalkyl-FZP, a much more potent (24,38) metabolite of FZP found in several species including rats (17); diazepam; clobazam, a 1,5-benzodiazepine; clonazepam; midazolam, an imidazobenzodiazepine; and triazolam, a triazolobenzodiazepine. Several doses of each drug were tested in groups of 12–16 FZP-treated and control rats. Since the choice of test dose is related to whether or not tolerance will be measured (29), an attempt was made to choose several doses, ranging from barely effective to doses that suppressed nearly all convulsive activity.

So that the observer would be blind to which rats had received chronic treatment, another person who was unfamiliar with the chronic treatment was asked to randomly assign codes to the rats just before they were to be tested. Each BZ dose was injected IP in a volume of 1 ml/kg. Nine minutes later, motor function was evaluated by observing the rat on a table top, and assigning an ataxia rating of 0 (no observable drug effect), 1 (slight ataxia), 2 (clear ataxia, falling or stumbling sideways), 3 (unable to stand, drags trunk on the table top), or 4 (loss of righting response). One minute later (ten min after BZ injection), 100 mg/kg of PTZ, in a volume of 1 ml/kg, was injected IP. This interval was based on estimates of the time-action profile of IP BZs, determined by observing the time course of intoxication after large doses of each drug in preliminary trials, and is consistent with the reported time-concentration profile of diazepam in brain following IP injection (7,15). The nature of convulsive activity, and the time to onset of myoclonus, front leg clonus, generalized clonus involving all four legs, and tonus was recorded for 20 min. A seizure score was assigned to each rat according to the most severe convulsion observed: 0, no convulsion, or tremors, face and ear twitches only; 1, myoclonic jerks; 2, front leg clonus, no loss of upright posture; 3, severe clonic seizure with loss of upright posture; 4, tonic-clonic seizure.

Drug Solutions

Diazepam and midazolam were administered in a vehicle consisting of 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol, 0.2 M Na benzoate and 0.02 M benzoic acid in distilled water. Clonazepam, desalkyl-FZP, clobazam, and the highest dose of diazepam were made in a similar solvent, but with 60% rather than 40% propylene glycol. Triazolam vehicle consisted of 5% polyoxyethylenesorbitan monooleate (Tween 80), 45% propylene glycol and 50% distilled water. FZP was diluted with saline from a 100 mg/ml aqueous stock solution, which was prepared and pH adjusted as previously described (25). PTZ was dissolved in normal saline and was prepared fresh daily.

Brain Benzodiazepine Levels

Brain BZ levels were measured to determine if tolerance was functional or pharmacokinetic. Groups of 6 FZP-treated and control rats were injected with either diazepam (5 mg/kg), FZP (20 mg/kg), desalkyl-FZP (2 mg/kg), or clonazepam (0.5 mg/kg). Ten min later, the rats were killed by decapitation. The brains were homogenized in 10 vol ice-cold absolute ethanol using a glass homogenizing tube and a Teflon pestle mounted in an electric drill (8). The resulting homogenate was centrifuged ($10,000 \times g$, 20 min); aliquots of 50 μl ethanol were evaporated overnight. The assay was based on the displacement of [^{3}H]flunitrazepam specifically bound to cerebral cortical membranes ("triple washed" P_2 membranes) freshly prepared from a naive rat. The preparation of the P_2 membranes and the assay technique were the same as used in previous studies (29,38). Tubes containing the residue from the brain extract were incubated 1 h at 4°C with 0.4 ml aliquots of membrane suspension. Membranes (0.6 mg protein/ml; bicinchoninic acid method, Pierce Chemical Co.) were incubated for 1 h with 2 nM [^{3}H]flunitrazepam in a total assay volume of 0.5 ml in the presence of either buffer alone, increasing concentrations of BZ standards, or reconstituted extract residue. The incubation was terminated by dilution with 5 ml ice-cold buffer and rapid filtration through Whatman GF/B filters under controlled suction, followed by two additional washes. Nonspecific binding was determined using 1 μM clonazepam. The radioactivity retained on the filters was counted by liquid scintillation spectroscopy. Specific binding was the difference between total and nonspecific binding. The displacement of specific binding produced by 10 concentrations of diazepam (1–1000 nM, in triplicate) was used to construct a standard displacement curve. The ability of each sample to displace [^{3}H]flunitrazepam was determined and the equivalent concentration of diazepam calculated (from the standard curve) by log-probit analysis with the aid of the Pharmacologic Calculation System (PCS) software. Based on this information and the weight of the brain, the amount of BZ was expressed as "diazepam equivalents" in units of μg diazepam/g brain.

Data Analysis

The anti-PTZ effect of each BZ, measured by seizure score, was evaluated using a two-way ANOVA, with dose and treatment as grouping variables. Time to onset of each convulsive behavior was also analyzed by 2-way ANOVA. Since BZs typically can protect against PTZ seizures at doses lower than those associated with ataxia (24), most of the doses used to test anticonvulsant activity produced little or no ataxia. Therefore, a single dose of each drug, which produced ataxia scores of 1 or 2 in all or almost all rats (either the largest or next to largest dose), was chosen for analysis of motor impairment. The difference between ataxia ratings in treated and control rats was evaluated by the Mann-Whitney U-test. The ability of brain extracts from treated and control rats to displace [^{3}H]flunitrazepam, ex-

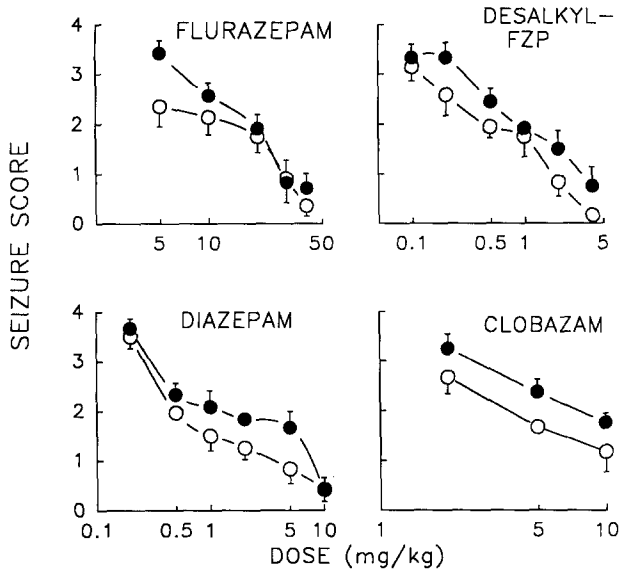

FIG. 1. Average PTZ seizure score in control rats (open circles) and in rats tested 48 h after a one week FZP treatment (filled circles). Tolerance, shown by a significant main effect for treatment, was found for FZP, desalkyl-FZP, diazepam and clobazam (n = 12–16 for each point).

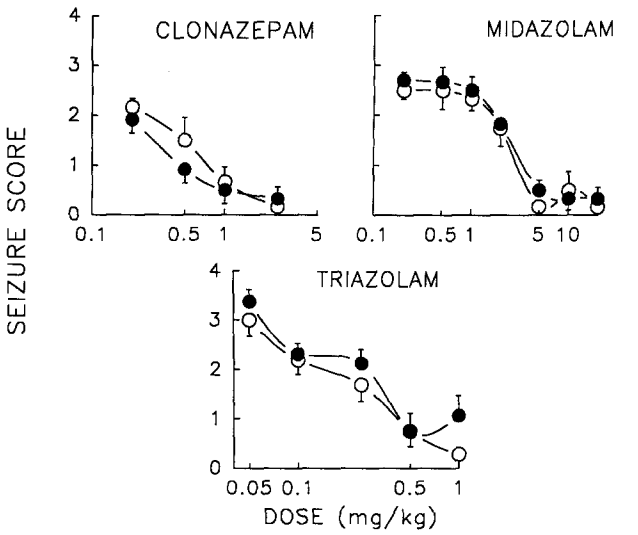

FIG. 2. Average PTZ seizure score in control rats (open circles) and in rats tested 48 h after a one week FZP treatment (filled circles). There was no significant effect of treatment, and no significant treatment × dose interaction for clonazepam, midazolam or triazolam (n = 12–16).

pressed in "diazepam equivalents," was compared by Student's *t*-test. In all cases, $p<0.05$ was considered statistically significant.

RESULTS

When injected 10 min before PTZ, each of the seven BZs suppressed convulsive activity in both control rats and rats that had been treated for a week with FZP. This anti-PTZ effect was dose-dependent for each BZ examined (for each BZ, $p<0.0001$ for dose effect). Tolerance to the anti-PTZ effect of four BZs (diazepam, FZP, desalkyl-FZP and clobazam) was shown by their decreased ability to suppress PTZ seizures in rats that had been treated for a week with FZP (Fig. 1). This was reflected by a significant treatment effect [diazepam, $F(1,132)=9.7$, $p<0.01$; FZP, $F(1,122)=4.0$, $p<0.05$; desalkyl-FZP, $F(1,149)=8.1$, $p<0.01$; clobazam, $F(1,65)=7.9$, $p<0.01$]. There was no significant interaction between treatment group and dose for any of these BZs. In contrast to the tolerance measured to these four BZs, there was no significant effect of chronic FZP treatment on the anti-PTZ effect of clonazepam, midazolam, or triazolam (Fig. 2). Thus, there was no significant main effect of treatment, nor a significant interaction between BZ dose and treatment group for these three BZs. There was some suggestion of tolerance to triazolam, though the difference did not achieve statistical significance [treatment effect, $F(1,146)=3.3$, $p=0.07$]. However, in the case of clonazepam, there appeared to be a (nonsignificant) trend toward an increase in the effectiveness of clonazepam in FZP-treated rats (Fig. 2).

The BZs also increased the latency to onset of PTZ convulsive activity. For all the BZs tested, except clonazepam and FZP, there was a significant main effect of dose on the time to onset of myoclonic jerks ($p<0.01$ for each BZ) and time to onset of clonus ($p<0.01$). A similar effect on the time to onset of tonus could not be evaluated because of the low number of rats having tonic seizures after BZ pretreatment. The BZ effect on the latency to onset of PTZ convulsive activity was not a sensitive measure of tolerance. In fact, there was a significant treatment effect only in the case of time to onset of PTZ-induced clonus after clobazam pretreatment, $F(1,65)=7.9$, $p<0.01$. There was no significant dose × treatment interaction in latency to convulsive activity for any of the BZs tested.

BZ-induced motor impairment was measured by ataxia score (Fig. 3). For each of the BZs tested, tolerance was shown by a significantly smaller ataxia score in FZP-treated as compared to control rats ($p<0.05$, Mann-Whitney U-test).

To determine if there might be a pharmacokinetic basis for the results, the amount of BZ in brain was determined in control

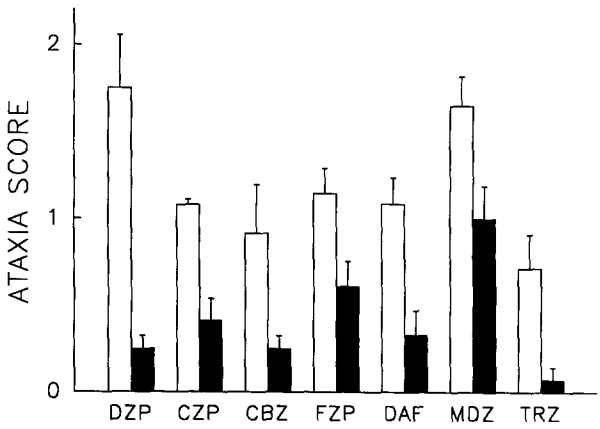

FIG. 3. Mean ataxia rating measured 9 min after BZ injection (n = 12–14 for each group). Open bars, control rats; closed bars, rats tested 48 hr after one week of FZP treatment. Vertical lines indicate one SEM. The drugs (and doses, in mg/kg) tested were: diazepam (DZP, 5), clonazepam (CZP, 1), clobazam (CBZ, 10), flurazepam (FZP, 40), desalkylflurazepam (DAF, 4), midazolam (MDZ, 10) and triazolam (TRZ, 1). Tolerance to BZ-induced ataxia was present for each drug ($p\leq0.05$, Mann-Whitney U-test).

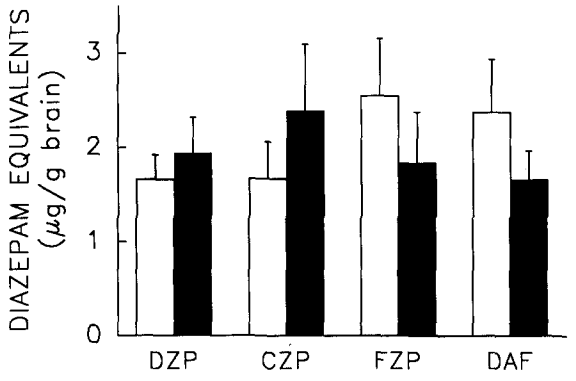

FIG. 4. Brain BZ levels, measured as BZ-like activity of brain extracts from control (open bars) and FZP-treated rats (filled bars). Rats were injected IP with diazepam (DZP, 5 mg/kg), clonazepam (CZP, 0.5 mg/kg), FZP (20 mg/kg) or desalkyl-flurazepam (DAF, 2 mg/kg) 10 min before being sacrificed. There was no significant difference between treated and control values for any drug. Bars represent mean (n=6)+ SEM.

and treated rats that were killed 10 min after IP injection of the test BZ. The activity of BZ in brain was measured by the ability of brain extracts to displace specifically bound 2 nM [^{3}H]flunitrazepam; results were expressed as "diazepam equivalents" (Fig. 4). For each BZ, there was no significant difference between control rats and those that had received one week FZP treatment.

DISCUSSION

The main finding of this study was that tolerance to the anti-PTZ effect of BZs was present for some BZs but not for others in rats tested 48 h after a one week FZP treatment. All the rats had received the same FZP treatment, and all were tested at the same time after this treatment. Thus, they would all be expected to respond as a single population to the test BZ. Indeed, FZP-treated rats were found to be tolerant to BZ-induced ataxia for each of the seven drugs tested. In contrast, tolerance to the anti-PTZ effect was found for only four of these BZs. Since there were differences found, it was concluded that the difference (i.e., the presence or absence of tolerance to the anti-PTZ effect) was a function of the BZs used for testing. If an inappropriate dose of BZ were chosen, tolerance might not have been detected (29). This did not appear to be the case except perhaps for triazolam (Fig. 2). Possibly, further testing with triazolam, especially at the higher dose range, might reveal tolerance after one week of FZP treatment. However, the data for midazolam and clonazepam appeared to be clear; there was no indication of tolerance to these BZs (Fig. 2). In fact, the data for clonazepam suggested that sensitization (1) may be present, though the difference between treated and control rats did not achieve statistical significance. The brain levels of BZs at the time of testing (Fig. 4) could not explain tolerance, or the differential presence of tolerance. The results suggest that the expression of tolerance may depend on subtle differences among these drugs in their receptor interactions, and the effect of chronic treatment on the $GABA_A$/BZ receptor. Since BZ receptor downregulation is no longer present 48 hr after terminating FZP treatment (25,35), it is probably not involved in the results of this study. However, more subtle $GABA_A$ receptor "remodeling" may have occurred to alter the interaction between BZs and the receptor. It has been found that changing the subunit composition of the $GABA_A$ receptor, even when the difference is between two highly homologous isoforms, can affect some receptor functions (e.g., effects of BZs) while having little effect on others (e.g., response to GABA) (18,40). A recent study (14), in which cDNA probes for human $GABA_A$ receptor α_1 and β_1 subunits were used, found changes in mRNA levels for the α, but not the β subunit, in diazepam-treated rats.

Failure to detect tolerance to the anti-PTZ action of three BZs 48 h after the week of FZP treatment might suggest that tolerance to these particular drugs does not occur in FZP-treated rats. However, another reasonable conclusion might be that greater tolerance must be produced before it can be detected using these BZs, so that more prolonged, or more intensive treatment might have allowed tolerance to be observed with all seven BZs. The importance of treatment duration was suggested by previous studies in which the development of tolerance during chronic treatments with different BZs were compared (9, 11, 30, 42), in contrast to the present study in which the chronic treatment was held constant. It may be possible that tolerance to all seven BZs in the present study examined might have been observed after a more prolonged FZP treatment. Conversely, shorter treatments might have caused tolerance to be observed with an even more limited number of BZs.

As alluded to above, most previous studies comparing anticonvulsant tolerance among BZs considered the effects of varying the drug used for chronic treatment. The present study dealt with a different issue, the patterns of BZ cross-tolerance in animals that had all received the identical chronic treatment regimen, and all of which presumably had the same alterations in neuronal and synaptic function as a consequence. Thus, in this study, variations among BZ agonists in the expression of tolerance must have been due to differences in their interactions with BZ receptors or the expression of that interaction by neurons. A few previous studies of BZ tolerance suggested that different patterns of tolerance might be determined by the drug used for testing. Ruhland (31) found various patterns of tolerance to three actions of diazepam, bromazepam, clobazam and metaclazepam (a 2-methoxy derivative) in separate groups of mice treated daily for 9 days with each of these BZs. For example, clobazam treatment afforded greater tolerance to the anti-PTZ effect of diazepam than to clobazam itself. No treatment afforded tolerance to the anti-PTZ effect of metaclazepam, though treatment with that drug did cause tolerance to the anticonvulsant action of the other drugs. In a study in dogs (6), in which PTZ seizure threshold elevation was used to measure BZ action, tolerance to diazepam was detected within a week. When dogs were treated with clorazepate (which is completely converted to desmethyldiazepam during absorption), they were tolerant to diazepam, but not to desmethyldiazepam. Like the present investigation, these studies also show that the expression of tolerance to the anticonvulsant effect of BZs depends in part on the BZ used for testing.

In previous studies in which tolerance to different BZs was compared (9, 11, 30, 42), variations in the expression of tolerance may likewise have influenced the results. For example, in our study of tolerance in amygdala kindled rats (30), tolerance appeared more rapidly during clobazam treatment than during clonazepam treatment. The results of the present study suggest the possibility that the same degree of tolerance may have developed during treatment with both, but was expressed differently, according to the BZ being used to measure the phenomenon. Tolerance to clobazam might have been present after a short clonazepam treatment, and clonazepam might have still been ef-

fective in rats that had become tolerant to clobazam. Future investigations of cross-tolerance between these BZs in amygdala kindled rats might answer this question, and would also address the question of whether the results found in the present study are unique to the anti-PTZ effect of BZs, or more generally applicable.

The basis for the differential expression of tolerance to BZ anti-PTZ activity was likely related to the drug-receptor interaction. Some drugs must either interact with the receptor in such a way that tolerance is not expressed, or produce their anti-PTZ effect by interacting with a different subset of BZ receptors than those involved with tolerance. This latter possibility would be analogous to the situation with multiple types of opioid receptors, and the role they play in determining patterns of opioid tolerance and dependence (21). Though there is evidence that BZ receptors do not constitute a homogeneous population (16) [see (13) for review], there is not a clear separation of receptor types as with opioid receptors. Furthermore, there is no direct evidence that the seven drugs evaluated in the present study interact with different populations of BZ receptors. For example, in one study (33) several BZs were evaluated, and their interactions with cerebellar and hippocampal BZ receptors were compared by their IC_{50}'s to displace specifically bound [^{3}H]flunitrazepam. There was no obvious relationship between the expression of tolerance (Figs. 1 and 2) and relative affinities for hippocampal and cerebellar BZ receptors (33).

One possibility is that the differing structures of the BZs could offer a clue as to what characteristics of triazolam, midazolam and clonazepam might set them apart from the other four drugs tested. However, comparing the structures of these seven drugs and whether or not tolerance could be measured, no clear pattern emerges. Though detailed analysis might prove otherwise, there is no immediately apparent structural explanation of why tolerance could be shown when FZP-treated rats were tested with some, but not with other BZs.

Not all BZs have the same intrinsic activity at BZ receptors, and some may be partial agonists. It has been suggested that the development of tolerance to BZs (or possibly the expression of tolerance) might be determined by the same structural features that determine a drug's efficacy, so that tolerance would not occur, or develop more slowly, for partial than for full BZ agonists (2, 9, 32, 39). There is some evidence that clonazepam might have less efficacy than diazepam (4,22). However, there is little direct evidence currently available comparing the efficacies of the other BZs used in the present study, and, most importantly, little information on their relative efficacies to antagonize PTZ seizures.

The results of the present study confirmed previous findings that the expression of BZ tolerance depends, among other things, on the particular drug action under study (3, 19, 28, 30). Thus tolerance to motor impairment caused by clonazepam, midazolam and triazolam was present even when tolerance to the anticonvulsant action was not. The results also showed that the expression of tolerance depends on the particular agonist being used to measure drug activity. Several questions remain to be answered. Foremost among these is the basis for differences among BZs in their ability to bypass whatever mechanism is responsible for tolerance to the anticonvulsant effect. It is also not known if the finding is unique to the action of BZs against PTZ seizures, or if it is more generally applicable. For example, it might be useful to know if a similar pattern of cross-tolerance to the anticonvulsant actions of these drugs would be found with other experimental seizures, or what patterns of cross-tolerance to drug-induced ataxia might occur if rats were tested after a briefer treatment period. Another important question to answer is whether or not similar results can be obtained in rats made tolerant by chronic treatment with other BZs. The answers to these questions might prove important during the treatment of epilepsy, when tolerance to the anticonvulsant effect often limits the usefulness of these agents.

ACKNOWLEDGEMENTS

Flurazepam, midazolam, desalkyl-flurazepam, clonazepam and diazepam were generously provided by Dr. Peter F. Sorter, Hoffmann-La Roche, Inc., Nutley, NJ. Clobazam was a gift from Val K. Wagner, Hoechst-Roussel Pharmaceuticals, Somerville, NJ. Triazolam was a gift from Warren King, Upjohn Co., Kalamazoo, MI. The expert technical assistance of Mary Ann Gonzalez and William M. Gilliam is greatly appreciated. Supported by grant R01-DA02194.

REFERENCES

1. Antelman, S. M.; Kocan, D.; Edwards, D. J.; Knopf, S. A single injection of diazepam induces long-lasting sensitization. Psychopharmacol. Bull. 23:430–434; 1987.
2. Boast, C. A.; Gerhardt, S. C. Lack of tolerance or withdrawal effects in mice after chronic administration of the non-sedating anxiolytic CGS 9896. Pharmacol. Biochem. Behav. 26:601–606; 1987.
3. Cannizzaro, G.; Nigito, S.; Provenzano, P. M.; Vitikova, T. Modification of depressant and disinhibitory action of flurazepam during short term treatment in the rat. Psychopharmacology (Berlin) 26: 173–184; 1972.
4. Chan, C. Y.; Farb, D. H. Modulation of neurotransmitter action: control of the γ-aminobutyric acid response through the benzodiazepine receptor. J. Neurosci. 5:2365–2373; 1985.
5. File, S. E. Strain differences in mice in the development of tolerance to the anti-pentylenetetrazol effects of diazepam. Neurosci. Lett. 42:95–98; 1983.
6. Frey, H.-H.; Philippin, H.-P.; Scheuler, W. Development of tolerance to the anticonvulsant effect of diazepam in dogs. Eur. J. Pharmacol. 104:27–38; 1984.
7. Friedman, H.; Abernethy, D. R.; Greenblatt, D. J.; Shader, R. I. The pharmacokinetics of diazepam and desmethyldiazepam in rat brain and plasma. Psychopharmacology (Berlin) 88:267–270; 1986.
8. Fujimoto, M.; Okabayashi, T. Good correlations between brain levels of benzodiazepines determined by radioreceptor assay and central nervous system activity. Chem. Pharm. Bull. 3:1014–1017; 1982.
9. Garratt, J. C.; Gent, J. P.; Feely, M.; Haigh, J. R. M. Can benzodiazepines be classified by characterising their anticonvulsant tolerance-inducing potential? Eur. J. Pharmacol. 145:75–80; 1988.
10. Gent, J. P.; Bently, M.; Feely, M.; Haigh, J. R. M. Benzodiazepine cross-tolerance in mice extends to sodium valproate. Eur. J. Pharmacol. 128:9–15; 1986.
11. Gent, J. P.; Feely, M. P.; Haigh, J. R. M. Differences between the tolerance characteristics of two anticonvulsant benzodiazepines. Life Sci. 37:849–856; 1985.
12. Greenblatt, D. J.; Shader, R. I. Long-term administration of benzodiazepines: pharmacokinetic versus pharmacodynamic tolerance. Psychopharmacol. Bull. 22:416–423; 1986.
13. Haefely, W.; Kyburz, E.; Gerecke, M.; Möhler, H. Recent advances in the molecular pharmacology of benzodiazepine receptors and in the structure-activity relationships of their agonists and antagonists. Adv. Drug Res. 14:165–322; 1985.
14. Heninger, C.; Saito, N.; Tallman, J. F.; Garrett, K. M.; Vitek, M. P.; Duman, R. S.; Gallager, D. W. Effects of continuous diazepam administration on $GABA_A$ subunit mRNA in rat brain. J. Molec. Neurosci. 2:101–107; 1990.

15. Hironaka, T.; Fuchino, K.; Fujii, T. Absorption of diazepam and its transfer through the blood-brain barrier after intraperitoneal administration in the rat. J. Pharmacol. Exp. Ther. 229:809–815; 1984.
16. Klepner, C. A.; Lippa, A. S.; Benson, D. I.; Sano, M. C.; Beer, B. Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors. Pharmacol. Biochem. Behav. 11:457–462; 1979.
17. Lau, C. E.; Falk, J. L.; Dolan, S.; Tang, M. Simultaneous determination of flurazepam and five metabolites in serum by high-performance liquid chromatography and its application to pharmacokinetic studies in rats. J. Chromatogr. 423:251–259; 1987.
18. Lûddens, H.; Pritchett, D. B.; Köhler, M.; Killisch, I.; Keinänen, K.; Monyer, H.; Sprengel, R.; Seeburg, P. H. Cerebellar $GABA_A$ receptor selective for a behavioral alcohol antagonist. Nature 346: 648–651; 1990.
19. Margules, D. L.; Stein, L. Increase of "antianxiety" activity and tolerance of behavioral depression during chronic administration of oxazepam. Psychopharmacology (Berlin) 13:74–80; 1968.
20. Marley, R. J.; Gallager, D. W. Chronic diazepam treatment produces regionally specific changes in GABA-stimulated chloride influx. Eur. J. Pharmacol. 159:217–223; 1989.
21. Martin, W. R. Multiple opioid receptors. A little about their history and some implications related to evolution. Life Sci. 28:1547–1554; 1981.
22. Massotti, M.; De Medici, D.; De Luca, C. Relative efficacies of benzodiazepine receptor agonists in affecting red nucleus electrical activity in rabbits. Psychopharmacolgy (Berlin) 102:479–484; 1990.
23. Miller, L. G.; Greenblatt, D. J.; Barnhill, J. G.; Shader, R. I. Chronic benzodiazepine administration. I. Tolerance is associated with benzodiazepine receptor downregulation and decreased γ-aminobutyric $acid_A$ receptor function. J. Pharmacol. Exp. Ther. 246: 170–176; 1988.
24. Randall, L. O.; Kappell, B. Pharmacological activity of some benzodiazepines and their metabolites. In: Garattini, S.; Mussini, E.; Randall, L. O., ed. The benzodiazepines. New York: Raven Press; 1973: 27.
25. Rosenberg, H. C.; Chiu, T. H. Tolerance during chronic benzodiazepine treatment associated with decreased receptor binding. Eur. J. Pharmacol. 70:453–460; 1981.
26. Rosenberg, H. C.; Chiu, T. H. Regional specificity of benzodiazepine receptor down-regulation during chronic treatment of rats with flurazepam. Neurosci. Lett. 24:49–52; 1981.
27. Rosenberg, H. C.; Chiu, T. H. Time course for development of benzodiazepine tolerance and physical dependence. Neurosci. Biobehav. Rev. 9:123–131; 1985.
28. Rosenberg, H. C.; Chiu, T. H.; Tietz, E. I. Studies on the mechanism of tolerance to benzodiazepines. In: Frey, H.; Koella, W. P.; Fröscher, W.; Meinardi, H., ed. Tolerance to the beneficial and adverse effects of antiepileptic drugs. New York: Raven Press; 1986:53.
29. Rosenberg, H. C.; Tietz, E. I.; Chiu, T. H. Tolerance to benzodiazepine anticonvulsant action: relationship to decreased receptor density. Neuropharmacology 24:639–644; 1985.
30. Rosenberg, H. C.; Tietz, E. I.; Chiu, T. H. Tolerance to the anticonvulsant effects of diazepam, clonazepam and clobazam in amygdala kindled rats. Epilepsia 30:276–285; 1989.
31. Ruhland, M. Metaclazepam—a new anxiolytic benzodiazepine with selective tolerance and crosstolerance to other benzodiazepines. Pharmacopsychiatry 18:22–23; 1985.
32. Scherkl, R.; Kurudi, D.; Frey, H.-H. Clorazepate in dogs: tolerance to the anticonvulsant effect and signs of physical dependence. Epilepsy Res. 3:144–150; 1989.
33. Sieghart, W.; Schuster, A. Affinity of various ligands for benzodiazepine receptors in rat cerebellum and hippocampus. Biochem. Pharmacol. 33:4033–4038; 1984.
34. Tietz, E. I.; Chiu, T. H.; Rosenberg, H. C. Regional GABA/benzodiazepine/chloride channel coupling after acute and chronic benzodiazepine treatment. Eur. J. Pharmacol. 167:57–65; 1989.
35. Tietz, E. I.; Rosenberg, H. C.; Chiu, T. H. Autoradiographic localization of benzodiazepine receptor downregulation. J. Pharmacol. Exp. Ther. 236:284–292; 1986.
36. Tietz, E. I.; Rosenberg, H. C.; Chiu, T. H. A comparison of the anticonvulsant effects of 1,4 and 1,5 benzodiazepines in the amygdala-kindled rat and their effects on motor function. Epilepsy Res. 3:31–40; 1989.
37. Tietz, E. I.; Rosenberg, H. C. Behavioral measurement of benzodiazepine tolerance and GABAergic subsensitivity in substantia nigra pars reticulata. Brain Res. 438:41–51; 1988.
38. Tyma, J. L.; Rosenberg, H. C.; Chiu, T. H. Radioreceptor assay of benzodiazepines in cerebrospinal fluid during chronic flurazepam treatment in cats. Eur. J. Pharmacol. 105:301–308; 1984.
39. Valerio, A.; Massotti, M. Electroencephalographic changes after short-term exposure to agonists of benzodiazepine receptors in the rat. Pharmacol. Biochem. Behav. 29:791–795; 1988.
40. von Blankenfeld, G.; Ymer, S.; Pritchett, D. B.; Sontheimer, H.; Ewert, M.; Seeburg, P. H.; Kettenmann, H. Differential benzodiazepine pharmacology of mammalian recombinant $GABA_A$ receptors. Neurosci. Lett. 115:269–273; 1990.
41. Wilson, M. A.; Gallager, D. W. Effects of chronic diazepam exposure on GABA sensitivity and on benzodiazepine potentiation of GABA-mediated responses of substantia nigra pars reticulata neurons of rats. Eur. J. Pharmacol. 136:333–343; 1987.
42. Young, N. A.; Lewis, S. J.; Harris, Q. L. G.; Jarrott, B.; Vajda, F. J. E. Differences in the development of tolerance to two different anticonvulsant benzodiazepines in the amygdaloid kindled rat. J. Pharm. Pharmacol. 40:365–367; 1988.