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INTERACTION OF [^{3}H]FLUNITRAZEPAM WITH THE BENZODIAZEPINE RECEPTOR: EVIDENCE FOR A LIGAND-INDUCED CONFORMATION CHANGE

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Abstract—The interaction of [^{3}H]flunitrazepam with benzodiazepine receptors in rat brain homogenates was studied in the presence of 2 μM endogenous GABA at 0° at pH 7.2. Equilibrium binding experiments showed a dominant component of high affinity with an equilibrium dissociation constant $K = 0.86 \pm 0.07$ nM which accounted for 75% of total binding and another component of lower affinity ($K \simeq 30$ nM). The dissociation kinetics of the [^{3}H]flunitrazepam complex at the high affinity site were strictly monophasic with a rate constant $k_{off} = (7.7 \pm 0.3) \times 10^{-4}$/sec. The association kinetics with the high affinity sites were studied with ligand concentrations $[L]_0$ in large excess over binding sites. The kinetics were in accordance with a single exponential with a reaction rate τ^{-1}. In the higher concentration range $[L]_0 \geq 10$ nM, τ^{-1} as a function of $[L]_0$ deviated from linearity and started to level off. The data are compatible with a two-step mechanism where R and L rapidly combine to form a pre-complex RL which then slowly isomerizes to the final complex C:

$$R + L \underset{}{\overset{K_1}{\longleftrightarrow}} RL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C$$

where $K_1 = ([R][L]/([RL])$ and $[RL]/[C] = k_{-2}/k_2 = K_2$. Nonlinear parameter estimation yielded $K_1 = 24.2 \pm 7.1$ nM, $k_2 = (2.8 \pm 0.5) \times 10^{-2}$/sec and $k_{-2} = (9 \pm 2) \times 10^{-4}$/sec. The isomerization step might reflect a ligand-induced conformation change of the high affinity site which is involved in the potentiation of GABA-ergic transmission produced by the benzodiazepines.

The discovery [1, 2] of high affinity binding sites for benzodiazepines in the brain provides a key to the molecular understanding of the action of these drugs (cf. review [3]). There is now clear evidence for a heterogeneity of the benzodiazepine binding sites in the brain. The recently discovered population of low affinity sites for [^{3}H]flunitrazepam and [^{3}H]diazepam in the mammalian brain [4–6] and in cortical cell cultures [6, 7] appears to have the pharmacological characteristics of the peripheral benzodiazepine receptors. These sites are specifically recognized by Ro 5-4864. It is generally accepted that they do not contribute to the central effects of the benzodiazepines [5, 6]. The 'central-type' benzodiazepine receptors themselves may also be differentiated by specific ligands (BZ_1 and BZ_2 receptors, for a review see [8]). The classical benzodiazepines do not distinguish between these BZ_1 and BZ_2 sites as their affinity for the two subpopulations of receptors is virtually the same [9, 10].

The interaction of the classical benzodiazepines with their pharmacologically relevant receptor(s) is currently interpreted using the bimolecular mechanism $R + L \rightleftharpoons C$ (e.g. [11, 12]). Indeed, the kinetically determined equilibrium constant obtained by dividing the respective rate constants for association (k_{on}) and dissociation (k_{off}) agrees with the K_D obtained from equilibrium binding experiments [11, 12]. We are reporting here a kinetic study with flunitrazepam which shows that at higher concentrations ($[L]_0 \geq 20$ nM) the one step mechanism must be expanded to include an intermediate complex RL: $R + L \rightleftharpoons RL \rightleftharpoons C$. This mechanism has been observed in several receptor–ligand systems with agonists or antagonists.

MATERIALS AND METHODS

[^{3}H]Flunitrazepam (sp. act. 88 Ci/mmole, radiochemical purity > 98%) and [^{3}H]Ro 5-4864 (70 Ci/mmole, purity 98%) were purchased from New England Nuclear (Dreieich, F.R.G.). Unlabeled Ro 5-4864 was a generous gift of Dr. H. Möhler, Hoffmann-La Roche (Basle, Switzerland).

Crude membrane fragments were prepared from whole rat brain by homogenization (1:20 vol.) followed by two centrifugations at 49,000 *g*. The membrane pellets were stored at −20°. Before use, the membranes were resuspended in 20 vol. of buffer and recentrifuged at 49,000 *g*. A 50 mM phosphate buffer containing 0.9% NaCl at a pH of 7.2 at 0° was used throughout. Protein was determined according to Bradford [13], using bovine serum albumin as a standard. One milligram original tissue (wet wt) corresponded to 0.022 mg protein in the final preparation.

Determination of residual GABA. Membrane pellets were extracted in 150 vol. of distilled water or buffer by thorough homogenization with a Potter Elvehjem homogenizer and subsequent centrifugation at 49,000 *g*. The GABA content of the supernatant was determined by competition with

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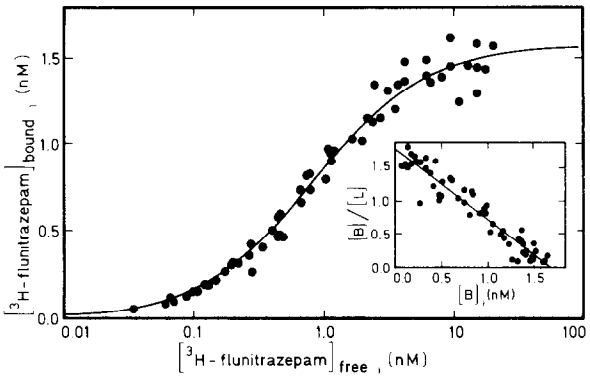

Fig. 1. High affinity binding of [^{3}H]flunitrazepam. Six individual binding experiments (58 data points) are shown as a plot of specifically bound [^{3}H]flunitrazepam concentration, [B], versus free ligand concentration [L]. Regression to the two site model equation (1) (solid curve) gave a single class of sites with $K = 0.83 \pm 0.1$ nM and $B_{max} = 1.6 \pm 0.05$ nM corresponding to 4.8 ± 0.2 pmoles/mg protein (for technical details see Materials and Methods). The insert shows a replot of the data according to Scatchard ([B]/[L] versus [B]) where the fit gives a straight line.

[^{3}H]muscimol (9.3 Ci/mmole, purity 98%, concentration in the assay 2–3 nM) in Triton treated previously frozen membranes [14] from rat cerebellum. In this membrane preparation, GABA displaced 2 nM [^{3}H]muscimol with an $EC_{50} = 17.7 \pm 2.6$ nM and a Hill coefficient $n_H = 0.96 \pm 0.08$ (mean of 7 calibration experiments).

Equilibrium binding data. These were obtained by incubating membrane fragments with various concentrations of the radioligand in triplicate for at least 1 hr at 0°. In experiments where only the high affinity site for flunitrazepam was studied (Fig. 1), tissue concentration was 15 mg/ml (wet wt), corresponding to 0.33 mg protein/ml. Bound ligand was determined by rapid filtration over Whatman GF/B glass fibre filters and two subsequent washes with 5 ml of ice-cold buffer. Nonspecific binding was determined in the presence of 10 μM of unlabeled flunitrazepam and was usually 1.5–2% of total radioactivity.

When the [^{3}H]flunitrazepam concentrations were increased to 370 nM (Fig. 2A), the radiolabel was diluted 1:10 in unlabeled flunitrazepam. The tissue concentration was 30 mg/ml, corresponding to 0.66 mg protein/ml. Nonspecifing binding was determined in the presence of 300 μM diazepam + 0.3% methanol and was less than 2.5% of total radioactivity. Control experiments had shown that nonspecific binding of 200 nM [^{3}H]flunitrazepam could be reliably determined in the presence of diazepam concentrations ranging from 30 to 300 μM and methanol from 0.03 to 0.3%.

In experiments with [^{3}H] Ro 5-4864 (Fig. 2B), the tissue concentration was 15 mg/ml. Nonspecific binding, determined either in the presence of 100 μM (unlabeled) Ro 5-4864 or 100 μM flunitrazepam, was 2–4% of total radioactivity and of the same order of magnitude or higher than specific binding.

Binding data were analysed by (nonlinear) regres-

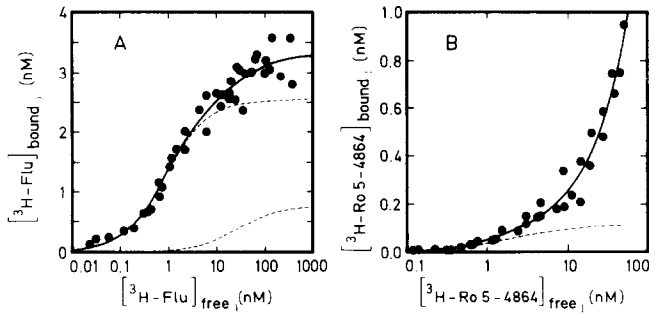

Fig. 2. Peripheral type benzodiazepine sites in the brain. (A) [^{3}H]Flunitrazepam binding up to high concentrations. Regression to the binding data (drawn out curve) gave two components: $K_h = 0.9$ nM, $B_{max,h} = 2.5$ nM ≙ 3.8 pmoles/mg protein and $K_l = 30$ nM, $B_{max,l} = 0.77$ nM ≙ 1.1 pmoles/mg protein. The broken curves show the individual binding components. (Simultaneous fit of three separate experiments, 41 data points; see Materials and Methods for details.) The second binding component was highly significant: $F_{calc} = 5.71 > F$ (99%, 2, 37) = 5.19, AIC (2 sites) = 26.16 < AIC (1 class) = 32.70 (see Materials and Methods). (B) [^{3}H] Ro 5-4864 binding. Simultaneous fit of three experiments to the two site model gave a small high affinity component ($K_h = 1.8$ nM, $B_{max,h} = 0.11$ nM corresponding to 330 fmoles/mg protein) and a large component with low affinity ($K_l > 100$ nM). The latter could not be reliably determined since at 100 nM [^{3}H] Ro 5-4864 there was no indication of saturation. The parameters of the high affinity component were remarkably stable towards arbitrary variations of the low affinity parameters. High affinity sites were statistically significant: $F_{calc} = 5.30 > F$ (95%, 2, 28) = 3.32 but F (99%) = 5.39; AIC (2 sites) = −82.2 < AIC (1 class) = −76.5.

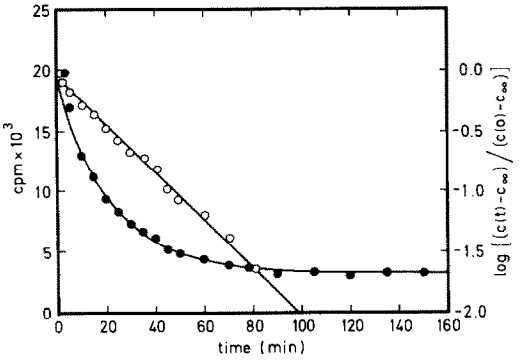

Fig. 3. Dissociation of the [^{3}H]flunitrazepam–receptor complex. A large excess of unlabeled flunitrazepam (5 μM) was added to a solution containing 1 nM [^{3}H]flunitrazepam and 0.8 nM high affinity sites (7.4 mg tissue/ml). (●) Dissociation of the labeled complex with time (left ordinate). (○) Normalized semilogarithmic presentation of the data [right ordinate; c_∞ = cpm at equilibrium, $c(t)$ = cpm at time t].

sion analysis of the following equation which describes binding to two independent classes of binding sites [15]:

$$B = B_{\max,1}\,L/(L + K_1) + B_{\max,2}\,L/(L + K_2) \quad (1)$$

Here, B denotes the sum of specific binding to classes 1 and 2 with densities $B_{\max,i}$ and equilibrium dissociation constants K_i (i = 1,2), L being the free ligand concentration. The statistical significance of a second class of binding sites in the fit to equation (1) was examined by the following two methods.

(i) The 'extra sum of squares principle' (F-test, [16, 17]). Here, an F-value is calculated as

$$F_{calc} = \frac{(SQ_1 - SQ_2)/(\nu_1 - \nu_2)}{SQ_2/\nu_2} \quad (2)$$

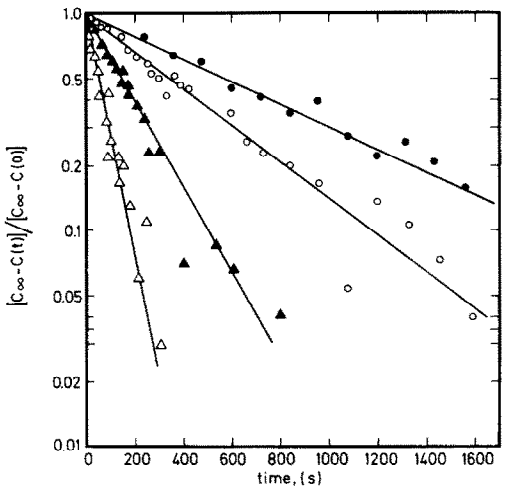

Fig. 4. Association kinetics of [^{3}H]flunitrazepam with benzodiazepine receptor at different ligand concentrations in excess. Data are presented in normalized semilogarithmic form (c_∞ = cpm at equilibrium, $c(t)$ = cpm at time t). From the slopes of the straight lines the reaction rates τ^{-1} were determined by linear regression: (●) $[L]_0$ = 0.2 nM, $\tau^{-1} = 1.2 \times 10^{-3}$/sec; (○) $[L]_0$ = 1.0 nM, $\tau^{-1} = 2.0 \times 10^{-3}$/sec; (▲) $[L]_0$ = 4.7 nM, $\tau^{-1} = 4.6 \times 10^{-3}$/sec; (△) $[L]_0$ = 17 nM, $\tau^{-1} = 1.4 \times 10^{-2}$/sec.

where SQ_1 is the residual sum of squares and ν_1 the number of degrees of freedom for the model with one class of sites and SQ_2, ν_2 denote the corresponding quantities for the fit to the two-site model (equation 1), so that $\nu_1 - \nu_2 = 2$. F_{calc} is then compared to the tabulated value of $F(\alpha\%, \nu_1 - \nu_2, \nu_2)$.

(ii) The 'minimum Akaike information criterion estimation' (MAICE) [18, 19], where the Akaike information criterion (AIC) is calculated for models with one and two classes of sites:

$$AIC_i = N \ln SQ_i + 2\,p_i \quad (3)$$

where N is the number of data points, SQ_i the residual sum of squares, and p_i the number of parameters for the respective models. The model with the smaller AIC is chosen [18, 19]. In all cases, both tests supported the use of the same model.

Dissociation rates. At time zero, a large excess of unlabeled ligand was added to 35 ml of [^{3}H]flunitrazepam–receptor complex. The decrease in radioactive complex was followed by filtration. The kinetics were plotted as cpm (t) − cpm (∞) on a logarithmic scale versus time (Fig. 3). The slope of the straight line yielded the dissociation rate constant $k_{off} = k_{-2}$. k_{off} did not depend on the degree of saturation of the labeled complex ([^{3}H]-flunitrazepam concentration varying from 1.0 to 5.0 nM) nor on the concentration of the unlabeled ligand in large excess (flunitrazepam varied from 0.1 to 5 μM).

Association rates. The kinetics of [^{3}H]-flunitrazepam binding were monitored by adding the ligand at time zero to 35 ml of the membrane fragment preparation (0°, pH 7.2). At appropriate times 1 ml samples were rapidly filtered over Whatman GF/B filters and the filters washed with 15 ml of ice-cold buffer. Filtration time was about 2–3 sec and washing time about 15–20 sec. The amount of non-specific binding was determined in the presence of 10 μM unlabeled flunitrazepam. The concentration of receptor sites was adjusted such that at the most 10% of the total ligand concentration was specifically bound. This assures pseudo-monomolecular conditions for the reaction kinetics. Data were plotted as cpm (∞) − cpm (t) versus time on semilogarithmic graph paper (Fig. 4).

Data analysis. Unweighted linear least squares analysis and error estimation were performed according to standard procedures [16]. Nonlinear parameter estimation was made on a Wang 2200 VP desk computer using the TOPFIT program package [20] including a program for binding data analysis (G. Heinzel, in preparation) and Metzlers NONLIN program [21] transcribed into Wang BASIC.

RESULTS

Equilibrium binding

Equilibrium binding of [^{3}H]flunitrazepam to benzodiazepine receptors was studied in a crude membrane preparation from whole rat brain at 0°, pH 7.2. When the free ligand concentration was $\leq$ 20 nM, analysis of the data revealed only a single class of non-interacting binding sites. This is shown in Fig. 1 where a simultaneous fit of six individual experiments to a model with two independent bind-

ing sites is presented (see equation 1). The fit degenerated to a single class of sites with an equilibrium dissociation constant $K = 0.83 \pm 0.1$ nM and a density of sites $B_{max} = 4.8 \pm 0.2$ pmoles/mg protein. The results are in good agreement with literature values [11, 22]. A replot of the data according to Scatchard (Fig. 1, insert) gave a straight line, indicating no deviation from binding to a single class of independent sites (see e.g. [23]). Separate analysis of the six experiments gave similar results, the averaged parameters being $K = 0.86 \pm 0.07$ nM and $B_{max} = 4.8 \pm 0.3$ pmoles/mg protein.

Recently, the existence in the central nervous tissue of an additional class of binding sites was reported with lower affinity for [^{3}H]diazepam and [^{3}H]flunitrazepam [4–7]. Indeed, experiments where the ligand concentration reached up to 350 nM clearly revealed two classes of binding sites (Fig. 2A). A fit of these data to the two-site model [equation (1)] without constraints gave $K_h = 0.9$ nM, $B_{max,h} = 3.8$ pmoles/mg protein for the high affinity component and $K_l = 30$ nM, $B_{max,l} = 1.1$ pmoles/mg protein for the low affinity component. The K_D value of the high affinity component is in excellent agreement with that determined in Fig. 1. The corresponding B_{max} value is somewhat lower which may be due to a difference in the membrane preparation. The K_D value for the interaction with the low affinity sites is again in excellent agreement with reported values [4, 7]. The B_{max} values determined in Fig. 2A show that in this preparation the low affinity sites make up about 23% of the total [^{3}H]flunitrazepam sites.

It has been shown that the low affinity [^{3}H]flunitrazepam sites have the pharmacological specificity of the peripheral benzodiazepine receptors [6, 7]. They are therefore specifically recognized by the centrally inactive ligand [^{3}H] Ro 5-4864 [4, 6, 7]. Figure 2B shows a simultaneous fit of three separate binding experiments with [^{3}H] Ro 5-4864 to the two-site model. It is seen that a small high affinity component ($K = 1.8$ nM, $B_{max} = 330$ fmoles/mg protein) is dominated by a large low affinity component which could not be saturated ($B_{max} > 5$ pmole/mg protein, $K > 100$ nM). The parameters of high affinity binding are in good agreement with the values reported by Schoemaker *et al.* [4] for [^{3}H] Ro 5-4864 binding to rat cerebral cortex. The low affinity component may be an experimental artifact due to high nonspecific binding of the radioligand (see Materials and Methods). At 1 nM [^{3}H] Ro 5-4864 where high affinity binding prevails the radiolabel was displaced by unlabeled Ro 5-4864 with an $EC_{50} = 5$ nM and by flunitrazepam with $EC_{50} = 100$ nM. The B_{max} value of the high affinity sites for Ro 5-4864 is three times lower than the density of the low affinity sites for flunitrazepam (Fig. 2A). However, determination of B_{max} of the low affinity flunitrazepam sites is difficult because of the high ligand concentrations needed to reach saturation (see scatter in Fig. 2A) and determination of B_{max} of the high affinity Ro 5-4864 sites is difficult because of problems with nonspecific binding (see Materials and Methods). Therefore, we do not believe that this difference in the B_{max} values is meaningful.

Another point concerns the residual concentration of endogenous GABA in the membrane preparation used here. GABA is known to be an allosteric modulator of benzodiazepine binding, increasing 2- to 3-fold the affinity of benzodiazepine agonists for the high affinity sites [24–28]. We have used this effect to estimate the residual GABA concentration near the receptor site in our (twice washed) membrane preparation. Table 1 shows the influence of GABA on high affinity binding of [^{3}H]flunitrazepam. Addition of a saturating amount of GABA to (virtually) GABA-free membranes increases the affinity for [^{3}H]flunitrazepam binding from 1.41 to 0.64 nM, i.e. 2.2-fold. In the routinely used membranes the affinity was 0.86 nM (Fig. 1). Therefore 73% of the (high affinity) benzodiazepine receptors were in the GABA-modified form. The modulation of the affinity for [^{3}H]flunitrazepam as a function of exogenous GABA concentration follows the Law of Mass Action with an $EC_{50} = 1.2$ μM (0°, data not shown), in excellent agreement with published data [28]. In this case, the Law of Mass Action is written as:

$$(K - K_+)/(K_- - K_+) = EC_{50}/(EC_{50} + [G]) \quad (4)$$

where K denotes the equilibrium dissociation constant for [^{3}H]flunitrazepam in twice washed membranes, K_+ that in the presence of 100 μM GABA, K_- that in extensively washed membranes, and [G] is the concentration of exogenous GABA. Inserting the values from Table 1 and $EC_{50} = 1.2$ μM gives [G] = 2.4 μM. This estimate is corroborated by direct determination of the GABA content of the membrane preparation (see Materials and Methods) which was 0.14 nmole/mg tissue. At a tissue concentration of 15 mg/ml this corresponds to a GABA concentration of 2.1 μM. It is concluded that the

Table 1. Influence of GABA on high affinity [^{3}H]flunitrazepam binding

Membrane preparation	GABA added	K (nM)	B_{max} (pmoles/mg protein)	n*
Extensively washed†	—	1.41 ± 0.15	4.5 ± 0.6	3
	100 μM	0.64 ± 0.08	4.8 ± 0.3	3
Washed twice (routine preparation)	—	0.86 ± 0.07	4.8 ± 0.3	6
	100 μM	0.62 ± 0.08	4.7 ± 0.4	4

* n = number of experiments.

† Osmotically shocked by homogenization with a Potter Elvehjem homogenizer in distilled water, frozen and subjected to five wash cycles. Most of the endogenous GABA is thereby removed [29].

membrane preparation contained about 2 μM residual GABA and that about 3/4 of the (high affinity) binding sites were in the GABA-modulated form.

Kinetics

The dissociation kinetics of the [^{3}H]flunitrazepam–receptor complex were monitored after addition of a large excess of unlabeled ligand to the labeled complex. Since the [^{3}H]flunitrazepam concentration was always less than 5 nM (see Materials and Methods), the low affinity sites did not contribute to the kinetics. A typical experiment is shown in Fig. 3 where the semilogarithmic plot of the data indicates that the dissociation kinetics were strictly monophasic. In five experiments a mean value of the dissociation rate constant was obtained, $k_{off} = (7.7 \pm 0.3) \times 10^{-4}$/sec, which is in good agreement with published values [11].

The association kinetics of [^{3}H]flunitrazepam with the benzodiazepine receptor were followed as a function of ligand concentration $[L]_0$ up to $[L]_0 = 20$ nM; at higher concentrations the kinetics were too fast to be reliably monitored by the slow filtration technique. With $[L]_0$ in large excess over the concentration of binding sites (at least 9-fold), strictly monoexponential kinetics were observed at all concentrations. It is shown in the Discussion that the low affinity sites do not contribute significantly to the observed kinetics. Furthermore it is proposed that the inhomogeneity of the high affinity sites (73% in the GABA-induced high affinity form) is not expected to lead to kinetic complications.

Some representative kinetic traces are shown in Fig. 4 in a normalized semilogarithmic form. The slopes of the straight lines were determined by linear regression and yielded the reaction rate, τ^{-1}. Examination of τ^{-1} as a function of $[L]_0$ (Fig. 5) gives insight into the mechanism of the receptor–ligand interaction.

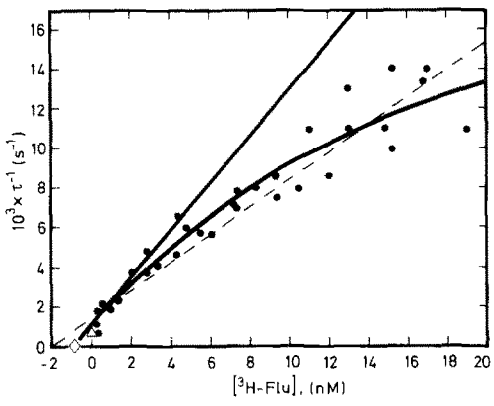

Fig. 5. Reaction rates τ^{-1} as a function of flunitrazepam concentration. (●) τ^{-1} from association kinetics; (△) k_{-2} from dissociation kinetics (see Fig. 3); (◇) K_D from equilibrium binding (Fig. 1). Drawn out straight line: data with $[L]_0 \leq 3$ nM are fitted to equation (5B) by linear regression: $k_{on} = k_2/K_1 = (1.20 \pm 0.05) \times 10^6$ M^{-1}/sec; $k_{off} = k_{-2} = (9.0 \pm 0.6) \times 10^{-4}$/sec; $r = 0.986$, $n = 19$. Broken straight line: fit of all data to equation (5B) gives: $k_{on} = (7.1 \pm 0.3) \times 10^5$ M^{-1}/sec, $k_{off} = (1.5 \pm 0.3) \times 10^{-3}$/sec; $r = 0.965$, $n = 43$. Drawn out curve: nonlinear regression to hyperbola, equation (6B), gives: $K_1 = 24.2 \pm 7.1$ nM, $k_2 = (2.8 \pm 0.5) \times 10^{-2}$/sec, $k_{-2} = (9 \pm 2) \times 10^{-4}$/sec.

For the bimolecular mechanism (equation 5A)

$$R + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C;\ K_D = k_{off}/k_{on} \qquad (5A)$$

τ^{-1} is given by equation (5B) (see Appendix, equation A2)

$$\tau^{-1} = k_{on}[L]_0 + k_{off} \qquad (5B)$$

τ^{-1} is a linear function of $[L]_0$ with slope k_{on} (association rate constant) and ordinate intercept k_{off} (dissociation rate constant). The abscissa intercept is given by the equilibrium dissociation constant $-K = -k_{off}/k_{on}$.

Subjecting the data shown in Fig. 5 to linear regression analysis (see broken straight line in Fig. 5) gives $k_{on} = (7.1 \pm 0.3) \times 10^5$ M^{-1}/sec and $k_{off} = (1.5 \pm 0.3) \times 10^{-3}$/sec. K is then calculated to be 2.1 ± 0.4 nM. These values for K and k_{off} are clearly incompatible with the results shown in Figs. 1 and 3. If the low concentration data ($[L]_0 \leq 3$ nM) alone are analysed by linear regression one obtains $k_{on} = (1.20 \pm 0.05) \times 10^6$ M^{-1}/sec, $k_{off} = (9.0 \pm 0.6) \times 10^{-4}$/sec and K is calculated to be 0.75 ± 0.1 nM (see drawn out straight line in Fig. 5). These values of k_{off} and K are in excellent agreement with those from Figs. 1 and 3. With increasing ligand concentration, however, τ^{-1} deviates from the straight line and starts to level off. The mechanism (5A) must be rejected on these grounds.

The most simple and most familiar mechanism which predicts a hyperbolic dependence of τ^{-1} on $[L]_0$ states that ligand and receptor combine in a rapid first step to a precomplex RL which then slowly isomerizes to the final complex C:

$$R + L \overset{K_1}{\longrightarrow} RL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C;\ K_1 = [R][L]/[RL],$$
$$K_2 = k_{-2}/k_2 = [RL]/[C] \qquad (6A)$$

If only the slow step can be monitored its relaxation rate τ^{-1} is given by equation (6B) (see Appendix, equation A7):

$$\tau^{-1} = k_{-2} + k_2[L]_0/([L]_0 + K_1). \qquad (6B)$$

The hyperbolic dependence of τ^{-1} on $[L]_0$ may be explained in the following way: at low concentrations, $[L]_0 \ll K_1$, equation (6B) reduces to the linear expression $\tau^{-1} \approx k_{-2} + k_2[L]_0/K_1$. Comparison with equation (5B) shows that $k_{on} = k_2/K_1$ and $k_{off} = k_{-2}$. Since $[L]_0 \ll K_1$, only a little RL is formed and the mechanism appears to be bimolecular. At high ligand concentrations, $[L]_0 \gg K_1$, all receptor is initially converted to RL and the isomerization to C dominates the kinetics. In this case the mechanism appears to be monomolecular.

Fitting the data in Fig. 5 to equation (6B) by nonlinear parameter estimation yielded: $K_1 = 24.2 \pm 7.1$ nM, $k_2 = (2.8 \pm 0.5) \times 10^{-2}$/sec and $k_{-2} = (9 \pm 2) \times 10^{-4}$/sec (see Fig. 5, solid curve for fit). $K_2 = k_{-2}/k_2$ is calculated to $(3.2 \pm 0.9) \times 10^{-2}$ so that at equilibrium about 3% of the total receptor–ligand complex is in the RL form. The overall

equilibrium dissociation constant may therefore be approximated by $K \approx ([L][R]/[C]) = K_1K_2 = 0.77 \pm 0.26$ nM. Obviously these values for K and k_{-2} ($= k_{off}$) agree well with those determined in Figs. 1 and 3.

The amplitude of the kinetics, δA = cpm (∞) − cpm ($t \rightarrow 0$), generally corresponded to the amount of complex formed at equilibrium. This indicated that the precomplex RL which had formed immediately after mixing ($t \rightarrow 0$) and which was initially present on the filter had (mostly) dissociated during the 15 sec washing period (see Appendix). The half-life of RL must therefore be shorter than this time interval, so that $k_{-1} > 0.1\ \text{sec}^{-1}$. This estimate is consistent with the assumption of the binding step being in fast pre-equilibrium with the isomerization step which requires $k_{-1} \gg k_2$. With $K_1 = k_{-1}/k_1 = 24$ nM one obtains $k_1 > 4 \times 10^6\ \text{M}^{-1}/\text{sec}$. It is possible that the binding step may be diffusion controlled [30].

DISCUSSION

Receptor heterogeneity. In this communication, the interaction of [^{3}H]flunitrazepam with a crude membrane preparation from whole rat brain is described. As far as the kinetic analysis is concerned one has to keep in mind that the receptor population is heterogeneous because of (i) a small fraction of low affinity sites and (ii) the presence of 2 μM endogenous GABA which converts about 75% of the (high affinity) sites to a 2.5-fold higher affinity. Since the different receptor subpopulations are non-interacting and since the association kinetics were studied under pseudo-monomolecular conditions, the kinetics will consist of decoupled parallel reactions (see Appendix, part 2).

Initially, the contribution of the low affinity sites to the association kinetics will be considered. As shown in the Appendix, one can estimate that they contribute 11% to the total binding kinetics at a ligand concentration of 20 nM. Considering the experimental accuracy of the (slow) filtration assay at reaction rates faster than 10^{-2}/sec, such a small component could only be detected if it were at least ten times slower than the main component. This was not observed (see Fig. 4, △). An unresolved kinetic component with maximum 11% of the total amplitude cannot, however, explain the deviation of the high concentration data shown in Fig. 5.

Concerning the presence of endogenous GABA it is noted that the GABA-mediated affinity increase is reflected in an increased association rate constant whereas the dissociation rate constant remains unchanged [24]. The membrane preparation contained 75% of the (high affinity) receptors in the GABA-modulated form. Therefore, one expects bi-exponential kinetics where one exponential will contribute 75% of the total amplitude and will have the reaction rate pertaining to the GABA-modified form, whereas the second component will have 25% of the total amplitude and a 2- to 3-fold slower reaction rate (see Appendix, equation A10). Clearly these two components cannot be resolved by the filtration assay. Instead, one obtains a mean reaction rate τ^{*-1} [31] which is 15% lower than the value of a population completely modified by GABA (see Appendix, A11). It is concluded that the kinetic results refer to the high affinity ('central type') benzodiazepine receptors and that the parameters will be close to those expected in the presence of saturating amounts of GABA.

Mechanism. The main result of this study is that, at higher ligand concentrations, the association kinetics deviate from those predicted by the bimolecular mechanism $R + L \rightleftharpoons C$. The results in Fig. 5 indicate a change in rate limiting step: at low ligand concentrations, the binding step is rate-determining. But, as its rate increases linearly with ligand concentration, a different kinetic step will become rate-limiting at higher concentrations. There are many kinetic mechanisms which will lead to the concentration dependence shown in Fig. 5. A biologically attractive hypothesis in agreement with the data is the existence of an endogenous inhibitor of benzodiazepine binding (e.g. 'GABA-modulin' [32, 33]) which must dissociate from the receptor before the benzodiazepines can bind. At sufficiently high ligand concentrations, dissociation of GABA-modulin will become rate-limiting. This mechanism predicts, however, biphasic kinetics [34] and strong assumptions must be made to make the theory fit the data (see Appendix, part 3, for quantitative treatment). It is mainly for this reason that we have interpreted the kinetic data within the framework of a ligand-induced conformation change. This is supported by recent thermodynamic analysis of benzodiazepine binding [11, 35, 36] which is in favour of a ligand-induced conformation change, at least at temperatures above 21° [35] or 10° [36].

The induced fit mechanism equation (6A) consists of a (unmeasurably) fast binding step followed by a slow isomerization. The binding step may be diffusion controlled. It contributes about −40 kJ/mole (K_1 = 24 nM) to the total energy of binding (ΔG = −47.5 kJ/mole, K = 0.8 nM). The equilibrium of the isomerization step lies to 97% on the side of the final complex. The half-life of the isomerization reaction is 24 sec (all values at 0°).

A ligand-induced conformation change has also been observed with other receptor–ligand systems, e.g. with acetylcholine receptors. In the case of agonists binding to the nicotinic acetylcholine receptor, the slow isomerization step [37–39] corresponds to the physiological event of receptor desensitization [40]. In binding of antagonists to the muscarinic acetylcholine receptor [41, 42] there is also a slow isomerization step, the physiological meaning of which is unknown. In the case of the benzodiazepines it is not known whether drugs act as agonists or antagonists to an eventual endogenous ligand at the receptor site and by which elementary steps binding is transduced into pharmacological effects. It has been hypothesized that the benzodiazepine-induced potentiation of GABA-ergic transmission may be brought about by a conformation change of the receptor upon binding [35, 43]. Following this notion one might speculate that the kinetics described here reflect this conformational change.

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APPENDIX

1. *Homogeneous receptor population*

For the bimolecular mechanism

$$R + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C,\ K = k_{off}/k_{on} \tag{A1}$$

the rate of formation of C is

$$\dot{C} = k_{on}\, R\, L - k_{off}\, C^*$$

Under pseudo-monomolecular conditions, $L_0 \gg R_0$, the free ligand concentration L does not change during the reaction: $L \approx L_0$. Consideration of the mass balance for the receptor sites, $R_0 = R + CC$, gives

$$\dot{C} = k_{on}\, L_0\, R_0 - (k_{on}\, L_0 + k_{off})\, C$$

Introducing the reaction rate τ^{-1} by

$$\tau^{-1} = k_{on}\, L_0 + k_{off} \tag{A2}$$

the differential equation reads

$$\dot{C} + \tau^{-1}\, C = k_{on}\, L_0\, R_0$$

and has the solution

$$C(t) = C_\infty\,(1 - e^{-t/\tau}),\ \text{with} \tag{A3}$$

$$C_\infty = \tau\, k_{on}\, L_0\, R_0 = R_0\, L_0/(L_0 + K) \tag{A4}$$

In the case of the sequential mechanism

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C,\ K_1 = k_{-1}/k_1,\ K_2 = k_{-2}/k_2 \tag{A5}$$

the rate of formation of C is given by

$$\dot{C} = k_2\, RL - k_{-2}\, C$$

If the (rapid) binding step is in fast pre-equilibrium with the slow isomerization, the slow time scale of formation of C allows one to estimate the concentration of RL by the Law of Mass Action: $K_1 = R \times L/RL$. Since $R_0 = R + RL + C$ and $L \approx L_0$, this is rearranged to

$$RL = (R_0 - C)\, L_0/(L_0 + K_1) \tag{A6}$$

Insertion of (A6) in the differential equation gives:

$$\dot{C} + \tau^{-1}\, C = R_0\, k_2\, L_0/(L_0 + K_1)$$

$$\text{where } \tau^{-1} = k_2\, L_0/(L_0 + K_1) + k_{-2} \tag{A7}$$

* The dot denotes the derivative with respect to time. Concentration brackets are omitted. Total concentration is denoted by the zero subscript.

The solution is again given by equation (A3) with

$$C_\infty = R_0\,\tau\,k_2\,L_0/(L_0 + K_1) \approx R_0\,L_0/(L_0 + K_1K_2) \quad \text{(A8)}$$

if $K_2 \ll 1$.

The amplitude of the kinetics, δA, is defined as δA = cpm $(t\to\infty)$ − cpm $(t\to 0)$. Omitting the blank value which cancels when the difference is taken we have ($K_2 \ll 1$): cpm $(t\to\infty) = (RL + C)_{t\to\infty} = C_\infty\ (1 + K_2) \approx C_\infty$ with C_∞ from equation (A8).

The value of cpm $(t\to 0)$ depends on whether or not the precomplex RL dissociates during the washing of the filter (≈15 sec).

(a) RL is stable: then cpm $(t\to 0) = (RL + C)_{t\to 0}$. Since $C(0) = 0$ we have $\delta A = C_\infty - RL_{t\to 0}$ with $RL(0) = R_0L_0/(L_0 + K_1)$ (see equation A6 with $C(0) = 0$). Inserting the values $K_1 = 24$ nM, $K_2 = 3.2 \times 10^{-2}$ and assuming $L_0 = 20$ nM we obtain $\delta A = R_0\,(0.96 - 0.45) \approx 0.5\,R_0$. Instead it was found experimentally that $\delta A \approx R_0$ at this concentration.

(b) RL completely dissociates during washing: then cpm $(t\to 0) = 0$ (blank omitted) and $\delta A \simeq R_0$ at $L_0 = 20$ nM.

2. *Two classes of independent sites (high and low affinity)*

Consider the case that ligand binding to either class of receptors R_i ($i = h, l$) follows the bimolecular mechanism of equation A1 and that $L_0 \gg R_{0,i}$. We then have

$$C_i(t) = C_{i,\infty}\,(1 - e^{-t/\tau_i})$$

with

$$\tau_i^{-1} = k_{on,i}\,L_0 + k_{off,i} \text{ and } C_{i,\infty} = R_{i0}\,L_0/(L_0 + K_i),\ i = h, l.$$

The specifically bound radioactivity on the filter is $C(t) = C_h(t) + C_l(t)$. Denoting the total concentration of receptors $R_0 = R_{h0} + R_{l0}$ and the fraction of high affinity receptors $\alpha = R_{h0}/R_0$, $C(t)$ is written

$$C(t) = R_0\left[\alpha\,\frac{L_0}{L_0 + K_h}(1 - e^{-t/\tau_h}) + (1 - \alpha)\frac{L_0}{L_0 + K_l}(1 - e^{-t/\tau_l})\right] \quad \text{(A9)}$$

$C(t)$ is the sum of two exponentials with reaction rates specific to either site and amplitudes given by the fraction of receptors, α or $1 - \alpha$, times the respective degree of saturation, $L_0/(L_0 + K_i)$. The contribution of high and low affinity sites to the total binding at $L_0 = 20$ nM is then ($K_h = 0.86$ nM, $K_l = 30$ nM, $\alpha = 0.77$): high affinity: $0.74\,R_0$, low affinity: $0.09\,R_0$. The relative contribution of the low affinity sites is therefore 11%.

The effect of endogeneous GABA is considered flunitrazepam at concentrations $L_0 \geq 10$ nM where it is largest. Denoting with τ_h the reaction time of a pure GABA-modified population and with τ_l that of an unmodified population we have $\tau_l = 2.5\,\tau_h$ [24]. With $\alpha = 0.75$ (see text) equation A9 is approximated by

$$C(t) \approx R_0\,[0.75\,(1 - e^{-t/\tau_h}) + 0.25\,(1 - e^{-t/2.5\,\tau_h})] \quad \text{(A10)}$$

The mean relaxation time τ^* of a biexponential expression with amplitudes δA_1, δA_2 and relaxation times τ_1 and τ_2 is defined as [31]:

$$\tau^{*-1} = \delta A_1/\tau_1 + \delta A_2/\tau_2.$$

For equation A10 one obtains

$$\tau^{*-1} = 0.85/\tau_h \quad \text{(A11)}$$

so that the observed relaxation rate is 15% lower than that expected in presence of saturating GABA.

3. *Endogenous inhibitor*

When an endogenous substance I competitively inhibits flunitrazepam binding, the mechanism is as follows:

$$\begin{array}{ccc} & L & \\ & + & \\ I + R & \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} & RI,\ K_i = k_{-i}/k_i,\ K = k_{off}/k_{on} \\ k_{on} \downarrow\uparrow k_{off} & & \\ & C & \end{array} \quad \text{(A12)}$$

Formation of C is given by

$$\dot{C} = k_{on}\,L \cdot R - k_{off}\,C \quad \text{(A13)}$$

It is assumed that the inhibitor is present in excess and the concentration of the free receptor R is always low. Then R can be calculated using the steady state approximation

$$\dot{R} = k_{-i}\,RI + k_{off}\,C - R\,(k_{on}\,L + k_iI) = 0$$

Eliminating RI by means of the mass balance $R_0 = R + RI + C$ yields the following expression for R

$$R = \frac{k_{-i}}{k_{on}\,L + k_i\,I + k_{-i}}\,R_0 - \frac{k_{-i} - k_{off}}{k_{on}\,L + k_i\,I + k_{-i}}\,C$$

Inserting this expression in equation A13 yields

$$\dot{C} + \tau^{-1}\,C = \alpha R_0$$

with the solution

$$C = C_\infty\,(1 - e^{-t/\tau}) \quad \text{(A14)}$$

$$\text{Here } \alpha = k_{on}\,L/(1 + I/K_i + k_{on}\,L/k_{-i}), \quad \text{(A15)}$$

$$\tau^{-1} = \frac{k'_{on}\,L + k_{off}}{1 + k'_{on}\,L/k_{-i}} = k_{-i}\frac{L + K'}{L + K'k_{-i}/k_{off}} \quad \text{(A16)}$$

with $k'_{on} = k_{on}/(1 + I/K_i)$, $K' = k_{off}/k'_{on}$ and $C_\infty = \alpha\tau R_0 = R_0\,L/(L + K')$.

Equations A14 to A17 show that the formation of C follows a single exponential when the steady state approximation for the free receptor concentration is used. As in the cases discussed earlier, the amplitude is given by the Law of Mass Action (compare A3, A4 and A8). It is noted that the equilibrium constant K' contains a term which takes into account the presence of the competitor: $K \to K' = K\,(1 + I/K_i)$.

The reaction rate τ^{-1} shows the required hyperbolic dependence on L (see A16). For the concentration limits one obtains from equation A16:

$$L \to 0\text{: } \tau^{-1} \to k_{off} \text{ and } L \to \infty\text{: } \tau^{-1} \to k_{-i}$$

The midpoint of the hyperbola is given by $L_m = k_{-i}/k'_{on} = K'\,k_{-i}/k_{off}$.

By analogy with the fit to equation A7 one obtains the parameter values $k_{off} = 9 \times 10^{-4}$/sec, $k_{-i} = 2.8 \times 10^{-2}$/sec, $L_m = k_{-i}/k'_{on} = 24$ nM, so that $k'_{on} = 1.2 \times 10^6\ M^{-1}$/sec and $K' = k_{off}/k'_{on} = 0.77$ nM in agreement with Fig. 5.