Mechanism-Based Modeling of Adaptive Changes in the Pharmacodynamics of Midazolam in the Kindling Model of Epilepsy

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Purpose. A mechanism-based model is proposed for the analysis of adaptive changes in the pharmacodynamics of benzodiazepines *in vivo*.
Methods. The pharmacodynamics of midazolam was studied in the kindling model of experimental epilepsy. Concentration-EEG effect data from kindled rats and their controls were fitted to the operational model of agonism. A stepwise procedure was used, allowing changes in the parameters efficacy (τ) and tissue maximum (E_m) either separately or in combination. The results were compared to data obtained *in vitro* in a brain synaptoneurosomal preparation.
Results. The relationship between midazolam concentration and EEG effect was non-linear. In kindled rats the maximum EEG effect was reduced by 27 ± 8.3 μV from the original value of 94 ± 4.4 μV. Analysis on the basis of the operational model of agonism showed that this decrease could be explained by a difference in the parameter system maximum (E_m) rather than efficacy (τ). In the *in vitro* receptor binding assay no changes in density, affinity or functionality of the benzodiazepine receptor were observed, consistent with the lack of a change in efficacy (τ).
Conclusions. The operational model of agonism provides a mechanistic basis to characterise adaptive changes in the pharmacodynamics of midazolam.

KEY WORDS: midazolam; epilepsy; functional adaptation; pharmacodynamics; operational model of agonism.

INTRODUCTION

Benzodiazepines are effective antiepileptic drugs, but development of tolerance limits their therapeutic value (1). Generally, tolerance is attributed to chronic treatment with the drug (2). However, we have shown that experimental epilepsy itself also causes a reduction of the maximum effect of benzodiazepines (3). In that study, using EEG as pharmacodynamic endpoint and midazolam as model compound, an empirical model (i.e., the Hill equation) was used to describe the concentration-effect relationship of benzodiazepines. This model yields estimates of *in vivo* potency and maximal effect, but provides only limited insight in the factors that govern these parameters, because the parameters are not only ligand-related, such as affinity and intrinsic efficacy, but also tissue related, such as receptor density and efficiency of the receptor-effector transduction (4,5). In contrast, mechanism-based pharmacodynamic models do allow identification of such components of drug action (5). A practical mechanism-based model for drug action is the operational model of agonism (6). In this model, an efficacy parameter (τ) has been defined that describes the efficiency of transduction of occupied receptors into pharmacological response. By comparison of the value of τ for the same drug under different circumstances, the influence of disease on receptor density and the efficiency of the receptor-effector coupling *in vivo* can be investigated. In addition the model contains the parameter E_m which represents the maximal primary response that can be achieved in the system by receptor activation.

In the present investigation we applied the operational model of agonism (6) to the concentration-EEG effect relationship data from the previous study (3). The data were re-analysed in a population mode, allowing changes in the parameters efficacy (τ) and tissue maximum (E_m) either separately or in combination. Furthermore, the results were compared to data obtained *in vitro* in brain synaptoneurosomal preparations from identically treated animals. Finally, the obtained data were used to predict quantitatively the changes in pharmacodynamics of other benzodiazepines with different intrinsic efficacy.

METHODS

Experimental Animals and Kindling Procedure

The experimental animals and kindling procedures have been described previously (3). Briefly, male Wistar rats, weighing 200–255 g, were housed individually in plastic cages, at a constant temperature of 21°C, with a normal 12-h light-dark cycle (lights on: 8.00 a.m. to 8.00 p.m.). Cortical electrodes for EEG recording were implanted under Hypnorm/Nembutal anaesthesia (7). For kindling a bipolar electrode with tip separation of 300 μm was implanted in the right basolateral amygdala (2.5 mm posterior and 5.4 mm lateral from bregma and 7.8 mm ventral from the brain surface). A skull screw (11 mm anterior, 2.5 mm lateral from lambda) served as reference electrode for afterdischarge measurement from the amygdala. After one week post-surgical recovery, the afterdischarge threshold was determined by stimulating at 5 minutes intervals, starting at 25 μA (2 sec, 50 Hz, 2 msec bipolar pulse train) and with 50 μA increments until afterdischarges were observed. Thereafter the animals were kindled twice daily at 150 μA above threshold until 6 or 7 sequential stage 5 seizures had been reached. The control rats were treated identically, but were not stimulated. On each day seizure severity and afterdischarge

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ABBREVIATIONS: α, upper asymptote of concentration-effect relationship; EC_{50}, midpoint of concentration-effect relationship; n_H, midpoint slope of concentration-effect relationship; E_0, no drug response; E_m, maximal achievable effect in the system; K_A, agonist equilibrium/dissociation constant; n, slope index; τ, efficacy parameter; K_E, concentration of agonist-receptor complex required to produce half-maximal effect; MVOF, minimum value of objective function.

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duration were recorded. Seizure severity was classified according to Racine (8). The protocol of this study was approved by the Committee on Animal Experimentation of Leiden University.

In Vivo Pharmacology

One day before experimentation, indwelling cannulas were implanted, in the right jugular vein for drug administration, and the right femoral artery for serial collection of blood samples. Twenty-four hours after the last stage 5 seizure, the conscious and freely-moving rats received an intravenous infusion of 10 $mg.kg^{-1}$ midazolam over 2 minutes (3). EEG recordings started at least 45 minutes before drug administration and lasted approximately 6 hours. Amplitudes in the β frequency band of the EEG (11.5–30 Hz) were calculated by Fast Fourier Transformation and used as a measure of drug effect intensity. Serial arterial blood samples were taken and immediately hemolyzed in MilliQ water. Concentrations of midazolam in blood were determined as described previously (9). Whole blood rather than plasma samples were sampled, to reduce the total volume of blood taken in these integrated PK/PD experiments. In a previous study (3) it has been demonstrated that the blood-to-plasma concentration ratio (P/B) and the degree of plasma protein binding (f_u) of midazolam are unchanged in kindled rats.

In Vitro Pharmacology

Isolation of Synaptoneurosomes

Synaptoneurosomes were isolated from brains of kindled or control rats according to the method of Schwartz *et al.* (10) with some modifications. Animals were decapitated and the forebrain was quickly removed and placed in ice-cold incubation medium with the following composition in mM: NaCl, 137; KCl 2.7; KH_2PO_4, 0.44; *d*-glucose, 10; $NaHCO_3$, 4.2; HEPES, 10; pH 7.4. The forebrain, excluding the hippocampus and cerebellum, was rapidly dissected and homogenized by hand in a glass-glass homogenizer (5 ml Braun, Salm en Kipp b.v., The Netherlands). The homogenate was diluted with 15 ml ice-cold buffer and filtered through two layers of nylon mesh of 155 μm pore size and through two nylon layers of 50 μm pore size (Plankton, Diemen, The Netherlands). Subsequently, the homogenate was filtered through an 8 μm Millipore filter (type SCWP 02500). The filtrate was centrifuged at 2300 rpm (1000 *g*) in a Sepatech 3090 rotor (Heraeus Sepatech, Kalkberg, Osterade, Germany), at 4°C for 15 min. The resulting pellet was resuspended in 10 ml ice-cold buffer and centrifuged at 3000 rpm for 10 min. The final pellet was resuspended in 4 ml ice-cold buffer and used within 90 minutes.

Radioligand Binding Studies

Saturation studies were performed in a solution of 350 μl, consisting of 75 μl synaptoneurosome preparation (89–216 μg protein), 100 μM GABA, [^{3}H] flunitrazepam (New England Nuclear-757, specific activity 84.0 $Ci.mmol^{-1}$) in a concentration range of 2 to 150 nM. Non-specific binding was determined in the presence of 1 μM midazolam. Incubations were terminated after 30 min by the addition of 5 ml ice-cold buffer, followed by rapid filtration over Whatmann GF/C filters, presoaked with 0.5% polyethylimine (Sigma, Zwijndrecht, The Netherlands). The filters were submerged in 5 ml Liquid Scintillation Cocktail (Emulsifier Safe, Packard, Downers Grove, Il, USA), and radioactivity was measured. Protein concentrations were determined by a commercial protein kit (P-5656, kit for protein determination, Sigma, Zwijndrecht, The Netherlands).

Measurement of $^{36}Cl^-$ Uptake

Uptake of $^{36}Cl^-$ in synaptoneurosomes was determined according to Schwartz *et al.* (10). Aliquots of 65 μl synaptoneurosomes were preincubated at 37°C for 2 min. Following the pre-incubation, uptake of labelled Cl^- was initiated by the addition 50 μl of a solution containing 0.105 $\mu Ci^{36}Cl^-$ (specific activity 79.4 $Ci.mmol^{-1}$, Nen Dupont de Nemours, The Netherlands), immediately followed by vortexing. A concentration-response curve for muscimol-induced $^{36}Cl^-$ uptake into synaptoneurosomes was determined in the range of 0–150 μM. Five seconds after the addition of $^{36}Cl^-$, influx was terminated by the addition of 3 ml ice-cold stop buffer containing 100 μM picrotoxin (Sigma, Zwijndrecht, The Netherlands) and quick filtering through Whatmann GF/C filters. Non-specific $^{36}Cl^-$ trapped on the filters was determined in medium containing 75 μM muscimol and 100 μM picrotoxin. The amount of radioactivity on the filters was determined by liquid scintillation counting. In order to investigate the coupling between the GABA and benzodiazepine receptor, muscimol-stimulated $^{36}Cl^-$ uptake was determined in the absence and presence of 1 μM midazolam.

DATA ANALYSIS

In Vivo Data

The blood concentration-time profiles were described by a bi-exponential function by use of the program Siphar (Simed CA, Creteil, France) (3). The fitted curve was then used to calculate the midazolam blood concentrations at the time points at which the EEG had been quantified. The Hill equation was fitted to all individual concentration-effect data points:

$$E_C = E_0 + \frac{\alpha \cdot C^{nH}}{EC_{50}^{nH} + C^{nH}} \quad (1)$$

in which E_C is the EEG effect at midazolam concentration C, α the upper asymptote, EC_{50} the midpoint location and n_H the midpoint slope. E_0 is the no-drug response, which was fixed to the value obtained by averaging data over the 30 min period preceding drug administration. Subsequently, the concentration-effect data were fitted to the following form of the operational model of agonism (6):

$$E_C = E_0 + \frac{E_m \cdot \tau^n \cdot C^n}{(K_A + C)^n + \tau^n \cdot C^n} \quad (2)$$

where E_m is the maximum effect achievable in the system, K_A is the agonist equilibrium/dissociation constant, n is the slope index for the occupancy-effect relationship and τ is the efficacy parameter, which is defined by the ratio of total receptor concentration ($[R_0]$) and the concentration of agonist-receptor complex required to produce half-maximal effect (K_E):

$$\tau = \frac{[R_0]}{K_E} \tag{3}$$

the value of the intrinsic activity of a drug (α) can be expressed in terms of system maximum E_m, efficacy τ and slope factor n (11) according to

$$\alpha = E_m \cdot \frac{\tau^n}{1 + \tau^n} \tag{4}$$

All fitting procedures, were performed by use of the nonlinear mixed effect modelling software package NONMEM (NONMEM project group, University of California, San Francisco). The statistical models used had the following general from:

$$E_{ij} = f([A]_{ij}, \theta_i) + \varepsilon_{ij} \tag{5}$$

where E_{ij} and $[A]_{ij}$ correspond to effect and concentration of midazolam, respectively, for the j^{th} datapoint in the i^{th} concentration-effect curve, f is a function (for example the Hill equation), θ_i represents the individual parameters (α, EC_{50} and n_H in the case of the Hill equation) belonging to the concentration-effect curve i and ε_{ij} is a random noise term from a normal distribution with mean zero and variance σ^2. The size of σ^2 is a measure of the intraindividual residual error in the model, i.e. the difference between the observed and predicted values.

The individual parameters (θ_i) were modelled as follows:

$$\theta_i = (\theta + \delta) + \eta_i \tag{6}$$

where θ is the population mean parameter value, δ is the effect of kindling on the population mean and η_i is a random term from a normal distribution with mean zero and variance ω^2. In case of a significant effect of amygdala kindling on the pharmacodynamic parameters (θ), δ will be significantly different from zero. The goodness-of-fit was evaluated on basis of the minimum value of objective function criterion (MVOF), representing the minimal value of the log-likelihood function.

Since the η_i values quantify the deviation of the individual parameters from the population mean, the variance ω^2 associated with a parameter θ provides a measure of the size of the interindividual variation in θ, which relates to biological variation and experimental errors. For convenience, interindividual variability was always expressed as coefficient of variation (c.v.).

In case of the Hill equation, the complete model takes the following form:

$$\alpha_i = (\alpha + \delta_\alpha) + \eta_i^\alpha \tag{7}$$

$$EC_{50_i} = (EC_{50} + \delta_{EC_{50}}) + \eta_i^{EC50} \tag{8}$$

$$n_{H_i} = (n_H + \delta_{n_H}) + \eta_i^{nH} \tag{9}$$

$$E_{ij} = E_i(0) + \frac{\alpha_i \cdot [A]_{ij}^{n_{H_i}}}{EC_{50}^{n_{H_i}} + [A]_{ij}^{n_{H_i}}} + \varepsilon_{ij} \tag{10}$$

Subsequently, the operational model was fitted to the data (equation 2) using the same additive normal error model for the intra-individual residual variation as described above (equation 5). In the modelling procedure the value for K_A was fixed to 82.12 ng.ml^{-1}, as determined previously in brain homogenates (12). In a stepwise procedure the operational model of agonism was first fitted simultaneously to the data from the kindled and control rats with the assumption that amygdala kindling had no effect on any of the three parameters E_m, τ and n. Subsequently the model was fitted to the data allowing changes in the values of both E_m and τ. Finally the model was fitted allowing only a change in E_m.

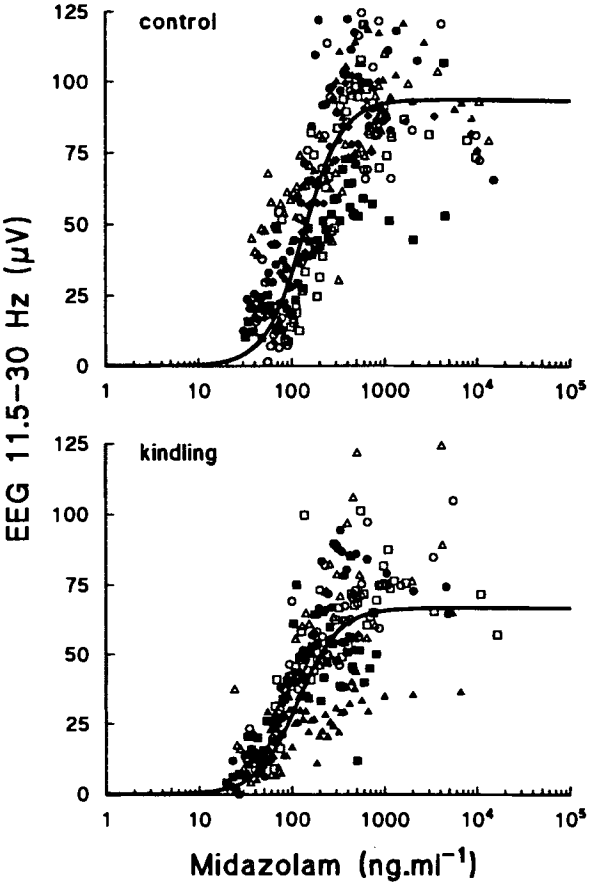

Fig. 1. Blood concentration-effect relationships for the EEG following intravenous infusion of 10 mg.kg^{-1} midazolam for 2 min. The solid line superimposed on the experimental data points (n = 6–7) was obtained by simultaneous fitting of the Hill equation to the data. Different symbols indicate different animals.

All fitting procedures were performed on a IBM-compatible personal computer (Pentium®, 133 MHz) running under Windows 95 using the Microsoft FORTRAN PowerStation 4.0 compiler with NONMEM version IV, level 2.0 (double precision) and Visual-NONMEM version 2.2.2. (RDPP, Montpellier, France).

In Vitro Data

The receptor binding characteristics of the radioligand[^{3}H]-flunitrazepam were determined by fitting the following equation to the data from the saturation experiment:

$$B = \frac{B_{max} \cdot [A]^n}{K_d^n + [A]^n} \tag{11}$$

in which B is the number of receptors occupied, B_{max} is the total number of specific binding sites, K_d is the ligand concentration at which 50% of the receptors is occupied, n is the slope factor and [A] is the ligand concentration.

Table 1. Hill Equation Parameter Estimates for *In Vivo* Effect of Midazolam after Amygdala Kindling (mean ± s.e., n = 6–7)

	Population mean	δ (Amygdala kindling)
intrinsic activity (α)	94 ± 4.4	−27 ± 8.3*
(μV)	(14.7%)	($-43.6 < \delta_\alpha < -10.4$)
EC_{50}	135 ± 18.6	−19 ± 14.2
($ng.ml^{-1}$)	(28.7%)	($-47.4 < \delta_{EC50} < 9.4$)
Hill factor	2.1 ± 0.3	−0.1 ± 0.4
	(54.2%)	($-0.9 < \delta_{nH} < 0.7$)

Note: The effect of amygdala kindling is reflected in the estimate of δ. In parenthesis are either shown the c.v. (population mean) describing the interindividual variation or the 95% confidence intervals of δ.
* $p < 0.05$ $\delta \neq 0$.

The muscimol-stimulated $^{36}Cl^-$ uptake was fitted with the following equation using a population approach with the same additive normal error model for the intra-individual residual variation as described above (equation 5):

$$v = \frac{v_{max} \cdot C}{EC_{50} + C} \qquad (12)$$

in which v is the $^{36}Cl^-$ uptake, v_{max} is the maximal $^{36}Cl^-$ uptake, EC_{50} is the muscimol concentration at which 50% of the uptake is obtained and C is the muscimol concentration. No Hill factor was required in this modelling as judged on basis of goodness-of-fit criteria. The effect of amygdala kindling was characterised by the term δ, reflecting the kindling-induced change in either v_{max} or EC_{50}, as explained before (equation 6).

RESULTS

Modelling of the Concentration-Effect Relationships

Midazolam produced a significant increase in the β activity of the EEG in amygdala kindled and control rats. Figure 1 shows the concentration-effect relationships (3). All individual concentration-effect data were fitted simultaneously to the Hill equation to provide estimates (mean ± s.e. and c.v. for inter-individual variation, Table 1) of the concentration-effect curve upper asymptote (α), midpoint location (EC_{50}) and midpoint slope (n_H). Amygdala kindling produced a reduction (δ) of 27 μV in the maximal effect, which was significantly different from zero (95% C.I. $-10.4 < \delta_\alpha < -43.6$, $p < 0.05$). The resulting values of α were 67 ± 7.7 μV versus 94 ± 8.3 μV for kindled and control rats, respectively. The results of the model fit for the population mean are illustrated in Fig. 1.

Mechanism-Based Modelling

When the operational model of agonism was fitted simultaneously to the data on the assumption of identical values of the parameters E_m, τ and n in kindled and control rats, the model converged, yielding estimates of each of the parameters (Table 2). When the model was fitted to the data allowing differences in the values of both E_m and τ, a considerable improvement of the goodness-of-fit was observed, as reflected in the reduction of the MVOF from 4051.9 to 4043.5 ($p < 0.05$). Kindling caused a significant reduction δ of 27.5 ± 7.2 μV ($p < 0.05$) of the value of E_m (value in control animals 109 ± 7.2 μV). In contrast no significant change was observed in the value of the efficacy parameter τ (value in control animals 1.71 ± 7.2 μV, reduction by kindling 0.06 ± 0.21 μV). When finally the operational model of agonism was fitted to the data allowing only a difference in the value of E_m, the goodness-of-fit was identical (MVOF = 4043.6) as was the case with the values of the parameters τ and n. The reduction in E_m in kindling was also very similar (30.5 ± 11.8 μV). The results of the model fit to the operational model of agonism, allowing only a difference in the value of E_m, are shown in Fig. 2.

Receptor Binding

The binding of [^{3}H]flunitrazepam in synaptoneurosomes was saturable and the non-specific binding was relatively low, i.e., 15 and 24% of the total binding at 20 and 70 nM [^{3}H]flunitrazepam, respectively. There was no influence of kindling on the [^{3}H]flunitrazepam receptor binding characteristics: a K_d of 28.7 ± 3.8 versus 37.5 ± 5 nM and B_{max} of 1393 ± 152 versus 1355 ± 150 $fmol.mg^{-1}$ was observed, for kindled and control animals, respectively.

Muscimol Stimulated $^{36}Cl^-$ Influx

Muscimol stimulated the $^{36}Cl^-$ flux in a concentration-dependent manner as shown in Fig. 3A. Table 3 shows the influence of kindling on the muscimol stimulated $^{36}Cl^-$ uptake

Table 2. Pharmacodynamic Parameter Estimates, Reflecting the Effect of Amygdala Kindling on the Different Parameters (mean ± s.e., n = 6–7)

	τ	δ_τ	n	E_m (μV)	δ_{Em} (μV)	MVOF
I	1.66 ± 0.05		4.81 ± 1.12	97 ± 7.1		4051.9
	(10%)		(61%)	(20%)		
II	1.71 ± 0.07	−0.06 ± 0.21	4.85 ± 1.03	109 ± 8.9	−27.5 ± 7.2**	4043.5*
	(9%)		(58%)	(13%)		
III	1.70 ± 0.04		4.84 ± 1.07	110 ± 9.6	−30.5 ± 11.8**	4043.6*
	(10%)		(56%)	(13%)		

Note: In the first run (I) it was assumed that amygdala kindling does not effect any of the pharmacodynamic parameters, subsequently a change in both τ and E_m (II) or E_m (III) was assumed. MVOF, minimum vale of objective function, reflects the goodness of fit. No statistical differences were observed between the MVOF values between II and III.
* $p < 0.05$ versus I.
** $p < 0.05$ $\delta \neq 0$.

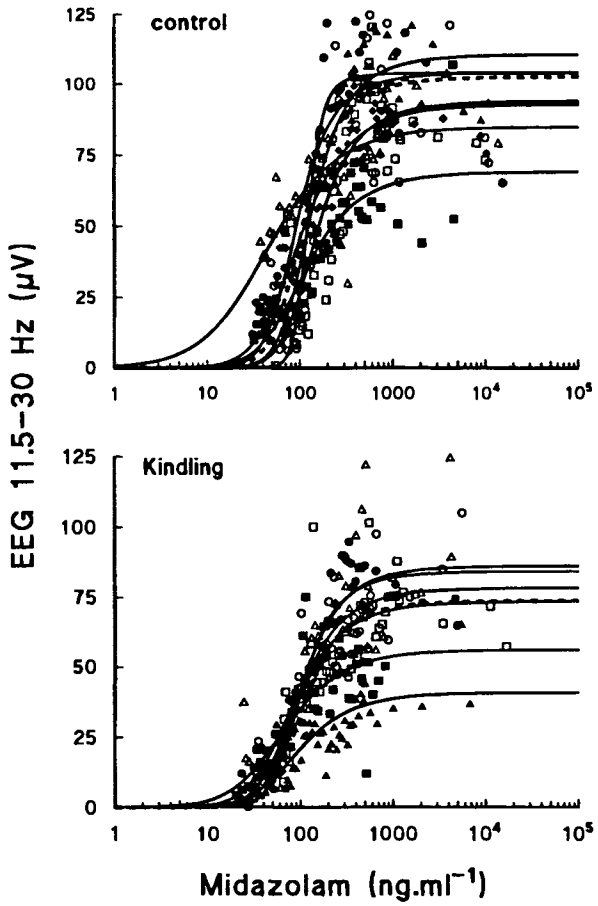

Fig. 2. Individual blood concentration-effect relationships for the EEG effect (β activity) following intravenous infusion of 10 mg.kg^{-1} midazolam for 2 min. The solid lines are individual post-hoc Bayesian estimates, obtained by fitting the data to the operational model of agonism reflecting a change in the system maximum E_m. The population mean estimates are represented by the thicker dashed lines. Different symbols indicate different animals.

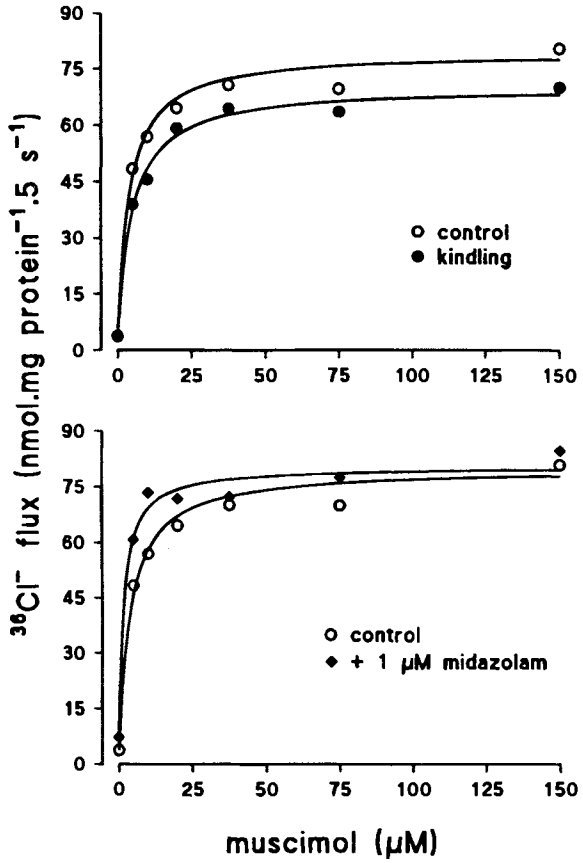

Fig. 3. Concentration-response curves for muscimol-stimulated $^{36}Cl^-$ uptake. The upper panel shows the influence of amygdala kindling, the lower graph reflects the modulation of the uptake by 1 μM midazolam in control animals. The lines represent the population fits (mean ± s.e., n = 9). The standard errors were omitted because they fall within the symbol.

characteristics. Kindling significantly reduced the maximal stimulated $^{36}Cl^-$ uptake (δ_{vmax} − 13.9 ± 4.2, 95% C.I. −22.2 < δ_{vmax} < −5.6), resulting in maximal uptakes of 66.2 versus 76.3 nmol.mg protein^{-1}. 5 sec^{-1} in kindled and control animals, respectively. In Fig. 3B the enhancement of muscimol-stimulated $^{36}Cl^-$ uptake by 1 μM midazolam is illustrated. EC_{50} values for muscimol were 1.79 ± 0.21 versus 4.04 ± 0.41 μM in the presence and absence of 1 μM midazolam, respectively. However, the degree of enhancement by midazolam was not affected in kindled animals as shown by the lack of changes in the EC_{50} of muscimol in the presence of 1 μM midazolam. (δ_{EC50} − 0.02 ± 0.26, 95% C.I. − 0.54 < δ_{EC50} < 0.50, Table 4).

DISCUSSION

In a previous investigation we have shown, that in three different experimental models of epilepsy there is a clear reduction in the maximum EEG effect of midazolam (3). Since the utilized EEG parameter (amplitudes in the 11.5–30 Hz frequency band) reflects activation of the $GABA_A$-benzodiazepine receptor complex (7) this finding indicates that benzodiazepine-modulated GABAergic inhibition *in vivo* is impaired in experimental epilepsy.

In the present investigation the *in vivo* midazolam concentration-EEG effect relationship has been analysed with a model that is based on receptor theory and that explicitly describes agonist concentration-effect relationships as a function of receptor affinity K_A, efficacy τ (which is related to receptor density

Table 3. Parameter Estimates for the Effect of Amygdala Kindling on the Muscimol Stimulated $^{36}Cl^-$ Uptake in Synaptoneurosomes (mean ± s.e., n = 9)

	Population mean	δ (Amygdala kindling)
basal uptake (nmol.mg protein^{-1}. 5 s^{-1})	3.8 ± 2.8 (15%)	0.1 ± 0.5
V_{max} (nmol.mg protein^{-1}. 5 s^{-1})	76.3 ± 1.3 (11%)	−13.9 ± 4.2*
EC_{50} (μM)	4.04 ± 0.41 (10%)	1.58 ± 0.59
$EC_{50}^{\#}$ (μM)	1.79 ± 0.21 (0%)	−0.02 ± 0.26

Note: δ reflects the effect of amygdala kindling on the population parameter estimates. $EC_{50}^{\#}$ is the potency of muscimol obtained in the presence of 1 μM midazolam.
* $p < 0.05$ $\delta \neq 0$.

and receptor-effector coupling), system maximum E_m and slope index n. This model is often referred to as the 'operational model of agonism' (6). It provides a unique basis to link information from *in vitro* bio-assays to parameters characterising pharmacodynamics *in vivo* (5), as has been demonstrated recently in the analysis of *in vivo* concentration-effect relationships of synthetic A_1 adenosine receptor agonists (13) and synthetic opiates (14), showing close correlations between values of the *in vivo* parameters K_A and τ and of the *in vitro* parameters affinity (K_d) and intrinsic efficacy (GTP-shift). In the present investigation this model has been applied for the first time to examine the mechanism of a disease-induced change in drug concentration-effect relationship.

As a first step in the data analysis, the available data on the plasma concentration and the EEG effect of midazolam in control and kindled rats (3) were reanalyzed, using a population approach. In this approach, data from all animals were analysed simultaneously to obtain the population mean values of the various parameters. When fitting the Hill equation to the data, values of the pharmacodynamic parameter estimates were obtained which were very close to those obtained on basis of an analysis of the individual curves according to the standard

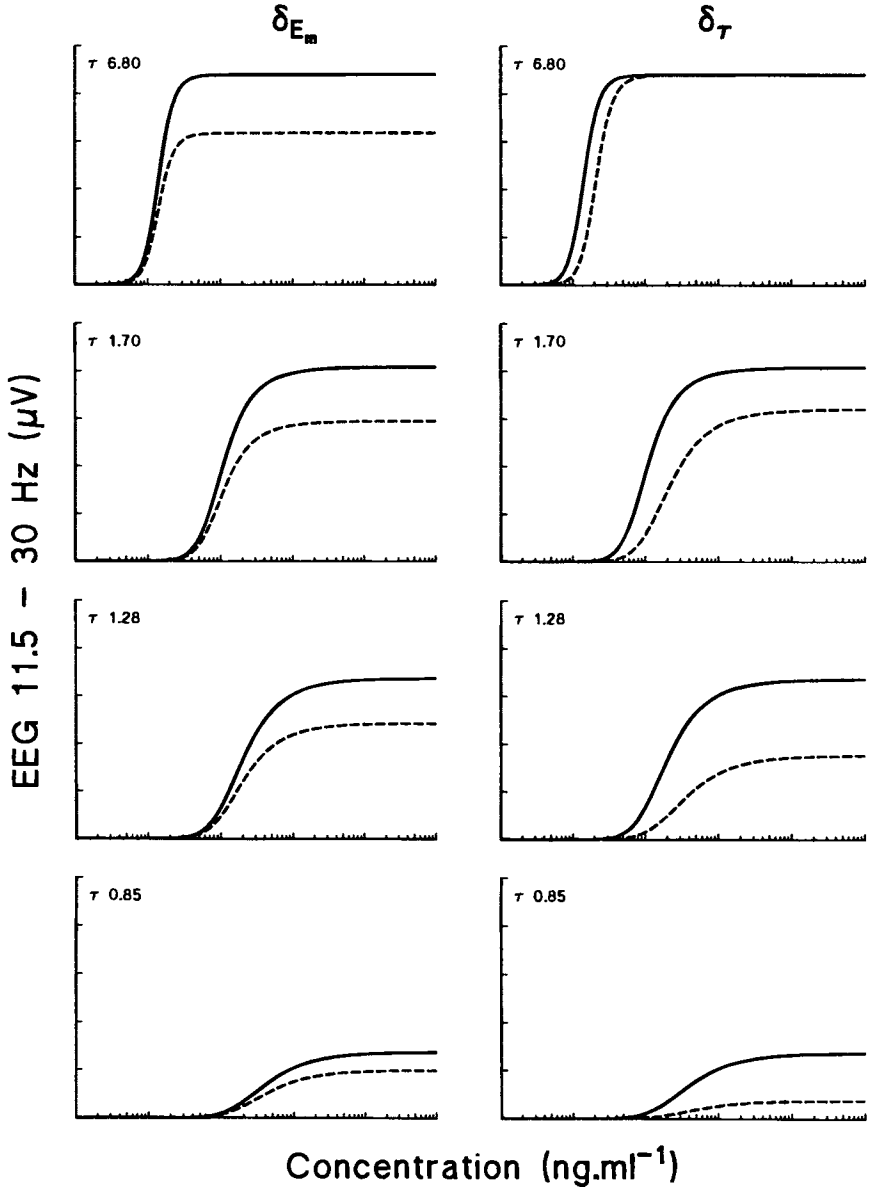

Fig. 4. Simulation of the concentration-effect relationship of four benzodiazepine agonists based on an operational model of agonism (Eq. 5). The solid lines were simulated with the values for E_m 110 μV, K_A 82.12 ng.ml^{-1} and slope factor 4.84. The values of the intrinsic efficacy were 6.80, 1.70, 1.28 and 0.85 respectively. This resulted in the following values of the maximal effect: 99.9, 93, 76 and 31% respectively. The dashed lines represent the simulated concentration-effect relationships in the situation of a 28% decrease in the system maximum (E_m, left panel) or a 28% decrease in the efficacy parameter (τ, right panel) for the different agonists.

two-stage approach. Specifically, a significant reduction in the maximum effect α was observed in kindled rats, confirming previous observations (3). A stepwise approach was subsequently used to fit the operational model of agonism to the data. Here the specific objective was to identify those changes in the parameters that can account for the observed change in the maximum effect α in a quantitative manner. A decrease in the value of α can in theory be explained by a change in each of the three parameters E_m, τ and n, either separately or in combination (6). A change in the value of the slope factor n however will always be associated with a change in the value of the Hill factor n_H (11). Since no changes in the value of n_H were observed in kindled rats a change in n can be ruled out. Therefore in the modelling only changes in the parameters E_m and τ were considered. Changes in the values of K_A were also not considered as this parameter is unrelated to α. Instead the value of K_A was fixed to 82.12 $ng.ml^{-1}$ as determined previously in radio ligand binding studies (12). The lack of an effect of kindling on the value of K_A is confirmed by the results from the ^{3}[H]-flunitrazepam binding experiments. The data from control and kindled rats were fitted simultaneously to the operational model of agonism in a number of runs, subsequently assuming that kindling i) had affected neither E_m nor τ, ii) had changed both E_m and τ and iii) had changed only E_m. This demonstrated convincingly that the reduction in benzodiazepine-modulated GABAergic inhibition in kindled rats can be exclusively attributed to a change in E_m. The lack of a marked change in τ indicates that benzodiazepine receptor density and function is unaffected by kindling. As a means to validate the results of the PK/PD modeling, this was studied in brain synaptoneurosomes *in vitro*. Using [^{3}H]flunitrazepam as a radioligand indeed no changes in benzodiazepine receptor affinity and density were observed. In the *in vitro* experiments a slightly lower value of the maximal muscimol-induced Cl^- flux was observed in kindled rats. It remains to be demonstrated that this is related to the observed reduction in tissue maximum E_m. More important is the observation that there were no changes in the functionality of the benzodiazepine receptor, as the enhancement of muscimol-stimulated $^{36}Cl^-$ uptake by 1 μM midazolam was unchanged. The lack of change in τ is consistent with these findings. Thus it appears that on basis of the mechanism-based model indeed realistic pharmacodynamic parameter estimates are obtained.

An important feature of the mechanism-based model is that it may be used for extrapolation and prediction. Many benzodiazepines are available that differ in both affinity and efficacy. Therefore, an intriguing question is whether in kindled rats the full maximum effect may still be reached by using a compound with a higher intrinsic efficacy. Simulation on basis of the operational model of agonism suggests that this is dependent on whether the reduction in maximum effect is caused by a change E_m or τ. In Fig. 4 simulated concentration-effect relationships are shown for 4 different benzodiazepines, each with a different intrinsic efficacy. In case of a change in E_m a reduction in maximum effect is always seen while the potency (EC_{50}) remains the same. In contrast, a change in τ, can affect both the maximum effect and the potency. For compounds with a high intrinsic efficacy, a decrease in τ will only reduce the potency, but for compounds with lower intrinsic efficacy, it will cause a more and more pronounced reduction both in maximal effect and potency. Thus, it is predicted that in kindled rats a benzodiazepine with a high intrinsic efficacy cannot restore the original maximal effect. Additional investigations with other benzodiazepines are required to substantiate these predictions.

In conclusion, we have applied a mechanistic model based on receptor theory to examine changes in the concentration-effect relationship of midazolam in an experimental model of epilepsy. The findings show that the reduced maximum effect can be explained by a reduction in the parameter tissue maximum (E_m) rather than efficacy (τ). This is consistent with the observed lack of changes in receptor affinity, density and functionality *in vitro*. It is shown that the utilized model may be useful for prediction of changes in concentration-effect relationships of other benzodiazepines.

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