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Research Article

Relationship Between Receptor Occupancy at 37°C and the Anticonvulsant Effect of Flunitrazepam in Rats

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In this investigation an attempt was made to evaluate quantitatively the relationship between benzodiazepine receptor occupancy and the anticonvulsant effect of flunitrazepam in rats. A graded measure of anticonvulsant effect was obtained on the basis of an elevation of pentylenetetrazol (PTZ) threshold concentrations. The concentration–anticonvulsant effect relationship could be described by the E_{max} model with an EC_{50} in cerebrospinal fluid of 2.9 ± 0.8 μg/liter and an E_{max} of 227 ± 22 mg/liter PTZ (mean ± SE). *In vitro* receptor occupancy was determined in a crude brain homogenate at 0 and 37°C, which yielded K_D values of 2.2 ± 0.2 and 26 ± 2 μg/liter, respectively. The results obtained in both experiments were combined by focusing on *free* flunitrazepam concentrations. This strategy resulted in a nonlinear relationship between receptor occupancy and anticonvulsant effect of flunitrazepam, with 90% of the maximum response achieved at a degree of receptor occupancy of approximately 50% at 37°C.

KEY WORDS: flunitrazepam; anticonvulsant effect; receptor occupancy; kinetics-dynamics.

INTRODUCTION

Several pharmacological effects of benzodiazepines have been shown to be elicited from interaction with specific receptors in the central nervous system (CNS). These receptors have also been convincingly implicated in the anticonvulsant effect of this drug class, as reflected, e.g., in protection against pentylenetetrazol (PTZ)-induced convulsions (1,2).

Most of these studies are based on receptor binding experiments *in vitro*, while the correlation between *in vivo* receptor occupancy and intensity of the anticonvulsant effect remains obscure. Apart from the difficulties encountered in the quantitation of CNS drug effects and several pharmacokinetic complications in *in vivo* pharmacodynamic studies (3), this lack of knowledge is also caused by methodological problems associated with estimation of the degree of receptor occupancy *in vivo* (4). The majority of receptor binding studies has been performed at 0–4°C in receptor-enriched preparations, which complicates extrapolation to physiological conditions. Profound influences of temperature and purification procedures on benzodiazepine receptor binding characteristics have been demonstrated (5,6).

The purpose of the present investigation was to characterize the relationship between receptor occupancy and the anticonvulsant effect of flunitrazepam. This goal was pursued by combination of two experimental strategies. First, the concentration–anticonvulsant effect relationship of *free* flunitrazepam in cerebrospinal fluid (CSF) was determined by using the PTZ threshold concentration as pharmacodynamic measure, which has the unique feature that it is a graded pharmacological response (7). Second, the degree of receptor occupancy was estimated *in vitro* as a function of the *free* flunitrazepam concentration in a crude receptor preparation at 37°C. From the results obtained in the two experiments, a relationship between the degree of receptor occupancy and the intensity of the anticonvulsant response could be derived.

MATERIALS AND METHODS

Animals and Materials

In all experiments male Wistar rats weighing 180–200 g were used. The animals were maintained on a normal laboratory diet and housed under a 12-hr light–dark cycle. Rats used in experiments in which compounds had to be infused were cannulated via the right jugular vein 1 day before the experiment. All pharmacodynamic investigations were performed between 10:00 AM and 1:00 PM to avoid potential perturbations due to a circadian rhythm in brain sensitivity (8).

[^{3}H]Flunitrazepam (sp act, 80 Ci/mmol) and PTZ were supplied by New England Nuclear and OPG (Utrecht, The Netherlands), respectively. Unlabeled flunitrazepam was a generous gift of Hoffmann-La Roche (Mijdrecht, The Neth-

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erlands). All other reagents were purchased from standard commercial sources.

Distribution of Flunitrazepam Between Plasma and CNS

The distribution of flunitrazepam among plasma, CSF, and brain was studied in 25 rats at 10 min after iv administration of varying doses of the drug (0.1–1.5 mg/kg). Due to failure in the collection of a CSF sample in 5 animals, complete data (i.e., plasma, brain, and CSF concentrations) are available for 20 rats. The dose range in this study was based upon some pilot experiments to obtain a plasma concentration range between 0 and 500 ng/ml. The injection solution consisted of 1–6 mg of flunitrazepam in 1 ml of polyoxyethylene glycol (PEG) 400 (Brocacef NV, Maarssen, The Netherlands), such that the amount of PEG administered to rats was maximally 50 μl. It was given as a short iv infusion (0.15 ml/min) with a precision syringe infusion pump. CSF (by puncturing the cisterna magna), blood (for plasma), and brain tissue were collected at 10 min after administration. Plasma, CSF, and brain tissue were stored at −35°C pending assay.

Concentration–Anticonvulsant Effect Relationships of Flunitrazepam

Experiments were conducted over 3 days (about 10 animals a day). Each day the same protocol was applied, with individual rats randomly allocated to the treatments. The baseline convulsion threshold was determined as described previously (9). Briefly, PTZ dissolved in normal saline was infused (1.5 mg/min) until the occurrence of the first myoclonic jerk, at which time a blood sample was obtained by bleeding of the retroorbital sinus under light ether anesthesia. The PTZ plasma threshold concentration was determined. Three days later flunitrazepam was administered in varying doses (0–1.5 mg/kg). After 10 min PTZ was infused until the predefined pharmacodynamic endpoint, since preliminary experiments had demonstrated that a distribution equilibrium among blood, CSF, and brain is reached within 10 min following an iv bolus dose of flunitrazepam, which is in agreement with literature data (20). On the basis of preliminary experimentation, the PTZ concentration in the infusate (20, 30, or 40 mg/ml) was adapted to the expected protective effect furnished by flunitrazepam, so that the duration of the infusion was kept in the range of 2–4 min. In an introductory experiment PTZ-induced convulsions were shown not to be associated with alterations in flunitrazepam distribution as has also been described for oxazepam (7). At the appearance of the first myoclonic jerk, which was detected by an observer who was unaware of the flunitrazepam dose administered and the PTZ concentration to be infused, rats were decapitated and trunk blood was collected in a heparinized tube. The total brain minus cerebellum was removed. In this experiment no CSF samples were collected. Plasma and brain tissue were stored at −35°C pending assay.

The anticonvulsant drug effect was expressed as the elevation of the PTZ plasma threshold concentration needed to elicit the first myoclonic jerk.

Assessment of *in Vitro* Receptor Occupancy

Brain membranes were prepared from drug-naive Wistar rats, which were housed under the conditions described above and had been fasting overnight. Rats were sacrificed by decapitation and the brains were excised from the craniums. Total brains minus brain stem were homogenized gently in 10 vol ice-cold 0.13 *M* sodium phosphate buffer (pH 7.4) in a Potter–Elvehjem homogenizer (Braun Potter S, Melsungen, F.R.G.). The homogenate protein concentration was determined according to the method of Lowry *et al.* (10). The tissue suspension was frozen rapidly and stored at −35°C. Binding characteristics were unchanged during storage for at least 3 months. All binding experiments were done in triplicate by a filtration assay.

At 0°C, incubation was performed for 30 min with [^{3}H]flunitrazepam at different concentrations in a range up to 50 ng/ml incubation volume to obtain saturation of the receptor sites from 10 to 95%. For the receptor binding studies, crude brain homogenate was diluted with 0.13 *M* sodium phosphate buffer (pH 7.4) to obtain a protein concentration of 1 mg/ml. The solvent of the flunitrazepam solution consisted of 0.5% alcohol in 0.13 *M* sodium phosphate buffer. In the final incubation mixture the alcohol concentration was fixed at 0.1% since former studies had shown that a concentration above 0.5% may influence the specific binding of [^{3}H]flunitrazepam. Free radioligand concentrations were determined by subtracting the concentration of totally bound [^{3}H]flunitrazepam at equilibrium from the concentration of [^{3}H]flunitrazepam present at the start of the assay (measured in an aliquot of incubation mixture). Incubation was terminated by transfer of the medium to a presoaked glass-fiber filter (Whatman GF/B) positioned over filtration manifolds (JSI-Multividor, Janssen Pharmaceutica, Beerse, Belgium) under mild suction. Filters were presoaked with 0.13 *M* sodium phosphate buffer (pH 7.4). Immediately thereafter the tissue-laden filter was washed twice with 5-ml aliquots of ice-cold phosphate buffer. The entire procedure was completed within 10 sec. The filters were submersed in 5 ml Scintillator 299 (Packard) scintillant and counted in a Tri-Carb 4640 (Packard) liquid scintillation counter at an average counting efficiency of 40%.

At 37°C, binding experiments (protein concentration 1 mg/ml) were performed after the temperature had been raised in 2 min from 0 to 37°C. At this temperature incubation was performed for only 5 min to avoid deterioration in binding properties (unpublished observations). Control experiments had shown that equilibrium was reached after 2–3 min of incubation at 37°C. [^{3}H]Flunitrazepam was added to achieve concentrations up to 30 ng/ml incubation volume.

Nonspecific binding was concurrently determined by incubation in the presence of an excess cold flunitrazepam (900 ng/ml). Control experiments showed that nonspecific binding reached the steady-state level after 1 min of incubation and remained constant throughout the experiment. Specific binding was assessed by subtracting the nonspecific binding from the total binding and is expressed as nanograms of flunitrazepam bound per milligram of protein. At both 0 and at 37°C nonspecific binding was found to vary linearly with the concentration. At 0°C, at a concentration equivalent to the K_D value, nonspecific binding amounted 4% of the

total binding. At 37°C nonspecific binding accounted for 70% of the total binding. Binding to filters accounted for less than 10% of the total nonspecific binding.

Drug Analysis

Details of the assay procedure for PTZ in serum and brain have been described previously (7).

Flunitrazepam concentrations in plasma, CSF, and brain were determined by a gas-chromatographic (GLC)–electron capture detection procedure based on a previously described method (11). Nitrazepam was added to the samples as internal standard. A fused silica WCOT capillary column (11 m * 0.32-mm i.d.) fitted with a chemically bonded film of CPSil 19CB (Chrompack, Middelburg, The Netherlands) was used. Temperatures of the injection port, oven, and detector were 300, 260, and 300°C, respectively. Calibration curves were linear with $r > 0.996$ and coefficients of variation less than 7% with respect to intra- and interassay precision. The detection limit was about 1 ng/ml using a 100-μl CSF or plasma sample.

Flunitrazepam concentrations in plasma were also determined by a radioreceptor assay (RRA), as has been described previously for oxazepam (7). The only modification was that plasma samples were subjected to the assay after extraction according to the same method as used in the chromatographic assay (11). The lower limit of sensitivity (20% inhibition of binding) is about 10 ng/ml. In a separate experiment the influence of PEG 400 on the binding assay was shown to be absent.

Data Analysis

Orthogonal least-squares regression analysis (12) was applied to the data regarding the distribution of flunitrazepam over the different body compartments and to the comparison of concentrations of flunitrazepam in plasma as determined by GLC and RRA, respectively. Regression lines were calculated in conjunction with 95% confidence intervals of slope and intercept.

The anticonvulsant effect of flunitrazepam, depicted as the difference between the two measured PTZ plasma threshold concentrations, was related to its plasma, CSF, and brain concentrations, respectively. CSF concentrations were calculated from plasma and brain concentrations, utilizing the values of the CSF/plasma and CSF/brain concentration ratios that were determined in the distribution study. Concentration–anticonvulsant effect data were fitted to the following equation with the nonlinear least-squares regression computer program ELSMOS (13,14):

$$E = \frac{E_{max} * C^N}{EC_{50}{}^N + C^N}$$

In this so-called sigmoid E_{max} model, E is the anticonvulsant response, EC_{50} is the flunitrazepam concentration associated with 50% of the maximum effect (E_{max}), and N is a parameter that determines the sigmoidicity of the curve. The output of the program yielded the pharmacodynamic parameters, which were fitted as independent variables, with their standard errors. The quality of the fit was judged from the mean of the standardized residuals and the multiple correlation coefficient (MCC).

Receptor-binding data were subjected to a Langmuir-type equation:

$$\text{number of receptors occupied} = \frac{B_{max} * C^N}{K_D{}^N + C^N}$$

in which B_{max} is the total number of specific binding sites, K_D is the apparent dissociation constant, and C is the free flunitrazepam concentration. The power parameter N and the B_{max} value as derived from the 0°C curve fitting procedure were assumed to be unchanged at 37°C (5,6). For the binding data obtained at 0°C also conventional Scatchard analysis was applied.

The concentration–anticonvulsant effect relationship and the results obtained in the assessment of the *in vitro* receptor occupancy at 37°C were combined to estimate the correlation between receptor occupancy and the anticonvulsant effect of flunitrazepam.

RESULTS

Distribution of Flunitrazepam

The correlations among flunitrazepam concentrations in plasma, brain, and CSF at 10 min after iv administration, as obtained by orthogonal regression analysis, are shown in Fig. 1. The intercepts of the regression lines did not deviate significantly from zero. From the slopes of the lines it is evident that the brain/plasma concentration ratio is about 1.5 (1.1–2.0), whereas the CSF/plasma and CSF/brain concentration ratios are 0.051 (0.034–0.067) and 0.035 (0.026–0.044), respectively (95% confidence interval in parentheses). The values of the coefficient of determination (r^2 value) for the brain/plasma, the CSF/plasma, and the CSF/brain concentration ratio were 0.696, 0.702, and 0.781, respectively.

Concentration–Anticonvulsant Effect Relationships of Flunitrazepam

The various concentration–effect relationships could be adequately fitted to the E_{max} model, the parameters of which are listed in Table I. By fitting according to the sigmoid E_{max} model, N appeared to be very close to unity. Subsequently the concentration–effect data were fitted to a simple E_{max} model. The MCC was in any case greater than 0.85, and the mean of the standardized residuals less than 0.007. Figure 2 represents the concentration–response relationships of flunitrazepam as derived from plasma (measured by both GLC and RRA) and brain and CSF (derived from corresponding

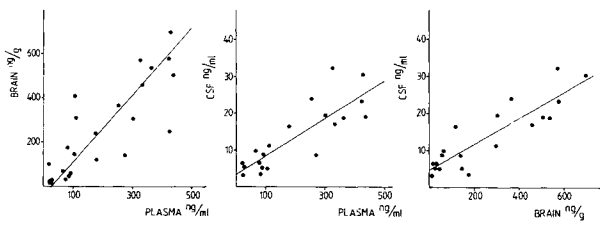

Fig. 1. Relationship among concentrations of flunitrazepam in plasma, CSF, and brain at 10 min following an intravenous bolus injection of 0–1.5 mg/kg.

Table I. Pharmacodynamic Parameters of the Concentration–Anticonvulsant Response Relationships of Flunitrazepam[a]

	EC_{50}	E_{max}
Plasma(GLC)	57 ± 15	227 ± 21
Plasma (RRA)	57 ± 16	228 ± 22
Brain	82 ± 23	225 ± 22
CSF (plasma)	2.9 ± 0.8	227 ± 22
CSF (brain)	2.9 ± 0.8	225 ± 22

[a] Concentration–effect data were fitted to the E_{max} model of the form $E = (E_{max} * C)/(EC_{50} + C)$. The parameters are reported as means ± SE. EC_{50} is expressed as ng/ml or ng/g (flunitrazepam) and E_{max} is expressed as μg/ml (PTZ).

plasma levels) concentrations. The shape of the curve obtained when CSF concentrations were derived from brain concentrations was very similar. Orthogonal least-squares regression analysis applied to plasma flunitrazepam concentrations measured by both GLC and RRA revealed a high correlation ($r = 0.98$), with a slope not significantly deviating from unity (Fig. 3).

In Vitro Receptor Occupancy

Figure 4 shows the specific binding of [^{3}H]flunitrazepam expressed as nanograms per milligram of protein as a function of the free [^{3}H]flunitrazepam concentration in the incubation medium at temperatures of 0 and 37°C. The free flunitrazepam concentration at 0°C was almost up to 50 ng/ml, which permitted calculation of the K_D and B_{max} value on the basis of Scatchard analysis: $K_D = 2.1 \pm 0.3$ ng/ml (6.7 ± 1.0 n*M*), $B_{max} = 0.41 \pm 0.05$ ng/mg protein (1.3 ± 0.2 pmol/mg protein; mean ± SE). The plot of the data corresponded to a straight line, which indicates that the ligand binds to a single class of noninteracting sites. The parameters found were very similar to those obtained by nonlinear regression analysis (MCC = 0.95), *viz.*, $K_D = 2.2 \pm 0.2$ ng/ml (7.0 ± 0.6 n*M*) and $B_{max} = 0.45 \pm 0.03$ ng/mg protein (1.4 ± 0.1 pmol/mg protein). The power parameter N was 1.0 ± 0.1. The latter two parameters were fixed for fitting K_D at 37°C: 26 ± 2 ng/ml (83 ± 6 n*M*; MCC = 0.84). Free [^{3}H]-flunitrazepam concentrations at 37°C reached values almost up to the calculated K_D value.

Figure 5 represents the relationship between the free concentration of flunitrazepam (≡ the CSF concentration) and the anticonvulsant response as well as the relationship between the free concentration of flunitrazepam and the degree of receptor occupancy at 37°C *in vitro*, both expressed as a percentage of the maximum value. From these data the relationship between the degree of receptor occupancy and

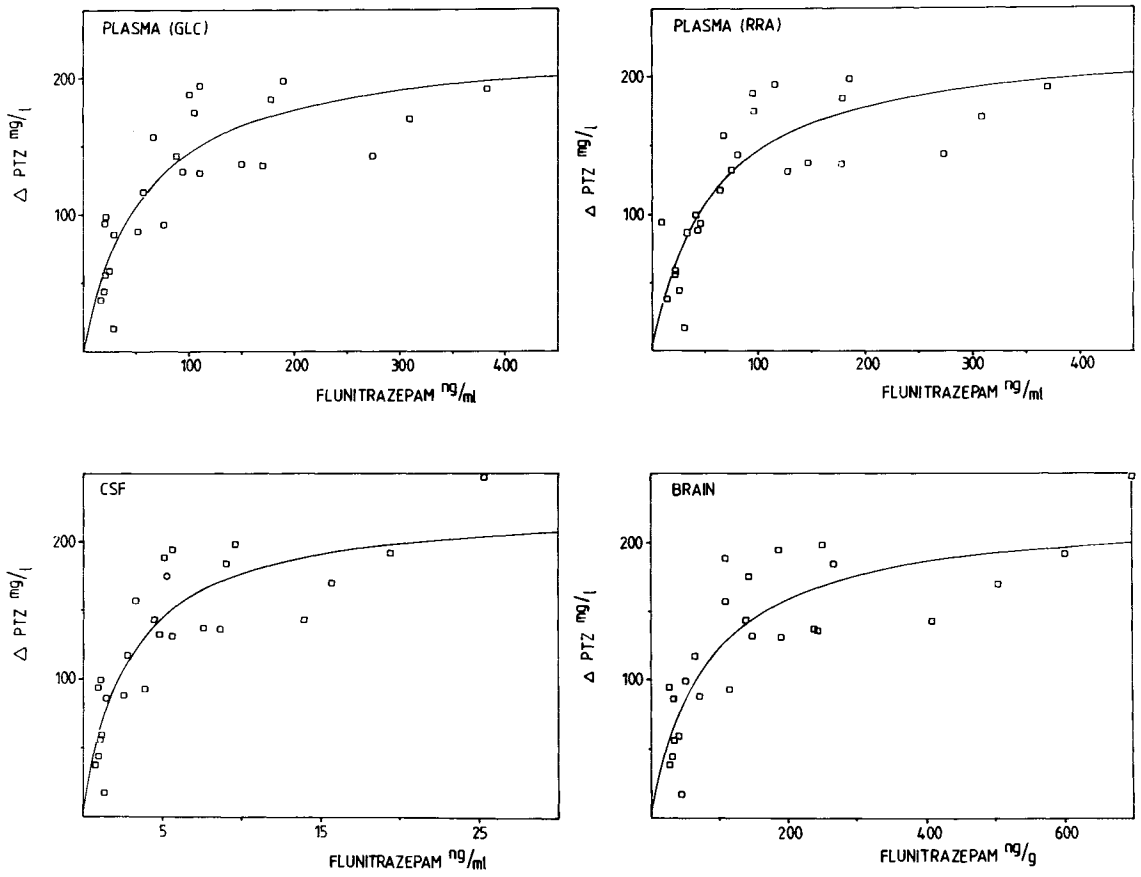

Fig. 2. Concentration–anticonvulsant effect relationships of flunitrazepam in plasma as determined by GLC (plasma GLC), in plasma as determined by RRA (plasma RRA), in cerebrospinal fluid (CSF), and in brain tissue (brain). Cerebrospinal fluid concentrations were calculated from plasma concentrations on basis of the CSF/plasma concentration ratio that was determined in the distribution study.

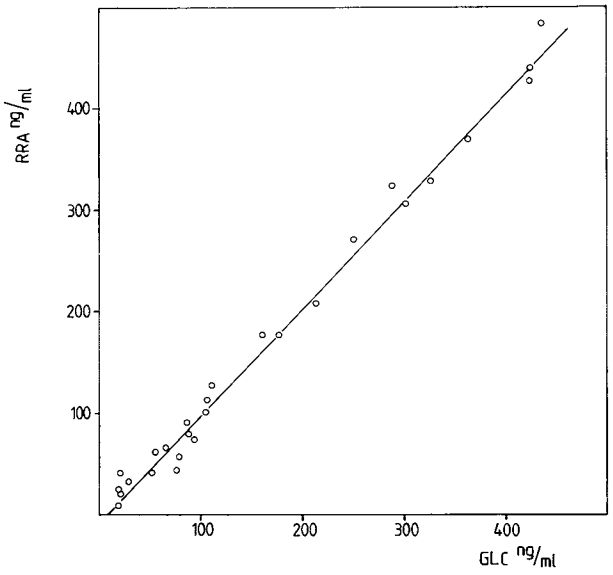

Fig. 3. Relationship of the concentrations of flunitrazepam in plasma as determined by GLC and RRA.

the anticonvulsant effect could be estimated as illustrated in Fig. 6.

DISCUSSION

The discovery of benzodiazepine receptors in the CNS has led to the hypothesis that the time course of receptor occupancy, i.e., pharmacokinetics at the site of action, would generally correlate more closely with the duration of effect than is the case for plasma pharmacokinetics (4,15). A direct correlation of pharmacological effects with the *in vivo* receptor occupancy is not easy, however, for a number of reasons.

First, many kinetic and dynamic variables may complicate the investigation of *in vivo* pharmacodynamics (3). By careful consideration of these perturbations in an animal study, it has proven possible to describe the concentration–anticonvulsant effect relationship of benzodiazepines (7). In the present study the same strategy has been applied to investigate the concentration–anticonvulsant effect relationship of flunitrazepam.

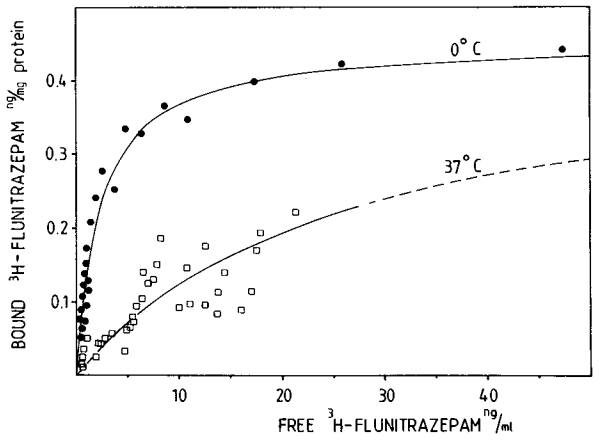

Fig. 4. Relationship between the free flunitrazepam concentration and the degree of receptor occupancy *in vitro* at 0 and 37°C.

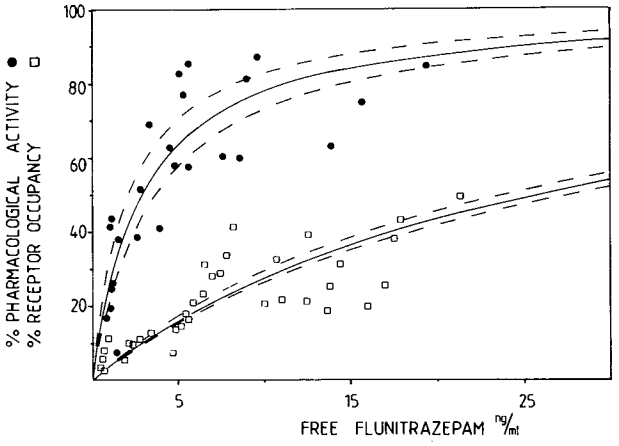

Fig. 5. Relationship between the free concentration of flunitrazepam (≡ CSF concentration) and the anticonvulsant effect and the relationship between the free flunitrazepam concentration and the degree of receptor occupancy *in vitro* at 37°C, both expressed as a percentage of the maximum value.

Second, the assessment of *in vivo* receptor occupancy is limited by methodological complications since after pharmacologically effective doses of benzodiazepines, only a small fraction of the total amount of drug present in brain is specifically bound to receptors (16). Several attempts have been described to measure the receptor occupancy by means of an *ex vivo* technique (15,17,18). This method requires receptor preparation (homogenization, centrifugation and washing) during which alterations in the amount of drug bound to receptors may occur.

In the present study an alternative approach was under-

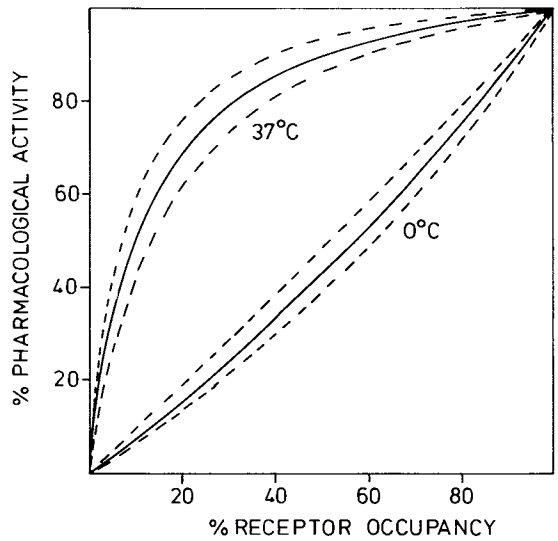

Fig. 6. Computer simulations of the relationships between the degree of receptor occupation and the anticonvulsant effect of flunitrazepam, at 0 and 37°C, respectively. The computer simulations were based upon the estimated values of the pharmacodynamic parameters and of the receptor binding parameters at 0 and 37°C, respectively. The results are presented as means ± SE.

taken to get insight into the receptor occupancy–anticonvulsant effect relationship of flunitrazepam.

Flunitrazepam was chosen as the model drug because it has favorable properties for this type of experiment; it has a high affinity for the receptor and recognizes a uniform population of receptors (19). Because of its high lipophilicity, equilibrium between plasma and brain is attained very rapidly after iv administration (20). Although the plasma concentration of flunitrazepam in rats after an iv bolus dose can be described by a two-compartment pharmacokinetic model (21), brain can be considered to belong to the central compartment, which facilitates the construction of concentration–effect relationships (3).

Anticonvulsant effect measurements were performed at 10 min after iv administration to minimize the possible contribution of (inter)active metabolites. Since at this time point drug concentrations change rapidly (21), the drug distribution between the different compartments was evaluated in a separate experiment. In the pharmacodynamic experiment animals were sacrificed immediately at the occurrence of the first myoclonic jerk and CSF concentrations were estimated via plasma and brain concentrations. From a kinetic-dynamic modeling point of view, CSF comprises an important biofluid with respect to CNS active drugs, as essentially *free* drug concentrations are measured in this compartment (3). Thus by focusing on CSF concentrations, *in vitro* plasma protein binding determinations can be avoided. This is considered a major advantage due to the many uncertainties that are associated with these determinations. An important feature of CSF is that for several compounds this compartment has been demonstrated to be pharmacokinetically indistinguishable from the site of action (3). Finally, CSF has been found accurately to reflect benzodiazepine concentrations in the brain extracellular fluid (21,22).

The concentration–anticonvulsant response relationships of flunitrazepam, as established by a continuously variable pharmacodynamic measure (7), could satisfactorily be described by the E_{max} model, in contrast to oxazepam, for which a *sigmoid* E_{max} model seemed more appropriate (7). Under the specified set of experimental conditions, the maximum flunitrazepam effect is about 225 mg/liter PTZ, compared to only 120 mg/liter PTZ for oxazepam. The brain/plasma EC_{50} ratio is consonant with the distribution ratio as illustrated in Fig. 1. The EC_{50} for CSF is about 3 ng/ml, irrespective of whether CSF concentrations are calculated via plasma or brain concentrations.

The fact that measurement of flunitrazepam concentrations at 10 min after iv administration obviates metabolism to active compounds as a confounding factor is demonstrated in Fig. 3. RRA provides an estimate of the total benzodiazepine-like activity present, which is a function of both the concentration and the affinity of each drug or active metabolite present for the receptor (23). No significant amounts of (inter)active metabolites are present in plasma, since the slope does not significantly deviate from unity.

The main concept in the present study is the assessment of the correlation between *free* drug concentrations and both the anticonvulsant effect and the receptor occupancy. In the light of the complexity of evaluating receptor binding parameters in living animals, receptor binding was investigated *in vitro*. However, in order to obtain more information relevant to the pharmacological properties of the benzodiazepines, the assay was carried out under relatively physiological conditions in a crude brain homogenate and at 37°C. The omission of centrifugation and washing steps in the preparation of the crude membranes is particularly of importance with respect to the presence of compounds such as γ-aminobutyric acid (GABA), GABA modulin, diazepam binding inhibitor, and other endogenous modulators of benzodiazepine binding (25–28). An interesting question is whether determination of receptor binding in brain slices would have presented an even more appropriate simulation of the physiological conditions. In brain slices however, relatively long incubation periods, up to 3 hr, are necessary to achieve equilibrium conditions (29). Therefore there is ample opportunity for receptor binding modifying compounds to diffuse from the cells into the medium, resulting in altered concentrations at the receptor site. Thus brain slices appear to be associated with similar limitations as crude brain homogenate. Receptor binding characteristics were evaluated at two different temperatures, *viz.*, 0°C, at which most *in vitro* binding assays have been carried out, and 37°C, because findings obtained at physiological temperature can differ markedly from those obtained at low temperature (5,6). Due to the unfavorable specific/nonspecific binding ratios of flunitrazepam in crude brain homogenate, we were unable to determine B_{max} at 37°C. This problem could not be solved by the use of brain tissue slices, since these show similar or even poorer specific/nonspecific binding ratios (29). Therefore in the present study B_{max} was considered not to change with temperature. The validity of this assumption is supported by literature data (5,6,30). Speth *et al.* (5) found in a receptor-enriched preparation a mean B_{max} value (±SE) for flunitrazepam of 114 ± 3 fmol/mg tissue at 0°C vs 120 ± 14 fmol/mg tissue at 37°C. In a detailed investigation on the temperature dependency of specific flunitrazepam receptor binding, Quast *et al.* (6) found the B_{max} values not to be different at various temperatures between 0 and 40°C, with mean values (±SD) of 4.5 ± 0.4 pmol/mg protein at 0°C and 4.4 ± 0.9 pmol/mg protein at 35°C. In addition, studies with another ligand (Ro 22-8515) that shows favorable specific/nonspecific binding ratios at 0 and at 37°C have also shown that B_{max} remains constant with temperature (30). The K_D values referred to in the previous reports are somewhat lower than those obtained in the present study, which may be due to the absence of endogenous receptor modulating compounds in the purified receptor preparations. Theoretically it is conceivable that the presence of receptor binding modifying substances may also affect the temperature-dependent behavior of the B_{max} value. However, this seems unlikely, since modulators of benzodiazepine binding appear to affect primarily the K_D and not the B_{max} value of benzodiazepine receptor binding (25,28).

The estimated relationship between receptor occupancy and anticonvulsant effect as presented in Fig. 6 is nonlinear. Only about 50% of the total number of benzodiazepine receptors need to be occupied to manifest 90% of the maximum anticonvulsant effect. In previous investigations linear correlations have been established between receptor occupancy and protection against PTZ-induced seizures following diazepam administration to mice and rats (17,18). In these studies, however, receptor occupancy was measured

with an *ex vivo* technique in a synaptosomal membrane preparation at 0–4°C and the anticonvulsant effect was determined quantally by recording seizure incidence in groups of animals. It seems likely that this finding of a linear relationship can be accounted for at least in part by the fact that the temperature dependency of benzodiazepine binding has not been taken into consideration, since in the present study also an almost-linear relationship was observed when receptor binding data obtained at 0°C were used (Fig. 6). Garattini and co-workers found about 50% inhibition of [^{3}H]diazepam binding injected *in vivo* at the times when the administered dose of several benzodiazepines afforded protection against PTZ in 50% of a test group (31,32). Using a similar technique Jochemsen *et al.* (33) indicated a receptor occupancy requirement of 18–35% for near-maximal anti-PTZ effect of metaclazepam. However, File *et al.* (34) found no correlation between receptor occupancy and anti-PTZ effects of diazepam across different mouse strains. Using a recently developed technique to visualize true *in vivo* receptor binding with [^{3}H]Ro 15-1788, Miller *et al.* (35) demonstrated a sigmoidal relationship between *in vivo* receptor occupancy and plasma and brain concentrations of clonazepam and lorazepam. The ED_{50} value for PTZ-induced seizures occurred at an occupancy of approximately 25 to 45%. With respect to the shape of the relationship between receptor occupancy and effect, it should be born in mind that differences might exist between different benzodiazepine agonists.

The present study has shown that the disparity between pharmacologically effective brain concentrations of flunitrazepam and the reported K_D value at 0°C can be largely reduced by determining the K_D value at 37°C in a crude membrane preparation and focusing on *free* drug concentrations, as a large amount of benzodiazepine in brain is nonspecifically bound. The described strategy of emphasizing free drug concentrations *in vitro* and *in vivo* provides a tool to estimate the occupancy of benzodiazepine receptors in relation to pharmacological effects.

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REFERENCES

1. H. Mohler and T. Okada. *Science* 198:849–851 (1977).
2. R. F. Squires and C. Braestrup. *Nature* 266:732–734 (1977).
3. J. Dingemanse, M. Danhof, and D. D. Breimer. *Pharm. Ther.* 38:1–52 (1988).
4. C. Braestrup and M. Nielsen. In R. W. Olsen and J. C. Venter (eds.), *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties,* Alan R. Liss, New York, 1986, pp. 167–184.
5. R. C. Speth, G. J. Wastek, and H. I. Yamamura. *Life Sci.* 24:351–358 (1979).
6. U. Quast, H. Mahlmann, and K.-O Vollmer. *Mol. Pharmacol.* 22:20–25 (1982).
7. J. Dingemanse, F. A. E. Sollie, D. D. Breimer, and M. Danhof. *J. Pharmacokin. Biopharm.* 16:203–228 (1988).
8. D. E. Woolley and P. S. Timiras. *Am. J. Physiol.* 202:379–382 (1962).
9. J. Dingemanse, J. B. M. M. van Bree, and M. Danhof. *J. Pharmacol. Exp. Ther.* (in press) (1989).
10. O. H. Lowry, H. J. Rosebrough, A. L. Farr, and R. J. Randall. *J. Biol. Chem.* 193:265–275 (1951).
11. R. Jochemsen and D. D. Breimer. *J. Chrom. Biomed. Appl.* 227:199–206 (1982).
12. E. A. van der Velde. In A. M. H. P. van den Besselaar, H. R. Gralnick, and S. M. Lewis (eds.), *Thromboplastin Calibration and Oral Anticoagulant Control,* Martinus Nijhoff, The Hague, 1984, pp. 25–39.
13. N. H. G. Holford and L. B. Sheiner. *Clin. Pharmacokin.* 6:429–453 (1981).
14. R. J. Francis. *Comp. Prog. Biomed.* 18:43–50 (1984).
15. W. E. Mueller and A. E. Stillbauer. *Pharmacol. Biochem. Behav.* 18:545–549 (1983).
16. T. Mennini and S. Garattini. *Life Sci.* 31:2025–2035 (1982).
17. S. M. Paul, P. J. Syapin, B. A. Paugh, V. Moncada, and P. Skolnick. *Nature* 281:688–689 (1979).
18. Y. Igari, Y. Sugiyama, Y. Sawada, T. Iga, and M. Hanano. *Drug Metab. Disp.* 13:102–106 (1985).
19. B. J. Ciliax, J. B. Penney, Jr., and A. B. Young. *J. Pharmacol. Exp. Ther.* 238:749–757 (1986).
20. Y. Igari, Y. Sugiyama, Y. Sawada, T. Iga, and M. Hanano. *J. Pharmacokin. Biopharm.* 11:577–593 (1983).
21. C. Becherucci, M. Palmi, and G. Segre. *Pharmacol. Res. Commun.* 17:733–747 (1985).
22. R. M. Arendt, D. J. Greenblatt, R. H. De Jong, J. D. Bonin, D. R. Abernethy, B. L. Ehrenberg, H. G. Giles, E. M. Sellers, and R. I. Shader. *J. Pharmacol. Exp. Ther.* 227:98–106 (1983).
23. E. A. Bering. *Fed. Proc.* 33:2061–2063 (1974).
24. D. B. Barnett and S. R. Nahorski. *Trends Pharmacol. Sci.* 4:407–409 (1983).
25. M. Karobath and G. Sperk. *Proc. Natl. Acad. Sci. USA* 76:1004–1006 (1979).
26. E. Costa, M. G. Corda, B. Epstein, C. Forchetti, and A. Guidotti. In E. Costa (ed.), *The Benzodiazepines. From Molecular Biology to Clinical Practice,* Raven Press, New York, 1983, pp. 117–136.
27. A. Ya. Korneyev and M. I. Factor. *Mol. Pharmacol.* 23:310–314 (1983).
28. E. Costa and A. Guidotti. *Biochem. Pharmacol.* 34:3399–3403 (1985).
29. K. J. Whitaker, E. L. Manchester, W. Jacobson, and M. Wilkinson. *Brain. Res. Bull.* 12:215–219 (1984).
30. H. E. Goeders, W. D. Horst, R. O'Brien, G. Bautz, and M. J. Kuhar. *Eur. J. Pharmacol.* 113:147–148 (1985).
31. S. Garattini, S. Caccia, and T. Mennini. In F. G. De las Heras and S. Vega (eds.), *Medicinal Chemistry Advances,* Pergamon, Oxford, 1981, pp. 171–178.
32. T. Mennini, S. Cotecchia, S. Caccia, and S. Garattini. *Pharmacol. Biochem. Behav.* 16:529–532 (1982).
33. R. Jochemsen, G. Kato, and M. Ruhland. *Drug Dev. Res.* 9:115–124 (1986).
34. S. E. File, D. J. Greenblatt, I. L. Martin, and C. Brown. *Psychopharmacology* 86:137–141 (1985).
35. L. G. Miller, D. J. Greenblatt, S. M. Paul, and R. I. Shader. *J. Pharmacol. Exp. Ther.* 240:516–522 (1987).