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Tolerance to the anticonvulsant effect of clonazepam in mice: no concurrent change in plasma concentration

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Clonazepam was administered for 10 or more days on three different dose regimens (0·5, 0·25 and 0·08 mg kg^{-1} twice daily) to mice given pentetrazol by slow intravenous infusion. Plasma concentrations of clonazepam were assayed by high performance liquid chromatography. Tolerance developed to the anticonvulsant effect of clonazepam at all doses but was incomplete and could be overcome by increasing the dose. With the 0·5 and 0·25 mg kg^{-1} regimens there was no significant change in the drug plasma concentrations during development of tolerance; on the lowest dose, levels were below the limits of accurate detection. Anticonvulsant tolerance does not seem to be the result of a disturbance in clonazepam metabolism.

The usefulness of clonazepam in the treatment of epilepsy is limited by the development of tolerance (Browne 1976; Pinder et al 1976). Recently, we have demonstrated the phenomenon of clonazepam tolerance against pentetrazol-induced clonic convulsions in mice, and shown it to be slower in onset and less pronounced than that produced by equipotent doses of clobazam (Gent et al 1985). A similar conclusion has since been reached from studies comparing clonazepam with diazepam in dogs (Scherkl et al 1985). Unfortunately, no reasonable explanation for these differences has yet been proposed.

Previous experiments in our animal model provided evidence that the induction of tolerance with clobazam was not the result of any major change in the metabolism of the drug. However, this interpretation was complicated by the formation of an active metabolite, *N*-desmethylclobazam (Gent et al 1984). In contrast, clonazepam is converted to a 7-amino derivative which has virtually no activity against pentetrazol-induced seizures in mice (Fukushhima et al 1977); thus, clonazepam is more suitable to assess the importance of metabolic changes in the development of tolerance. In view of this we have extended our studies with clonazepam by comparing its anticonvulsant effect with its plasma concentration.

Many chromatographic assay techniques are too insensitive to measure accurately the low plasma concentrations of clonazepam which provide protection against seizures, and the small volumes of mouse plasma necessitated the substantial modification of an existing assay method.

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Materials and methods

Animals. Male Tuck No. 1 mice, 25–40 g, were housed in groups of 5 and maintained on a 12 h light–dark cycle with free access to a standard diet and water.

Materials. Clonazepam (Roche Products Ltd, UK) was dissolved in a vehicle of composition: propylene glycol 0·4 ml, ethanol 0·1 ml, benzyl alcohol 0·015 ml, sodium benzoate 50·0 mg, benzoic acid 2·25 mg and distilled water to 1·0 ml. Pentetrazol (Sigma London Chemical Co. Ltd, Poole, UK) was dissolved in 165 mM NaCl solution at a concentration of 15 mg ml^{-1}.

All reagents for the assay were obtained from BDH Chemicals (Poole, UK) and filtered before use; clobazam (Hoechst, UK) was the internal standard.

Convulsive test procedure. Pentetrazol was infused into a tail vein of the unrestrained mouse at 0·3 ml min^{-1} until a clonic convulsion was elicited. The minimum convulsant dose (MCD) to induce this was obtained for each mouse and the mean (± s.e.m.) calculated for each group.

Treatment schedule. 70 mice were randomly assigned to two equal groups. The 35 animals in one group (experimental) were injected i.p. with 0·5 mg kg^{-1} clonazepam twice daily (0800 and 1900 h) for 16 days; the rest of the animals, acting as controls, were given vehicle alone on the same schedule. All mice received an injection volume of 2·5 ml kg^{-1}. On the first day and subsequently every 3 days, subgroups of 5 experimental and 5 control mice were taken at random and tested with pentetrazol 1 h after the morning injection of clonazepam (or vehicle) which was given s.c. on these occasions. Immediately following seizures, blood samples were taken from the mice dosed with clonazepam (see Haigh et al 1984). After experiencing a clonic convulsion animals were not used again. On the final day, and in addition to the routine testing, the 5 remaining control mice were given an acute dose of clonazepam before being tested with pentetrazol; the remaining animals on repeated clonazepam treatment received double their normal dose.

The study was repeated using two different clonazepam dose regimens, 0·25 and 0·08 mg kg^{-1} twice daily. The 0·25 mg kg^{-1} study was continued for only 10 days and thus fewer mice were used.

Clonazepam assay. Plasma concentrations of clonazepam were assayed by reversed-phase HPLC using a method adapted from Good & Andrews (1981). BondElut C_{18} bonded-phase extraction columns (Analytichem. Int., California) were prewashed with two column volumes of each of acetonitrile, distilled water and 0·1 M sodium carbonate-bicarbonate buffer (pH 10·1). Aliquots (500 µl) of plasma, containing 125 ng of clobazam as internal standard, were applied to each column and left to equilibrate for 2 min before being washed with two volumes of the buffer, two volumes of distilled water and one volume of acetonitrile–water (25 : 75 v/v). Benzodiazepines were eluted from each column using 750 µl methanol which was collected in 2 ml conical tubes. The eluant was evaporated to dryness at 55 °C using a stream of N_2 and the residue reconstituted in 200 µl mobile phase. Samples (50 µl) were injected onto a 3 µm Apex ODS, 150 × 4·6 i.d., analytical column (Jones Chromatography, Glamorgan, UK) with a Rheodyne (Cotati, California) Model 7125 injector. Chromatography was at ambient temperature using a Waters Associates (Hartford, UK) Model 510 solvent delivery system with a Knauer (Berlin, FRG) variable wavelength detector set at 254 nm. The mobile phase was acetonitrile–water (45 : 55 v/v) at a flow rate of 0·7 ml min^{-1}. Under these conditions clonazepam and clobazam (internal standard) were eluted with retention times of 5·9 and 8·0 min, respectively.

A plot of peak height ratio against clonazepam concentration for standards made up in plasma was linear in the range 20–500 ng ml^{-1} (r = 0·980). The recovery of clonazepam at 150 ng ml^{-1} was 86·4 ± 5·9% (mean ± s.d.; n = 10) and the detection limit of the assay was 15 ng ml^{-1}. Intra-assay reproducibility (CV = 1·7%; n = 10) and inter-assay reproducibility over a 10 day period (CV = 5·9%; n = 10) were determined by extracting plasma samples containing 100 ng ml^{-1} clonazepam.

Results

In all the studies, the 'protection' afforded by clonazepam was calculated as the difference between the mean MCD of pentetrazol for corresponding control and experimental groups and expressed in mg kg^{-1} pentetrazol. Changes in protection and plasma concentrations of clonazepam were analysed by single classification analysis of variance.

Twice daily administration of 0·5 mg kg^{-1} clonazepam for 16 days resulted in a significant reduction in protection (F = 10·23; $P < 0·001$; Fig. 1). Independent comparisons of the group means showed that protection fell significantly between the first and fourth days, from 103·6 ± 7·2 (mean ± s.e.m.) to 71·3 ± 7·9 mg kg^{-1} pentetrazol (F = 15.31; $P < 0·001$); thereafter no significant change in protection occurred (F = 1·32; $P > 0·25$). Plasma concentrations of clonazepam in individual animals ranged from 45–111 ng ml^{-1} but there was

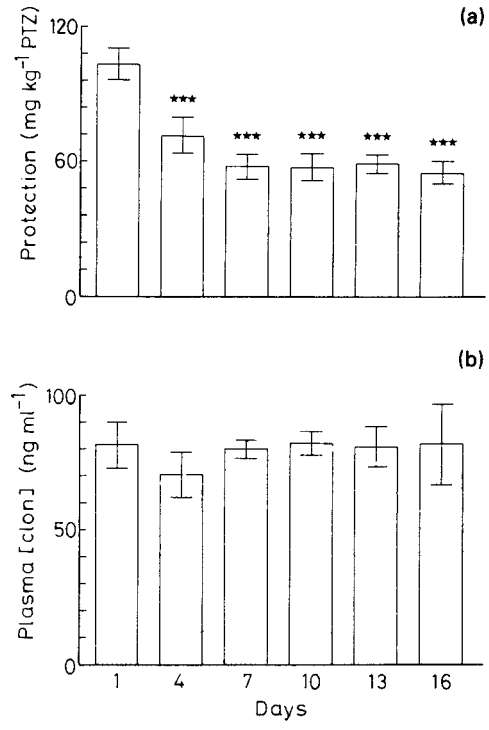

FIG. 1. Development of tolerance to the anticonvulsant effect of 0·5 mg kg^{-1} clonazepam (i.p., twice daily) in mice. Bars show changes in protection (a) with concomitant plasma clonazepam concentrations (b) during 16 days of treatment (mean ± s.e.m.; n = 5; 10 mice per estimate of protection). *** $P < 0·001$ compared with day 1; single classification analysis of variance.

no significant difference between the mean plasma values on each day (F = 0·41; $P > 0·25$: Fig. 1).

The repeated administration of a lower dose of clonazepam (0·25 mg kg^{-1}; Table 1) also caused a significant reduction in protection during 10 days of treatment (F = 9·91; $P < 0·001$), although the fall only became significant by the seventh day (F = 12·88; $P < 0·05$). Furthermore, on the tenth day it was significantly less than that on the fourth day (F = 12·30; $P < 0·005$) indicating that the anticonvulsant effectiveness of clonazepam declined continually throughout the study. Plasma concentrations of clonazepam varied from 16–48 ng ml^{-1} in these animals, but the mean values did not vary significantly (F = 1·59; $P > 0·2$). With the lowest dose of clonazepam (0·08 mg kg^{-1}; Table 1), the plasma concentrations were below the limits of accurate detection (<15 ng ml^{-1}). Protection changed significantly during the study (F = 2·92; $P < 0·05$) as a result of the significant reduction between the first and fourth days (F = 5·98; $P < 0·025$); no further fall was observed throughout the 16 days (F = 0·42; $P > 0·25$).

The mean MCD of pentetrazol in groups of control mice varied between 42·8 ± 1·5 and 47·8 ± 2·6 mg kg^{-1}

Table 1. Development of tolerance to the anticonvulsant effect of clonazepam (0·25 mg kg^{-1}; 0·08 mg kg^{-1}) during twice daily i.p. administration to mice. Values represent mean ± s.e.m. (n = 5; 10 mice per estimate of protection).

	0·25 mg kg^{-1}		0·08 mg kg^{-1}	
Day	Protection (mg kg^{-1} PTZ)	Plasma [Clon] (ng ml^{-1})	Protection (mg kg^{-1} PTZ)	Plasma [Clon] (ng ml^{-1})
1	71·6 ± 5·6	36·0 ± 4·5	42·9 ± 2·9	<15
4	62·3 ± 2·7	25·4 ± 2·5	32·4 ± 4·4*	<15
7	49·9 ± 4·5**	37·9 ± 4·8	29·1 ± 3·6**	<15
10	41·1 ± 4·3***	32·4 ± 5·5	28·5 ± 4·2**	<15
13	—	—	33·1 ± 4·3*	<15
16	—	—	31·8 ± 2·3*	<15

* $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$ compared with day 1 (single classification analysis of variance).

in the three studies and did not change significantly during the course of any treatment ($F \leq 1·44$; $P > 0·25$).

On the final day of each study the protection afforded by the appropriate dose of clonazepam given acutely to an extra group of control mice was not significantly different from that observed in naive animals on the first day ($P > 0·5$; Student's t-test). The protection afforded by clonazepam in tolerant animals was significantly increased on the last day of each study by doubling the clonazepam dose ($P < 0·01$; Student's t-test).

Discussion

In these experiments tolerance developed to the anticonvulsant effect of clonazepam administered on three different dose regimens; in each case the tolerance could be significantly overcome by doubling the clonazepam dose. The reproducibility of the results in control animals indicated that neither the vehicle itself nor the procedures employed had any marked effect on the estimation of the MCD of pentetrazol. Moreover, post-study control groups showed that repeated vehicle treatment played no part in the loss of anticonvulsant activity.

The results support and extend our previous findings with clonazepam (Gent et al 1985). Tolerance developed within days of starting treatment but appeared to be incomplete; significant residual protection was afforded even after 16 days administration. In epilepsy, tolerance to clonazepam usually develops within 6 months and is often incomplete (Browne 1976). Not unexpectedly tolerance occurred faster in mice than in man and the change in seizure threshold was a more sensitive indicator of tolerance than is the recurrence of seizures in man.

The reduction in anticonvulsant activity during two of these studies was not the result of a fall in the plasma concentrations of clonazepam and thus could not be attributed to a disturbance of metabolism. A similar view has been expressed for clobazam tolerance in the mouse (Gent et al 1984) and diazepam tolerance in the dog (Frey et al 1984), although in those studies the interpretation of plasma levels was complicated by the formation of active *N*-demethylated metabolites. Recently, also using an i.v. infusion of pentetrazol as the convulsive stimulus, Scherkl et al (1985) demonstrated tolerance in dogs after twice daily clonazepam (0·5 mg kg^{-1} orally) for 1–2 weeks. During treatment the elimination rate of clonazepam actually decreased, providing further evidence against a metabolic basis for tolerance. Thus it seems unlikely that clonazepam tolerance is the result of pharmacokinetic adaptation.

It is not clear how well plasma concentrations of benzodiazepines correlate with brain levels. Studies indicate a close correlation for some benzodiazepines (Caccia et al 1980), but as these compounds are protein-bound (Sellers et al 1982) measuring total plasma concentrations would not reveal changes in unbound, and thus active, clonazepam levels. In addition, studies have shown that the permeability of the blood-brain barrier may be altered by chronic drug treatment (Preskorn et al 1981), making it desirable to assay both plasma and brain concentrations before drawing conclusions regarding metabolic tolerance. However, there is now good evidence that the development of anticonvulsant tolerance to diazepam is not accompanied by a reduction in brain concentration (Gonsalves & Gallager 1986).

The obvious alternative to a pharmacokinetic basis for tolerance is a pharmacodynamic (functional) mechanism. While there is no evidence of any alteration in the binding affinity of benzodiazepine receptors, reports of changes in receptor number are both numerous and inconsistent (see Tietz et al 1986). The most logical explanation for a loss of pharmacological activity would be a reduction in the maximal binding capacity, and indeed receptor down-regulation has been observed in the forebrains of mice treated with clonazepam (Crawley et al 1982). However, this receptor subsensitivity resulted from doses of clonazepam ranging from 1 to 7·5 mg kg^{-1} day^{-1} for 3 weeks, whereas in the present study tolerance was demonstrated at doses as low as 0·08 mg kg^{-1} twice daily for only 4 days. In view of this disparity, and in the absence of any behavioural assessment of tolerance development concomitant with the change in receptor number, the relevance of receptor down-regulation remains unclear.

Functional tolerance might be associated with an adaptive process more subtle than simple receptor down-regulation; for example, a reduction in the postsynaptic sensitivity to GABA has been reported (Gallager et al 1984). However, this also required prolonged, high dose benzodiazepine treatment and is thus similarly incompatible with the temporal characteristics of tolerance in the present study.

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Functional evidence for altered activity of gabaergic receptors following chronic desipramine treatment in rats

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The antinociceptive effect of subcutaneous 4,5,6,7-tetrahydroisoxazol[5,4-*c*]pyridin-3-ol (THIP) or (±)-baclofen, measured as reaction time of rats placed on a plate heated to 55 °C, was assessed after a single or the last repeated (18 consecutive days) dose (5 mg kg^{-1} once daily) of subcutaneous desipramine. Baclofen (10 mg kg^{-1})-induced antinociception was reduced by acute and unaffected by chronic desipramine treatment. On the contrary, THIP (20 mg kg^{-1})-induced antinociception was unaffected by acute and reduced by chronic desipramine.

Recent biochemical evidence suggests the involvement of GABA in the mechanism of action of antidepressants. A change in the number of brain GABA-A and GABA-B receptors has been observed in rats and mice following chronic treatment with antidepressant drugs (Pilc & Lloyd 1984; Lloyd et al 1985; Suzdak & Gianutsos 1985). We thought it worthwhile to check the responsiveness of GABA receptors in animals repeatedly administered with an antidepressant, by the use of a functional test. This was done in animals chronically treated with desipramine, by assessing the antinociceptive effect of 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP) and (±)-baclofen, which are supposed to bind to GABA-A and GABA-B receptors, respectively (Hill & Bowery 1981).

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Method

Male Wistar Morini rats, 250–300 g, received subcutaneous THIP or baclofen 24 h after a single or the last repeated (18 consecutive days) dose (5 mg kg^{-1} once daily) of subcutaneous desipramine. The antinociceptive effect was assessed by recording the reaction time of a rat placed on a plate heated to 55 °C. On the basis of preliminary experiments: (a) the test was carried out 60 and 90 min after baclofen and THIP administration, respectively, and (b) the doses of the compound were selected to induce subthreshold and maximal antinociceptive effects.

Results and discussion

Baclofen-induced antinociception was reduced by acute and unaffected by chronic desipramine treatment (Fig. 1). Since reduction in noradrenergic function potentiates baclofen's antinociceptive effect (Sawynok 1984), the increased noradrenergic activity brought about by acute administration of desipramine might explain the reduced effect of baclofen. On the other hand, chronically administered desipramine induced desensitization of noradrenergic receptors (Racagni et al 1983) might be responsible for the restoration of baclofen antinociception.

Unlike baclofen, THIP-induced antinociception was unaffected by acute and reduced by chronic desipram-