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Kinetics of [^{3}H]Muscimol Binding to the $GABA_A$ Receptor in Bovine Brain Membranes†

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ABSTRACT: The binding of the GABA receptor agonist [^{3}H]muscimol to membrane preparations from bovine cerebral cortex has been investigated in equilibrium and kinetic experiments. Equilibrium binding curves are biphasic and suggest that [^{3}H]muscimol binds to both high-affinity (K_d approximately 10 nM) and low-affinity (K_d approximately 0.5 μM) sites. Binding to each class of sites is inhibited by GABA and by the specific $GABA_A$ receptor antagonist bicuculline. The kinetics of [^{3}H]muscimol binding have been measured by using both manual filtration assays and an automated rapid filtration technique which permits the measurement of ligand dissociation on subsecond time scales. Association and dissociation curves are biphasic at all concentrations of [^{3}H]muscimol studied, even under conditions of low receptor saturation when no significant occupancy of the low-affinity sites would be expected. These results cannot be simply explained by the presence of two populations of binding sites in the membrane preparations but suggest the existence of two forms of the monoliganded receptor. Dissociation constants for these two forms have been estimated to be 16 and 82 nM at 23 °C. At higher ligand concentrations, kinetic measurements have suggested that the binding of [^{3}H]muscimol to low-affinity sites is accompanied by a slow conformational change of the receptor–ligand complex.

γ-Aminobutyric acid (GABA)[1] is a major inhibitory neurotransmitter in the central nervous system, and the response to its binding to the $GABA_A$ receptor is a hyperpolarization of the postsynaptic membrane resulting from an increased permeability to chloride ions (Curtis & Johnson, 1974; Krnjevic, 1974). In addition to its importance in neurotransmission, the $GABA_A$ receptor is also the target for a number of clinically important drugs including the benzodiazepines and barbiturates [reviewed in Haefely et al. (1979), Tallman et al. (1980), Olsen (1982a), and Squires (1988)]. There is therefore considerable interest in the study of the ligand binding properties of the GABA receptor and attempts to correlate these binding characteristics with effects on receptor function.

There have been many studies of the binding of radioactive GABA and its analogues to membrane preparations from mammalian brain (Enna & Snyder, 1975, 1977; Beaumont et al., 1978; Wang et al., 1979; Olsen et al., 1981; Browner et al., 1981; Skerritt et al., 1982; Supavilai et al., 1982; Olsen & Snowman, 1982, 1983; Burch et al., 1983; Yang & Olsen, 1987). At equilibrium, a heterogeneity in the binding of GABA analogues has been observed, and models involving multiple independent sites (Wang et al., 1979; Olsen et al., 1981; Browner et al., 1981; Burch et al., 1983) or interconvertible states of a single site (Olsen et al., 1981; Olsen & Snowman, 1982, 1983; Burch et al., 1983) have been proposed. In these studies, the higher affinity component of GABA binding was characterized by a dissociation constant of 10–20 nM with one or more lower affinity components being observed in the range of 100 nM–1 μM (Olsen, 1982b; Falch & Krogsgaard-Larsen, 1982). It has not been unambiguously demonstrated that the high- and low-affinity states arise from the binding of agonist to one type of receptor protein. There is strong evidence for receptor heterogeneity, and recently, cDNAs encoding three distinct $GABA_A$ receptor α-subunits have been isolated from bovine brain (Schofield et al., 1987; Levitan et al., 1988). These α-subunits are differentially expressed in the central nervous system, and, when expressed with the β-subunit in *Xenopus* oocytes, they produced receptor subtypes which could be distinguished by their sensitivity to GABA (Levitan et al., 1988). Recently, Fuchs et al. (1988) have also reported the isolation of three α-subunits from rat brain which could be distinguished by their mobility on SDS–polyacrylamide gels. The observed heterogeneity in

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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binding of radiolabeled agonists to brain membrane preparations may therefore be due to binding to different receptor populations. It is also likely that each GABA receptor may have multiple binding sites with different affinities for agonists since the receptor, purified to apparent homogeneity, appears to carry both high- and low-affinity agonist binding sites in addition to sites for benzodiazepines (Sigel & Barnard, 1984; Schoch et al., 1984).

In electrophysiological experiments [see, e.g., Gallagher et al. (1978)] and in $^{36}Cl^-$ flux assays using brain slices (Wong et al., 1984) or isolated membrane preparations (Harris & Allan, 1985; Allan & Harris, 1986; Cash & Subbarao, 1987b), the concentrations of GABA required to elicit the chloride flux response were much higher than those required to occupy the high-affinity binding sites measured in vitro. The relationship between ligand occupancy of binding sites and effects on functional responses remains to be established. Complications in the interpretation of equilibrium binding data arise from the observations that the GABA receptor, like other neurotransmitter receptors, may desensitize upon prolonged exposure to GABA (Mathers, 1987). Thus, the binding that is measured at equilibrium is likely to be to a nonconducting state of the receptor.

The GABA receptor must therefore exist in at least three distinct conformational states, i.e., a resting state in which the channel is closed, an open-channel conducting form, and a desensitized nonconducting state. Transitions between these states are time dependent and depend on the time of exposure to the neurotransmitter. In order to identify ligand-induced conformational changes of the GABA receptor, we have studied the kinetics of binding of [^{3}H]muscimol, a GABA receptor agonist, to membrane preparations from bovine cerebral cortex.

Materials and Methods

Membrane Preparations. Membranes were prepared from bovine cerebral cortex and were well washed to remove endogenous GABA and frozen–thawed to reduce complications from cold-labile GABA reuptake sites (Enna & Snyder, 1975). Bovine brain which had been stored frozen at −80 °C was partially thawed, and strips of cerebral cortex were removed. All operations were performed on ice, and buffers were at 0–4 °C. The tissue was minced with a chilled razor blade and homogenized by using a motor-driven glass–glass homogenizer in 10 volumes of 50 mM Tris-HCl, pH 7.4, 0.32 M sucrose, 1 mM EDTA, and 0.02% NaN_3 to which had been added 0.1 mM PMSF and 0.02 mg/mL soybean trypsin inhibitor. After centrifugation for 10 min at 2500 rpm in a Sorvall GSA rotor, the supernatant was filtered through six layers of cheesecloth. The filtrate was centrifuged for 45 min at 40 000 rpm in a Beckman type 45Ti rotor, and the supernatant was discarded. The pellets were resuspended as above in 10 volumes of 50 mM Tris-HCl, pH 7.4, and 0.02% NaN_3, and the mixture was centrifuged at 40 000 rpm for 30 min. This washing procedure was repeated once, and the final pellets were resuspended in 5 volumes of 50 mM Tris-HCl, pH 7.4, and 0.02% NaN_3. The mixture was frozen overnight at −80 °C, thawed, and recentrifuged. Following resuspension to give a protein concentration of 10–20 mg/mL, the membranes were divided into aliquots and frozen at −80 °C. Immediately before use, an aliquot was thawed, diluted in the appropriate buffer, recovered by centrifugation, and washed by centrifugation at least once. Protein concentrations in the final samples were measured by the method of Lowry et al. (1951). Unless otherwise stated, the buffer used in all experiments was 50 mM Tris-HCl, pH 7.4.

Equilibrium Binding Assays. The binding of [^{3}H]muscimol (New England Nuclear) was measured in filtration assays using either a Millipore filtration manifold or a Hoefer filtration apparatus; 100-μL aliquots of membranes were added to different concentrations of [^{3}H]muscimol to give a final volume of 800 μL and a final protein concentration of 0.375–0.5 mg/mL. Nonspecific binding was measured in the presence of 10 μM unlabeled muscimol (Sigma Chemical Co.). After incubation in the dark for 60 min at 4 °C, 0.5 mL of each sample was filtered under vacuum through Whatman GF/C filters, and the filters were washed with two 4-mL volumes of cold buffer. The filters were dried, extracted overnight in 5 mL of 3a70 scintillation fluid (Research Products International), and counted for ^{3}H. Duplicate 100-μL aliquots of the incubation mixture were also removed and similarly counted for ^{3}H in order to provide estimates of the total ligand added. Equilibrium binding experiments were routinely carried out at 4 °C rather than at room temperature as used in the kinetic experiments. Preliminary kinetic experiments showed that when the membranes were incubated for longer than 30 min at room temperature there was a reduction in [^{3}H]muscimol binding activity.

Manual Kinetic Assays. To measure the kinetics of [^{3}H]muscimol association, an aliquot of membranes was rapidly diluted into the appropriate concentration of [^{3}H]-muscimol, and at various times after dilution, aliquots of 0.5 mL were removed and filtered, and the filters were washed and counted for ^{3}H as described above. For measurement of dissociation, membranes were first incubated with [^{3}H]muscimol for 15 min at room temperature (23 ± 2 °C), and dissociation was measured by filtering 0.5–1.0-mL aliquots at appropriate times after the addition of 10 μM unlabeled muscimol. Unless otherwise stated, all kinetic experiments were carried out at room temperature.

Rapid Kinetics of [^{3}H]Muscimol Dissociation. To measure dissociation on subsecond time scales, a Biologic rapid filtration system (Biologic, Meylan, France) was used as described by Dupont (1984). After formation of the complex between membranes and [^{3}H]muscimol as described above, 1.0-mL aliquots were applied to a GF/C filter mounted in the filtration apparatus, and excess buffer was removed under vacuum. Dissociation was induced by forced filtration for the desired time period either with buffer alone or with 10 μM unlabeled muscimol in buffer. For all samples, corrections were made for the results of parallel control experiments which were carried out using membranes that had been equilibrated with [^{3}H]muscimol in the presence of excess unlabeled muscimol.

Data Analysis. All equilibrium and kinetic data were analyzed by nonlinear regression techniques using the algorithm of Marquardt (1961) as previously described (Dunn et al., 1980). Parameters are expressed as the mean ± standard deviation of replicate experiments.

Equilibrium binding data were first fit directly by the general model described by Klotz (1974) in which no assumptions are made about the binding mechanism and the independence of binding sites:

$$B/R = \frac{K_{11}L + 2K_{11}K_{12}L^2}{1 + K_{11}L + K_{11}K_{12}L^2}$$

in which B/R is the number of moles of ligand bound per mole of protein and K_{11} and K_{12} are the *stoichiometric* equilibrium constants describing formation of the monoliganded and diliganded complexes, i.e.

$$R + L \rightleftharpoons RL$$
$$K_{11} = [RL]/[R][L]; \quad [RL] = K_{11}[R][L]$$

$$RL + L \rightleftharpoons RL_2$$
$$K_{12} = [RL_2]/[RL][L];\ [RL_2] = K_{12}[RL][L]$$

In this model, no distinction is made between different monoliganded species with ligand bound at different possible sites, but rather the total sum of these are considered as constituents of RL. Similarly, for RL_2, all different species with two ligands bound contribute to the concentration of RL_2.

[^{3}H]Muscimol binding data were also represented by Scatchard plots, and the equation used for curve fitting was the two-site model described by Rodbard and Feldman (1975) which assumes the presence of two distinct independent sites:

$$B/F = \tfrac{1}{2}\{(R_1 - B)/K_{d1} + (R_2 - B)/K_{d2} + \sqrt{[(R_1 - B)/K_{d1} - (R_2 - B)/K_{d2}]^2 + 4R_1R_2/K_{d1}K_{d2}}\}$$

in which B and F represent bound and free [^{3}H]muscimol, respectively, R_1 and R_2 are the concentrations of the two classes of binding sites, and K_{d1} and K_{d2} are the corresponding dissociation constants.

In all experiments of the kinetics of association, concentrations of receptor sites and [^{3}H]muscimol concentrations were chosen such that there was no appreciable depletion of added ligand during the course of binding; i.e., conditions were pseudo first order. Association data were fit by either the single-exponential equation

$$L_{bound} = A_1(1 - e^{-k_1 t}) + A_{NS}$$

or the two-exponential equation

$$L_{bound} = A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t}) + A_{NS}$$

where A_{NS} represents nonspecific binding which is unobservably fast, A_1 and A_2 are the amplitudes of the fast and slow processes, respectively, and k_1 and k_2 are the corresponding rate constants. Dissociation rates were fit by either a single-exponential equation

$$L_{bound} = A_1 e^{-k_1 t} + A_{NS}$$

or a double-exponential equation

$$L_{bound} = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_{NS}$$

Other equations are described in the text and figure legends.

Results

Equilibrium Binding of [^{3}H]Muscimol to Bovine Brain Membranes. The equilibrium binding of [^{3}H]muscimol to membranes from bovine cerebral cortex does not follow a simple binding isotherm. As shown in Figure 1A, specific binding did not have a simple hyperbolic dependence on ligand concentration, and a fit of the data by the general model previously described (Klotz, 1974) yielded values for the stoichiometric constants describing formation of monoliganded and diliganded complexes of 9.88×10^7 M^{-1} for K_{11} ($1/K_{11}$ = 10.1 nM) and 4.46×10^6 M^{-1} for K_{12} ($1/K_{12}$ = 0.22 μM). A Scatchard plot of these data is shown in Figure 1B and is clearly biphasic, suggesting either that there are multiple binding sites with different affinities (Klotz & Hunston, 1971) or that binding is negatively cooperative (Koshland, 1970). If one makes the assumption that binding is to noninteracting sites, these data may be described by two components: a high-affinity site with a K_d of 7 nM and a density of 3.5 pmol/mg and a low-affinity site with a K_d of 300 nM and an apparent density of 6.2 pmol/mg. It should be emphasized that these latter values are subject to large error since the maximum ligand concentration that can reasonably be used in these experiments is about 500 nM and thus the low level of occupancy of low-affinity sites precludes accurate measurement of binding site parameters. The assumption of independent sites is implicit in the Scatchard model (Scatchard, 1949), and although an apparently good fit of the Scatchard plot to a two-site model was obtained, many different binding mechanisms may be responsible for the observed curvature. These mechanisms cannot be discriminated on the basis of equilibrium binding data alone since, for example, different conformational states of the receptor cannot be detected by such measurements. An equilibrium binding study will detect only one apparent K_d value for all monoliganded complexes and one for the diliganded complexes etc. [see Connors (1987)]. Decomposition of a curved Scatchard plot to give valid microscopic dissociation complexes therefore requires further information on the nature of the binding mechanism.

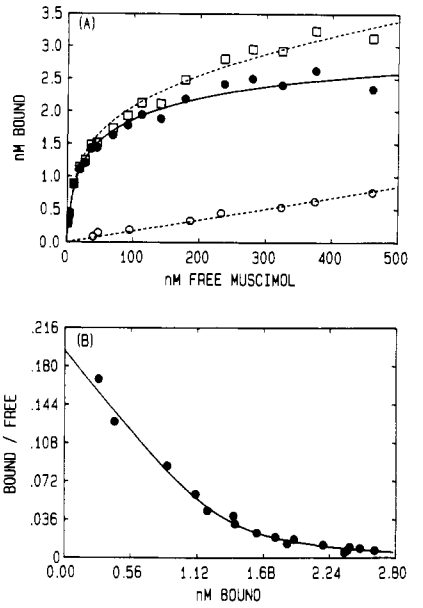

FIGURE 1: Equilibrium binding of [^{3}H]muscimol to bovine brain membranes. Membranes (0.375 mg of protein/mL) were incubated with various concentrations of [^{3}H]muscimol for 30 min at 4 °C after which bound and free ligands were separated by filtration. (A) Estimation of stoichiometric equilibrium constants for [^{3}H]muscimol binding. Binding was measured in the absence (□) or presence (○) of 10 μM unlabeled muscimol, allowing estimation of specific binding. Specific binding (●) was fit by the equation $B = R(K_1L + 2K_1K_2L^2)/(1 + K_1L + K_1K_2L^2)$. Best-fit values were R = 1.53 nM, $K_{11} = 9.88 \times 10^7$ M^{-1}, and $K_{12} = 4.46 \times 10^6$ M^{-1}. (b) Representation of equilibrium binding data [same data as in (A)] in a Scatchard plot. Data were fit by the two-site model described in the text, and the best-fit values were K_{d1} = 7 nM, R_1 = 1.31 nM (3.5 pmol/mg), K_{d2} = 300 nM, and R_2 = 2.32 nM (6.2 pmol/mg).

Specificity of [^{3}H]Muscimol Binding for GABA$_A$ Receptors. The specificity of the observed high-affinity [^{3}H]muscimol binding for GABA$_A$ receptors has been demonstrated by the ability of unlabeled muscimol, GABA, and the specific GABA$_A$ receptor antagonist bicuculline to displace all of the bound labeled ligand. Examples of such competition curves carried out at a [^{3}H]muscimol concentration of 2 nM are illustrated in Figure 2. Similar experiments carried out at a [^{3}H]muscimol concentration of 200 nM have demonstrated that the low-affinity binding component is also displaced by bicuculline and GABA (data not shown).

Kinetics of [^{3}H]Muscimol Binding to Bovine Brain Membranes. The kinetics of 5 nM [^{3}H]muscimol binding to brain membrane preparations are shown in Figure 3. The observed rate of dissociation is not fit with precision by a single-exponential process but rather is better fit by a model involving two exponential phases. The apparent rate constants obtained for the fast and slow phases were 0.068 and 0.011 s^{-1}, re-

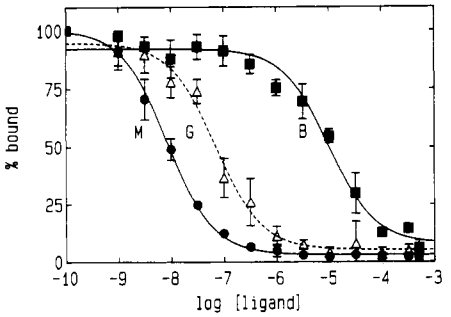

FIGURE 2: Effects of unlabeled muscimol (M), GABA (G), and bicuculline (B) on the binding of [^{3}H]muscimol to bovine brain membranes. Membranes (0.5 mg of protein/mL) were incubated with 2 nM [^{3}H]muscimol and various concentrations of the other ligands for 60 min at 4 °C, after which bound radiolabel was estimated by filtration. Data shown are average values obtained from experiments using three different membrane preparations, and error bars represent the standard deviation of the replicates. Data were fit by an equation assuming a single population of binding sites: % bound = (total − NS)/(1 + [I]/IC_{50}) + NS where total is the total bound, NS is the nondisplaceable component, and [I] is the concentration of the competing ligand. Best-fit values for IC_{50} were 8.5 nM (M), 71.1 nM (G), and 9.9 μM (B).

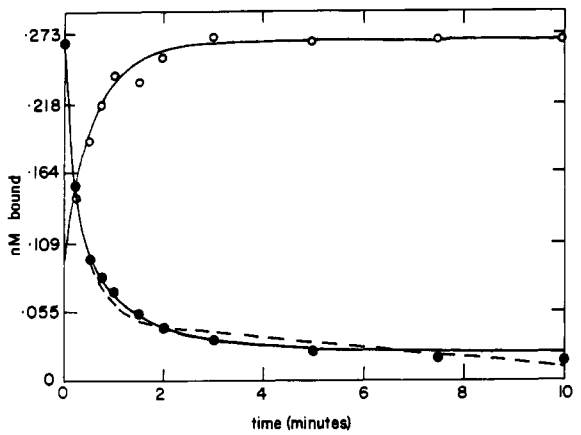

FIGURE 3: Kinetics of binding of [^{3}H]muscimol (5 nM) to brain membranes (0.5 mg of protein/mL) measured by using manual filtration assays at 25 °C. Association data were biphasic, but the fast phase was complete by the first measured time point (15 s). These data were fit by a single-exponential equation, giving A_1 = 0.183 nM and k_1 = 0.029 s^{-1}. Dissociation was initiated by the addition of 10 μM unlabeled muscimol to membranes and [^{3}H]muscimol which had been equilibrated for 15 min at room temperature (23 °C). Data were fit either by a single-exponential model (dashed line), giving A_1 = 0.213 nM and k_1 = 0.046 s^{-1}, or by a two-exponential model (solid line), giving A_1 = 0.162 nM, k_1 = 0.068 s^{-1}, A_2 = 0.0817 nM, and k_2 = 0.011 s^{-1}.

spectively. Similar experiments have been carried out using five different membrane experiments, and average values were 0.06 ± 0.02 and 0.008 ± 0.003 s^{-1} with the magnitude of the displacement occurring in the faster phase representing 66 ± 7% of the total displaceable [^{3}H]muscimol. The association reaction was also more complex than would be expected for a simple bimolecular association. A fraction of the binding appeared to be complete by the first measurable time point (15 s), and this was followed by a slower exponential process with an apparent rate constant of 0.029 s^{-1} (0.03 ± 0.01 s^{-1}, n = 6). It seems, therefore, that association is also biphasic but that the first phase is unobservably fast in these filtration assays.

At first sight, these experiments appear to yield the expected kinetic pattern; i.e., the observation of two kinetic components is consistent with the equilibrium evidence (Figure 1) for the existence of two classes of binding sites. However, there is

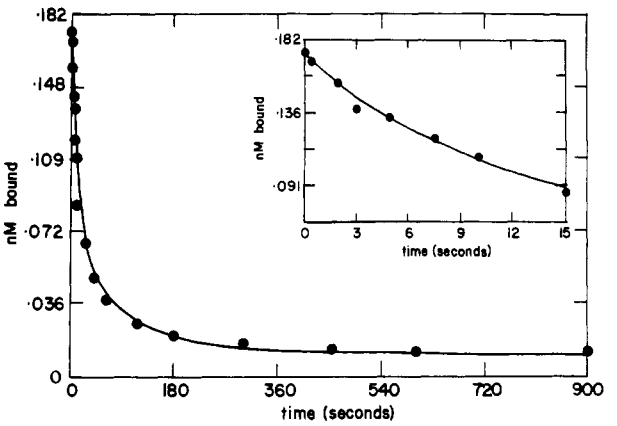

FIGURE 4: Dissociation of [^{3}H]muscimol measured by a combination of manual filtration assays and an automated rapid kinetic technique. Membranes (0.5 mg of protein/mL) were equilibrated with 10 nM [^{3}H]muscimol for 15 min at 23 °C. For dissociation times less than 10 s, 1-mL samples were removed and filtered for the desired dissociation time with buffer containing 10 μM unlabeled muscimol as described in the text. The extent of dissociation at longer times was measured by initiating dissociation by the addition of 10 μM unlabeled muscimol and filtering 1-mL samples at various time intervals. The data obtained by using the two techniues were used to construct a composite curve. The solid line is a best fit to a two-exponential process, giving A_1 = 0.092 nM, k_1 = 0.12 s^{-1}, A_2 = 0.059 nM, and k_2 = 0.027 s^{-1}. The inset shows an expansion of the data in the short time region.

an important quantitative discrepancy between the kinetic and the equilibrium data. At the concentration of [^{3}H]muscimol used in the kinetic experiments (5 nM), no appreciable occupancy of the low-affinity sites (K_d = 300 nM) would be expected. These results therefore indicate a heterogeneity in [^{3}H]muscimol binding at low ligand concentration.

Automated Rapid Filtration Technique To Study [^{3}H]-Muscimol Dissociation. One of the problems inherent in using manual filtration assays to measure the kinetics of radiolabeled ligand binding is the lack of time resolution. Realistically, the first time point cannot be made less than 10 s following the initiation of association or dissociation. Washing of the filters requires an additional 5 s, and during this time, some loss of bound ligand from rapidly dissociating sites is inevitable. In Figure 3, for example, the half-time for the dissociation of the faster component was 10.2 s, and therefore more than half of this component would have dissociated by the end of the first measurement. We have therefore used a rapid filtration technique (Dupont, 1984) to measure dissociation on time scales between 100 ms and 10 s. A combination of these rapid measurements and the results from manual filtration assays have facilitated the quantitative analysis of the entire time course of ligand dissociation as shown in Figure 4.

Effect of [^{3}H]Muscimol Concentration on the Kinetics of Dissociation. The kinetics of dissociation have been measured over a [^{3}H]muscimol concentration range of 1–300 nM. At all concentrations, dissociation appeared to be biphasic. The total amplitude was qualitatively consistent with the results obtained in the equilibrium binding experiments. This suggests that the increase in temperature from 4 °C used in the equilibrium experiments to 23 °C used in the kinetic experiments did not cause a major perturbation in the binding mechanism. Direct fitting by the general model described under Materials and Methods gave values for the stoichiometric equilibrium constants of 8.11 × 10^7 M^{-1} for K_{11} and 3.78 × 10^5 M^{-1} for K_{12} (data not shown). A Scatchard plot constructed from the kinetic amplitude data was biphasic, suggesting two classes of sites with apparent K_d values of 15.8 and 825 nM (Figure 5A). However, the amplitudes of the

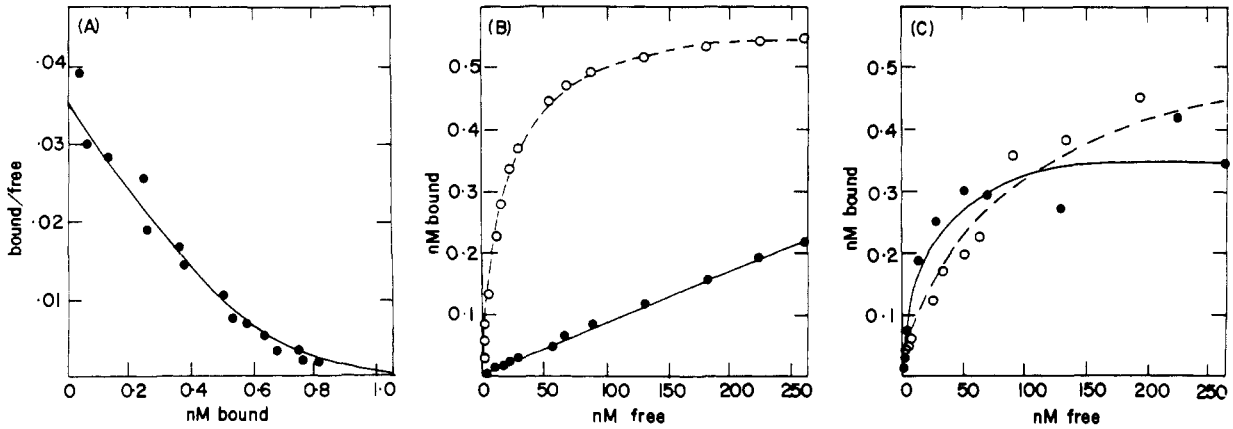

FIGURE 5: Effects of [^{3}H]muscimol concentration on the amplitudes of the fast and slow phases of ligand dissociation (23 °C). Data were obtained from composite dissociation curves as shown in Figure 4. The total amplitude measured in the kinetic experiments was used to construct a Scatchard plot (A). Values obtained for the best-fit parameters were K_{d1} = 15.8 nM, R_1 = 0.58 nM, K_{d2} = 825 nM, and R_2 = 0.88 nM. These data were used to calculate theoretical values for the amplitudes of the fast and slow phases (B) which would be predicted if the two phases were due to dissociation from independent sites of low and high affinity suggested from the curvilinear Scatchard plot. In (C), the actual concentration dependence of the amplitudes of the fast (●) and slow phases (○) is shown. Solid lines were calculated from the best-fit parameters obtained from curve fitting of the data obtained at [^{3}H]muscimol concentrations less than 75 nM by the equation $B = R_0[L]/(K_d + [L])$. From the fast-phase amplitude data (●), R_0 and K_d were estimated to be 0.37 nM and 11.8 nM, respectively. Corresponding values for the slow-phase data (○) were 0.505 nM and 82 nM.

two kinetic components were not consistent with the extent of occupancy predicted for a model in which there are independent binding sites of low and high affinity. Figure 5B shows theoretical curves calculated for the magnitudes of the two phases of dissociation assuming independent sites and that the observed fast and slow phases arise from dissociation from the putative low- and high-affinity sites, respectively. The actual data are shown in Figure 5C and demonstrate clear discrepancy from this simple model. Biphasic dissociation was observed even when it is expected that only the high-affinity sites would be occupied. The simplest explanation for these data is that dissociation is being observed from two different complexes (Scheme I) where $k_{-1} > k_{-2}$; i.e., dissociation from RL represents the faster of the two phases observed in the dissociation kinetics. These data alone do not allow distinction between the possibilities that R and R* represent two different receptors, two distinct sites on the same receptor, or two conformations of a single receptor site. From the relative amplitudes of the two phases, it would, however, appear that the two species are present in approximately equal amounts. Another interesting observation from the present data is that the midpoint in the titration of the amplitude of the faster dissociating component occurs at a lower ligand concentration than that of the slower component (Figure 5C). It appears therefore that, contrary to intuition, the faster dissociating species represents the complex with higher affinity.

Scheme I

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL \qquad K_{d1} = k_{-1}/k_1$$

$$R^* + L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R^*L \qquad K_{d2} = k_{-2}/k_2$$

The rates of the two phases of dissociation have been measured over a range of muscimol concentrations and were found to be independent of whether dissociation was initiated by dilution alone or by the addition of an excess of unlabeled muscimol (data not shown). The rate of the fast phase was independent of concentration, and an average value of 0.057 ± 0.021 s^{-1} at 23 °C was obtained. The slow phase rate was also independent of ligand concentration below 100 nM and had an average value of 0.010 ± 0.006 s^{-1}. Corresponding values obtained from experiments at 4 °C were 0.044 ± 0.023 and 0.007 ± 0.003 s^{-1}. In agreement with the amplitude data described above, the similarity of the dissociation rates measured at two temperatures suggests that temperature changes do not profoundly affect the reaction mechanism. An obvious alteration in the dissociation kinetics was apparent at higher ligand concentrations, where the slow phase became increasingly difficult to measure due to an apparent reduction in the amplitude. This is discussed further below.

Kinetics of [^{3}H]Muscimol Association. At low ligand concentrations, the kinetics of [^{3}H]muscimol association are also biphasic, but the faster component is "lost" due to the lack of time resolution of the filtration assays (see Figure 3). Unfortunately, the rapid filtration technique is not suited to measurements of the kinetics of association since the technique precludes washing of the filters and the result is a high background due to unbound radiolabel trapped within the filter. Data obtained for the association kinetics are therefore limited to the slower component and only to the low concentration region where the time scales involved are accessible to measurement by manual filtration assays. The rate of the slower component increased linearly with ligand concentration as shown in Figure 6A, suggesting a simple bimolecular association:

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL$$

Under pseudo-first-order conditions, such a model predicts that the observed rate constant, k_{app}, will increase with ligand concentration, [L], according to

$$k_{app} = k_1[L] + k_{-1}$$

In data pooled from three separate experiments, values obtained for k_1 and k_{-1} were (3.6 ± 0.9) × 10^6 M^{-1} s^{-1} and 0.022 ± 0.004 s^{-1}, respectively, giving an estimated dissociation constant (k_{-1}/k_1) of 6 ± 2 nM. Unfortunately, the errors inherent in such kinetic measurements preclude the ready correlation of this process with either the fast phase (k_{-1} = 0.057 ± 0.021 s^{-1}) or the slow phase (k_{-1} = 0.01 ± 0.06 s^{-1}) of dissociation measured directly. Additional information may, however, be obtained from studying the magnitudes of the different kinetic processes.

Although the faster phase of association could not be measured directly, it was possible to obtain some information

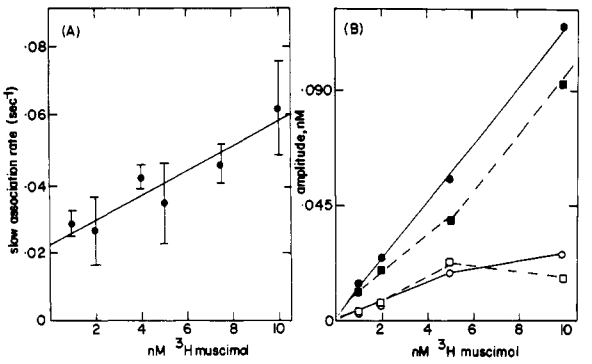

FIGURE 6: (A) Effect of [^{3}H]muscimol concentration on the slow rate of association. Data were obtained by using manual filtration assays at 23 °C as described in Figure 4 except that the protein concentration was 0.1–0.2 mg/mL to reduce the extent of depletion of added ligand; i.e., conditions were chosen to be pseudo first order. Data were pooled from three different preparations of bovine brain membranes. Assuming a simple bimolecular associaton and that $k_{app} = k_1[L] + k_{-1}$, estimates for k_1 of 3.6×10^6 M^{-1} s^{-1} and for k_{-1} of 0.022 s^{-1} were obtained from the slope and intercept, respectively, of a linear least-squares fit. (B) Effect of [^{3}H]muscimol concentration of amplitudes of the fast (○) and slow phase (●) of association compared to amplitudes of the fast (■) and slow (□) phase of dissociation. Data were obtained from experiments as in Figures 3 and 4, and the protein concentration was 0.5 mg/mL.

regarding its amplitude from the extrapolation of the slow component to $t = 0$ and using appropriate corrections for nonspecific binding. Figure 6B compares the amplitudes of the two association phases with those of the two dissociation processes. Clearly, the amplitude of the faster phase of dissociation displays a similar concentration dependence to the slower rate of association. This suggests that these processes may be coupled, and in terms of Scheme I above

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL$$

where k_1 has been estimated to be $(3.6 \pm 0.9) \times 10^6$ M^{-1} s^{-1} (Figure 6A) and k_{-1} has been estimated from direct measurement of the faster dissociation rate as 0.057 ± 0.021 s^{-1}, giving $K_{d1} = 16 \pm 7$ nM. Further support for the validity of making such estimates from the kinetic data comes from a direct fit of the amplitude of the faster dissociation to a simple binding isotherm which gave an estimated K_{d1} of 12 nM (Figure 4C). In this analysis, only data obtained at [^{3}H]-muscimol concentrations less than 75 nM were used to reduce complications arising from occupancy of the lower affinity binding sites.

At low concentrations of [^{3}H]muscimol, there was also good agreement between the magnitudes of the slower dissociation and faster association phases, implicating their involvement in the formation of R*L in Scheme I. An apparent dissociation constant for this binding may again be estimated from the concentration dependence of the magnitude of the dissociation phase, and an estimate of 82 nM for K_{d2} was obtained. Since the rate of association could not be measured directly (see above), it is not possible to estimate the dissociation constant from the rate data. However, it appears that a simple bimolecular association, which adequately describes the formation of RL, is not sufficient to describe the binding of [^{3}H]muscimol to R*. The dissociation rate has been directly measured as 0.010 ± 0.006 s^{-1} at 23 °C. Assuming that complex formation is a simple bimolecular reaction, the association rate constant can be estimated from k_{-2}/K_{d2} to be 1.8×10^5 M^{-1} s^{-1}. Such a rate should readily be measurable by using the manual filtration assays employed in this study. However, since this association was too rapid to be observed experimentally, it must be concluded that the actual binding step is more complex. A possible explanation is that rapid binding is followed by a fast conformational transition to the equilibrium R*L complex:

$$L + R^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (R^*L)' \underset{k_{-2}'}{\overset{k_2'}{\rightleftharpoons}} R^*L$$

giving $K_{d2} = K_2K_2'/(1 + K_2')$ where $K_2 = k_{-2}/k_2$ and $K_2' = k_{-2}'/k_2'$. In general, this model predicts that the rate of ligand association displays a complex dependence on ligand concentration (Strickland et al., 1975), but if one makes a simplifying assumption that the intermediate (R*L)′, if it exists, is not significantly populated, then the apparent association rate constant (k_{app}) may be approximated by $k_2k_2'/(k_{-2} + k_2')$ (King & Burgen, 1976). In the limiting case where $k_2' \gg k_{-2}$, this reduces to $k_{app} = k_2$ which is the diffusion-limited rate constant. However, in the other limiting case where $k_2' \ll k_{-2}$, then $k_{app} \approx k_2'/K_2$; i.e., the effective forward rate depends on both the affinity constant for the intermediate complex and the rate of the isomerization to R*L.

Kinetics at High [^{3}H]Muscimol Concentration. At concentrations of [^{3}H]muscimol above about 15 nM, the rate of the slower phase of association also became too rapid to measure since maximum binding was reached within the first 15 s. However, as the concentration was increased above 100 nM, a slower component of rate approximately 0.06 s^{-1} was observed (Figure 7A). This phase was difficult to analyze quantitatively both because of its small amplitude relative to the total bound ligand in this concentration region and because of the technical difficulties inherent in measuring low specific binding with a large excess of free ligand. However, within the limitations described, it appears that the rate of this phase is virtually independent of ligand concentration although its amplitude did seem to increase with concentration. These findings, in addition to the concentration range in which the slow phase becomes apparent, suggest that this process represents a rate-limiting conformational transition accompanying ligand occupancy of lower affinity sites.

At high ligand concentration (>200 nM), the overall rate of complex dissociation appeared to *increase* as shown in Figure 7B. The experiment depicted in this figure was carried out at 4 °C where the slower rates involved better illustrate this phenomenon. Similar results have been obtained at room temperature, and although it is again difficult to rigorously characterize kinetic parameters at such high ligand concentration, it appears that the change in the kinetics is due to an increase in the rate of the slower phase of dissociation, resulting in its becoming indistinguishable from the faster phase. This suggests that occupancy of low-affinity sites accelerates the rate of ligand dissociation from R*L.

Discussion

Previous measurements of the binding of GABA receptor agonists to membrane preparations from mammalian brain have revealed a heterogeneity in binding (Enna & Snyder, 1975; Beaumont et al., 1978; Wang et al., 1979; Olsen et al., 1981; Browner et al., 1981; Olsen & Snowman, 1982; Yang & Olsen, 1987). In general, when binding to native membrane preparations is investigated, it is difficult to determine whether the observed high- and low-affinity binding components represent specific interactions with the one type of receptor protein or arise from the presence of different receptor species. There is abundant evidence for the existence of multiple types of GABA/benzodiazepine receptors (Bowery et al., 1984), although in most ligand binding studies the observed binding has displayed the pharmacological specificity of $GABA_A$ re-

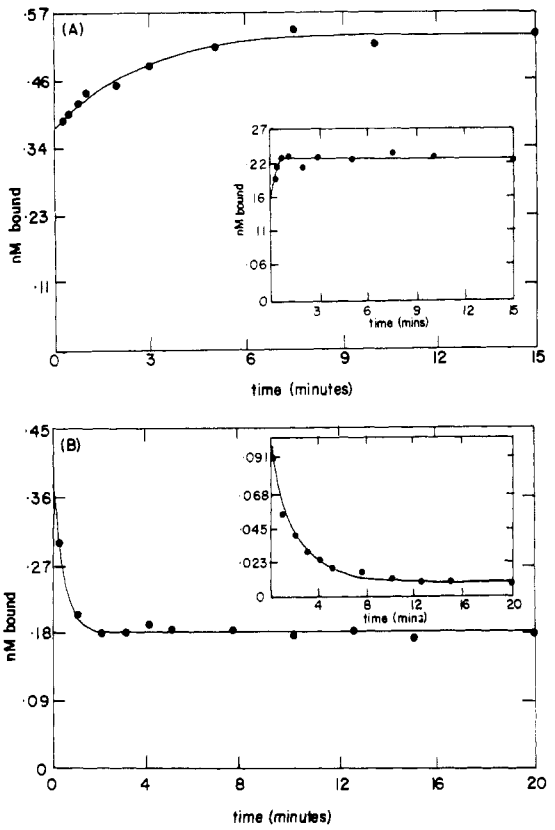

FIGURE 7: Kinetics of [^{3}H]muscimol binding at high ligand concentration. (A) Association of 250 nM [^{3}H]muscimol with bovine brain membranes at 23 °C. The solid line is fit by a one-exponential model, giving A_1 = 0.31 nM and k_1 = 0.0063 s^{-1}. This figure shows a slow phase in association that is observable only at high (>100 nM) ligand concentration. The inset shows data obtained at a [^{3}H]muscimol concentration of 30 nM, showing that at this concentration association reactions are unobservably fast. (B) Increase in the rate of dissociation observed at a [^{3}H]muscimol concentration of 300 nM. Kinetic parameters from a single-exponential fit were A_1 = 0.415 nM and k_1 = 0.0378 s^{-1}. The inset shows dissociation from the complex formed with 10 nM [^{3}H]muscimol for comparison. Best-fit values for a two-exponential model were A_1 = 0.11 nM, k_1 = 0.039 s^{-1}, A_2 = 0.10 nM, and k_2 = 0.00546 s^{-1}. In this experiment, the temperature was 4 °C. Similar results are observed at room temperature.

ceptors [see, e.g., Olsen et al. (1981)]. Muscimol is a very potent agonist of the $GABA_A$ receptor (Krogsgaard-Larsen et al., 1979) and is also a poor substrate for GABA reuptake mechanisms (Johnston, 1976). It is likely therefore that the binding of [^{3}H]muscimol is specific for $GABA_A$ receptor sites.

Recently, Levitan et al. (1988) reported the isolation of cDNAs encoding three distinct but homologous α-subunits of the bovine brain $GABA_A$ receptor. Coexpression of any one of these subunits with the β-subunit in *Xenopus* oocytes produced receptor subtypes which displayed different sensitivities to GABA, with half-maximal doses varying from 1.3 to 42 μM. As previously discussed (Levitan et al., 1988), the differences in GABA sensitivity could be due to changes in affinity for GABA, changes in the coupling between GABA binding and channel gating, or changes in the rates of desensitization. The relationship between the receptor subtypes expressed in oocytes and native receptors remains to be established. However, since it is likely that different receptor subtypes exist in bovine cerebral cortex, the simplest explanation for the present observations of kinetically distinguishable receptor–agonist complexes may be that they arise from receptor heterogeneity.

In addition to multiple receptors, there are also several lines of evidence to support the notion that one receptor complex carries both high- and low-affinity sites. The $GABA_A$ receptor has been purified to apparent homogeneity from bovine brain (Sigel et al., 1983; Sigel & Barnard, 1984; Schoch et al., 1984). The purified protein which displayed two major subunits of M_r 53 000 and 57 000 when analyzed by SDS–PAGE electrophoresis (Sigel et al., 1983) retained the ability to bind [^{3}H]muscimol with high affinity. Lower affinity sites were also detected in direct [^{3}H]muscimol binding studies (Sigel & Barnard, 1984; Sigel et al., 1985), and, in addition, their presence was inferred from the finding that micromolar concentrations of GABA were necessary to modulate the equilibrium binding of benzodiazepines (Sigel & Barnard, 1984; Schoch et al., 1984), an observation that is consistent with earlier studies using native membrane preparations (Tallman et al., 1978). The purified protein therefore appears to carry both high- and low-affinity sites although the functional significance of multiple sites is unclear.

Equilibrium binding studies of neurotransmitter receptors that undergo desensitization suffer from the disadvantage that they yield information only on the properties of the inactive desensitized state. In several preparations, the $GABA_A$ receptor has been shown to desensitize upon exposure to agonists. This desensitization has been observed in some but not all electrophysiological studies [see Mathers (1987) and Cash and Subbarao (1987a)], in synaptoneurosome preparations (Schwartz et al., 1986a,b; Dunn et al., 1989b), in reconstituted membrane preparations (Dunn et al., 1989a), and in brain homogenates (Cash & Subbarao, 1987a,b). It is unlikely, therefore, that the affinity of the receptor for muscimol at equilibrium is the same as in the resting state. The chloride channel is an integral part of the GABA receptor protein (Schofield et al., 1987), and since protein conformational changes must be involved in the opening and closing of this ion channel, it is likely that the bimolecular association of GABA with its receptor is followed by functionally important transitions.

In the present study, we have investigated the binding of [^{3}H]muscimol in both equilibrium and kinetic experiments. In agreement with other authors, a heterogeneity in equilibrium binding was observed. As discussed under Results, it is not possible to distinguish among the many potential models involving multiple sites or interconvertible states on the basis of equilibrium data alone. However, when these results are considered along with specific kinetic information, some predictions may be made about the underlying molecular mechanisms. An important clue into the nature of the binding mechanism came from the observation that association and dissociation reactions were biphasic even under conditions of low levels of receptor occupancy. Quantitative analysis of these different phases has suggested that there are two forms of the monoliganded receptor (Scheme I). Surprisingly, it was found that the tighter binding complex (RL) is associated with the slower phase measured in the association kinetics (k_1 = 3.66 × 10^6 M^{-1} s^{-1}) and is also the faster dissociating species (k_{-1} = 0.022–0.057 s^{-1}). Resolution of this faster phase has been greatly facilitated by using an automated rapid filtration technique to measure dissociation on subsecond time scales, thus significantly improving the time resolution compared to previous studies (Yang & Olsen, 1987). The equilibrium constant for the tighter binding complex (K_{d1}) could be estimated both from the rate data (k_{-1}/k_1 = 6–16 nM) and from the direct fit of the amplitude of the faster phase of dissociation (12 nM; Figure 5). Less information is available about the parameters characterizing the R*L complex since, although the dissociation rate constant could be directly measured as 0.010 ± 0.006 s^{-1} (23 °C), the association rate was too rapid

to be measured in manual filtration assays. The overall dissociation constant of this complex could be estimated to be 82 nM from the amplitude of the slower phase of dissociation (Figure 5). This estimate is, however, subject to error because of subsequent lower affinity binding.

From the kinetic amplitude data, it appears that, in terms of Scheme I, R and R* are present in approximately equal amounts. This raises the possibility that, rather than representing distinct receptors, R and R* may represent two sites on the one receptor. Under these circumstances, a value for K_{d2} may also be estimated from the stoichiometric equilibrium constant, K_{11}, since this is related to the microscopic dissociation constants describing formation of the monoliganded species by

$$K_{11} = 1/K_{d1} + 1/K_{d2}$$

(Connors, 1987). Using measured values of 8.11×10^7 M^{-1} for K_{11} and 16 nM for K_{d1}, K_{d2} may be calculated to be 54 nM, i.e., in reasonable agreement with the estimate from the amplitude data above. Further studies using purified protein are required to determine if the two monoliganded complexes identified in the present study are due to binding to two receptor subunits or to independent receptor molecules.

In addition to the two relatively high-affinity complexes described above, the present data also suggest the presence of a lower affinity component of binding. Due to the problems inherent in measuring such low-affinity binding, these sites are more difficult to characterize. However, a study of the kinetics of [^{3}H]muscimol association at high ligand concentration (Figure 7A) has provided evidence for a slow conformational transition ($k_{app} \approx 0.06$ s^{-1}) accompanying occupancy of low-affinity sites. Another obvious change at high ligand concentration was that the overall rate of dissociation became faster (Figure 7B). Within experimental error, it was not possible to detect a new phase in the dissociation kinetics due to dissociation from the low-affinity sites, and at high ligand concentration, the dissociation kinetics could be fit by a single-exponential process. This change from the biphasic kinetics observed at lower concentration appeared to be due to an increase in the rate of the slower phase (suggested to be due to dissociation from R*L in Scheme I) so that this could not be distinguished from the more rapid process. This suggests that there must be some coupling between R*L and occupancy of low-affinity sites. This is immediately suggestive of negative cooperativity, i.e., a decrease in affinity with increasing site occupancy. However, there is an inconsistency with such a mechanism in that identical dissociation kinetics were observed when dissociation was triggered by dilution alone or by addition of an excess of unlabeled muscimol. Thus, the mechanism underlying site–site interaction must be more complex, and a more detailed description of the kinetics of [^{3}H]muscimol binding at high ligand concentration, in addition to further information on the stoichiometry of binding sites in the purified protein, will be required to elucidate the molecular basis for such apparently disparate results.

While this work was in progress, Yang and Olsen (1987) reported the results of their studies of [^{3}H]muscimol binding to mouse brain membrane preparations. These authors also observed a biphasicity in the association and dissociation kinetics at all ligand concentrations. However, there are several inconsistencies between the results obtained in the present study and the earlier report. In contrast to our results, Yang and Olsen did not find a correspondence between the amplitudes of the two phases of association and dissociation. They also observed significant changes in [^{3}H]muscimol binding with changes in temperature from 0 to 23 °C and an increase in the rate of dissociation when this was initiated by addition of excess cold muscimol rather than dilution alone. Neither of these effects was observed in the present study. A further discrepancy is that in the earlier work the rate of the slower phase of association was virtually independent of [^{3}H]muscimol concentration between 5 and 100 nM, an effect that we did not observe until the appearance of the slow phase at higher ligand concentrations. A major difference between the two studies is that Yang and Olsen used fresh brains and membrane preparations that had never been frozen, in contrast to the frozen–thawed preparations of membranes from frozen bovine brain that we used here. It remains to be established whether the conflicting results arise from the different membrane preparations or are a result of a species difference.

The $GABA_A$ receptor is formed by two subunits, α (apparent M_r 53 000) and β (apparent M_r 57 000), in an apparent stoichiometry $\alpha_2\beta_2$ (Schofield et al., 1987). Photoaffinity labeling experiments have demonstrated that the benzodiazepine [^{3}H]flunitrazepam labels primarily the α-subunits (Sigel et al., 1983; Schoch et al., 1984) although labeling of other subunits has also been observed [reviewed in Sieghart (1988)]. Recently, it has been shown that [^{3}H]muscimol labels mainly the β-subunits (Casalotti et al., 1986). However, Blair et al. (1989) have recently reported that individual α- and β-subunits, when expressed separately in *Xenopus* oocytes, can form GABA-sensitive ion channels with retention of many of the regulatory properties of the native receptor. This suggests that both α- and β-subunits carry binding sites for agonists and other ligands, a notion that is supported by the sequence homology of the subunits (Schofield et al., 1987).

There is electrophysiological evidence to suggest that opening of the $GABA_A$ receptor ion channel requires the simultaneous binding of two agonist molecules (Sakmann et al., 1983), and this has led to the suggestion that there is (at least) one GABA binding site on each β-subunit (Casalotti et al., 1986). From their studies of the kinetics of channel activation and desensitization of the $GABA_A$ receptor in brain homogenates, Cash and Subbarao (1987b) have suggested that these two processes are mediated by different binding sites. Since each process seems to require the binding of two agonist molecules, it has been suggested that each $GABA_A$ receptor may carry four binding sites for agonists. This is an intriguing possibility in view of the evidence for the existence of multiple agonist binding sites in the nicotinic acetylcholine receptor and the likelihood that channel activation and desensitization are mediated by different sites (Dunn & Raftery, 1982a,b; Raftery et al., 1983). The present study of ligand binding to the $GABA_A$ receptor represents a first step toward the elucidation of some of the molecular mechanisms underlying the functional responses of this receptor.

Registry No. Muscimol, 2763-96-4.

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