Modulation of Glutamate-Induced Uncompetitive Blocker Binding to the NMDA Receptor by Temperature and by Glycine†

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ABSTRACT: The effect of temperature on the binding of [^{3}H]-*N*-[1-(2-thienyl)cyclohexyl]piperidine ([^{3}H]TCP) to the ion channel of the *N*-methyl-D-aspartate (NMDA) receptors was studied in washed rat brain-cortex membranes. Raising the temperature from 5 to 33 °C resulted in a significant increase in the association rates of [^{3}H]TCP binding measured in the presence of 1 μM glutamate and 1 μM glycine, but was less effective in the absence of the added agonists. No such effects of temperature on the dissociation rates of [^{3}H]TCP–receptor complexes were observed. In the absence of agonists, neither the association nor the dissociation binding components varied with temperature, suggesting a diffusion-controlled limitation of access of the ligand to its site within the nonactivated NMDA receptor. No evidence was found for a temperature-dependent change in the density of [^{3}H]TCP binding sites or for heterogeneity of [^{3}H]TCP binding sites associated with the NMDA receptor, even though when approaching equilibrium the binding kinetics in the presence of glutamate and glycine deviated from an ordinary bimolecular reaction scheme. The data were fitted instead to a two-exponent binding function, comprising the sum of a fast and a slow binding component. Their corresponding time constants exhibited an increase with temperature, and the increase of each one was correlated significantly with the corresponding decrease in the equilibrium binding constant; however, there was no temperature-related change in the relative proportions of the two components, with the fast binding component (α) accounting for 50–70% of the site population. Varying glutamate concentrations (without glycine) resulted in corresponding dose-dependent increases of both the fast and the slow time constants, with no change in α (28–35%). Glycine increased α from its glutamte-induced level to 60%, but did not change the fast and slow time constants. The results suggest homogeneity of [^{3}H]TCP-binding domains within the NMDA receptor channel but variability of total channel opening time. The observed effects of glutamate and of glycine on the kinetic components are consistent with this suggestion.

The *N*-methyl-D-aspartate (NMDA)[1] class of excitatory amino acid receptors (Watkins & Evans, 1981) has recently attracted much interest as a result of studies that pointed to their involvement in long-term potentiation (Collingridge & Bliss, 1987) and neuronal plasticity (Collingridge, 1987; Kleinschmidt et al., 1986) on the one hand and pathological neuronal degeneration (Choi, 1988; Olney & Sharpe, 1969; Simon et al., 1984) and epileptic seizures (Turski et al., 1985) on the other. This class of excitatory amino acid receptors corresponds to ligand-activated cation channels that conduct monovalent ions and Ca^{2+} (McDermott et al., 1986; Ascher et al., 1988; McDermott & Dale, 1987). Endogenous amino acids such as L-glutamate and L-aspartate and agonists such as NMDA trigger channel opening, which is potentiated by the amino acid glycine acting as an allosteric effector of the NMDA receptor (Johnson & Ascher, 1987). Glycine increases the frequency of channel openings (Johnson & Ascher, 1987) and reduces desensitization of the receptor by increasing the rate of its recovery from a desensitized state (Mayer et al., 1989). The NMDA-receptor ion channel is blocked in a voltage-dependent manner by extracellular Mg^{2+} (McDermott et al., 1986; Ascher et al., 1988; Mayer et al., 1984) as well as by the anticonvulsant drug MK-801 and the dissociative anesthetics phencyclidine (PCP) and related drugs (Anis et al., 1983; Honey et al., 1985; Huettner & Bean, 1988). Zn^{2+} also inhibits NMDA receptors but acts at a site distinct from the agonist, the glycine, or the Mg^{2+} and PCP sites (Westbrook & Mayer, 1987).

While it is not known whether the psychotropic effects of PCP-like drugs (Domino & Luby, 1981; Contreras et al., 1986) are associated with the NMDA receptor, the evidence indicates that these drugs have a neuroprotective action (Choi, 1988; Woodruff et al., 1987) and act by blocking the open state of the NMDA-receptor ion channel (Anis et al., 1983; Honey et al., 1985; Huettner & Bean, 1988). Biochemical experiments have demonstrated that NMDA-receptor agonists enhance the binding of [^{3}H]TCP (Loo et al., 1986; Bonhaus et al., 1987; Johnson et al., 1988; Snell et al., 1987) and of [^{3}H]MK-801 (Foster & Wong, 1987; Reynolds et al., 1987; Javitt et al., 1987; Fagg, 1987) to rat brain membranes, an effect that is potentiated by glycine. Other studies have shown that NMDA-receptor agonists increase the rate of [^{3}H]TCP (Kloog et al., 1988a; Bonhaus & McNamara, 1988) and of [^{3}H]MK-801 (Kloog et al., 1988b; Javitt & Zukin, 1989a) association with and dissociation from the NMDA receptor.

A simple model that could explain these data suggests that a diffusion-limited binding of uncompetitive blocker to the closed NMDA-receptor channel (in the absence of agonists)

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[1] Abbreviations: PCP, phencyclidine; TCP, *N*-[1-(2-thienyl)cyclohexyl]piperidine; Glu, L-glutamate; Gly, glycine; NMDA, *N*-methyl-D-aspartate; AP-5, D-(−)-2-amino-5-phosphovaleric acid; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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is followed by a rapid binding step, which would itself act as the rate-limiting step of the binding when agonists are present (Kloog et al., 1988a,b). Fitting of [^{3}H]TCP-binding kinetics to a conceptually similar model, viz., the "guarded receptor" model (Starmer & Grant, 1985), also indicated that NMDA-receptor agonists control the accessibility of the ligand to its site (Bonhaus & McNamara, 1988).

In light of these models, which correspond well to the mode of channel block produced by PCP-like drugs, we were interested to examine the effects of temperature on glutamate- and glycine-induced [^{3}H]TCP binding to the NMDA receptor. Our results support the notion that the channel contains a hidden receptor site for [^{3}H]TCP which becomes accessible due to the action of glutamate and that channel opening is a nonuniform phenomenon that can be modulated by glycine.

Experimental Procedures

Materials

[^{3}H]TCP (28.6 Ci/mmol, >98% pure) was purchased from Israel Nuclear Center, Negev, Israel. PCP and TCP were prepared as described previously (Haring et al., 1983). L-Glutamate and glycine were from Sigma, St. Louis, MO, and D-(−)-2-amino-5-phosphovaleric acid (AP-5) and NMDA were from Cambridge Research Biochemicals.

Methods

(*a*) *Tissue Preparation.* Repeatedly washed rat cerebral cortex membranes (from Charles Rivers CD rats) were prepared essentially as described in detail elsewhere (Snell et al., 1987; Foster & Wong, 1987; Kloog et al., 1988a). Briefly, tissues were homogenized in double-distilled water and subjected to incubation for 30 min at 37 °C, centrifugation, three washings with 10 mM HEPES buffer, pH 7.4, containing 2 mM EDTA, and freezing with liquid nitrogen. Membrane preparations were kept at −70 °C, thawed at low temperature on the day of the experiment, washed twice before use (Kloog et al., 1988a), and resuspended in 20 mM HEPES buffer to yield 1.6 mg of protein/mL. Each membrane preparation was examined for sensitivity and specificity by measurement at 25 °C of the AP-5 (100 μM) blockade and of the enhancement of [^{3}H]TCP binding by NMDA (100 μM). Only 3 of more than 25 different preparations were discarded because of their unusual rapid basal binding of [^{3}H]TCP.

(*b*) *Binding Assay.* The binding assay was performed essentially as described previously (Haring et al., 1987; Kloog et al., 1988a), except that the buffer used was 20 mM HEPES, pH 7.4, containing the following antiproteases: 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 5 units/mL aprotinine, and 5 μg/mL pepstatin A. The reaction mixture consisted of 50 μL of the membrane preparation (80 μg of protein) and 150 μL of 20 mM HEPES buffer containing [^{3}H]TCP or [^{3}H]TCP together with 10 μM $MgCl_2$, 1 μM L-glutamate, and 1 μM glycine (unless otherwise indicated). Reactions were initiated by the addition of the membrane preparation to the cocktail of ligands, and mixtures were incubated at the indicated temperature for the desired time period. Nonspecific binding was determined in samples containing the labeled ligands and 100 μM unlabeled PCP. Dissociation of [^{3}H]TCP–receptor complexes was measured by the isotopic dilution technique: unlabeled PCP (100 μM) was added to the preformed complexes, and the reaction was terminated at zero time or at the times indicated in the figures. All other binding assay procedures were as described previously (Kloog et al., 1988a,b).

(*c*) *Data Analysis.* Equilibrium binding data expressed in the form of Scatchard plots were subjected to linear regression analysis. Kinetic binding data were expressed in terms of the two-step mechanisms of [^{3}H]TCP binding to the NMDA-receptor channel as described previously (Kloog et al., 1988a,b), assuming that the ligand outside the channel (L_o) diffuses into the interior (L_c) with forward and backward diffusion constants k_a and k_b, respectively, and then binds to the hidden receptor site (R) with association and dissociation rate constants of k_1 and k_{-1}, respectively:

$$L_o \underset{k_b}{\overset{k_a}{\rightleftharpoons}} L_c + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL$$

According to the model, high concentrations of L-glutamate and glycine will allow fast equilibrium of L_o and L_c, thus enabling a diffusion-unlimited, apparently one step, association of [^{3}H]TCP with its receptor sites; thus

$$([RL]_{eq} - [RL])/[RL]_{eq} = e^{-k_{obs}t} \quad (1)$$

where RL and RL_{eq} are the receptor–ligand complexes at time t and at equilibrium, respectively, and $k_{obs} = (k_1k_a/k_b)L + k_{-1}$. Where data could not be fitted to the single-exponential term shown in eq 1, we used a curve-fitting procedure for the sum of two exponential terms as described previously (Kloog et al., 1978).

The residual sums of squares obtained from the fits to one and to two exponents were compared (F test). Association kinetics in the absence of added NMDA-receptor ligands were analyzed according to the two-step model on the assumption that the diffusion process is the rate-limiting step of [^{3}H]TCP binding; thus

$$1 - \frac{(k_{-1}/k_1)(k_b/k_a)[RL]}{[L]([R_T] - [RL])} = e^{-k_bt} \quad (2)$$

On the basis of previous data (Kloog et al., 1988a,b) and as shown here (see Results), no additional assumptions were necessary for the characterization of [^{3}H]TCP binding under these conditions.

Results

The effect of temperature on the binding of [^{3}H]TCP to its receptors in repeatedly washed cortical membranes was determined at 5, 15, 25 and 33 °C. Over this temperature range identical values of specifically bound ligand were recorded at 18 and at 24 h in the presence of 1 μM glutamate and 1 μM glycine, indicating that the ligand–receptor complexes had reached equilibrium. Binding isotherms determined with 5–100 nM [^{3}H]TCP indicated the following: (a) binding site homogeneity (at all temperatures), as reflected by linear Scatchard plots (see Figure 1A for analysis at 15 and 33 °C); (b) similar site densities (B_{max}) for [^{3}H]TCP as determined at 5, 15, 25, and 33 °C (2.2 ± 0.2, 2.9 ± 0.4, 2.6 ± 0.5, and 2.4 ± 0.4 pmol/mg of protein, respectively); and (c) increase in affinity with temperature, as shown by the decrease of [^{3}H]TCP equilibrium binding constants (K_d) (Table I). The van't Hoff plot of these data was linear (Figure 1B; thus, the phase-transition temperature apparently exerted no effect on [^{3}H]TCP binding and ΔH was +75 cal/mol. The calculated ΔS at 25 °C was 35.7 cal mol^{-1} °C^{-1}.

The K_d and B_{max} values for [^{3}H]TCP determined in the different membrane preparations showed insignificant variations. By contrast, the kinetics of [^{3}H]TCP binding—and especially those determined in the absence of added glutamate and glycine (basal binding)—varied with each preparation, and we therefore performed a set of kinetic experiments for each membrane preparation separately. Differences of up to

Table I: Effects of Temperature on the Equilibrium Binding Constants and on the Kinetics of [^{3}H]TCP Binding to the NMDA-Receptor Channel

T (°C)	equilibrium binding K_d (nM)	association kinetics in the presence of Glu + Gly k_{obs1} (min^{-1})	k_{obs2} (min^{-1})	α	basal association kinetics k_b (min^{-1} × 10^{-3})
5	36 ± 5	0.27 ± 0.07	0.19 ± 0.005	0.51 ± 0.09	3.85–5.05
15	24 ± 3	0.39 ± 0.06	0.027 ± 0.005	0.60 ± 0.12	5.05–3.50
25	12 ± 2	0.60 ± 0.05	0.041 ± 0.006	0.65 ± 0.07	3.95–10.10
33	8 ± 2	0.88 ± 0.11	0.060 ± 0.010	0.60 ± 0.13	4.43–6.60

[a] Values represent the means ± SD of three separate binding isotherms obtained at each temperature in the presence of 1 μM Glu + 1 μM Gly. The association kinetics in the absence (basal) and in the presence (induced) of 1 μM Glu + 1 μM Gly were determined with 5 nM [^{3}H]TCP as described in the legends to Figures 2 and 3. Association kinetics in the presence of the agonists were fitted to the sum of two exponential terms specifying a fast and a slow binding component with time constants k_{obs_1} and k_{obs_2}, respectively, α being the proportion of the rapidly associating component. Basal association kinetics were fitted to eq 2. All kinetic experiments were performed three or four times at each temperature. The values shown are the means ± SD of parameters evaluated in these experiments. For SD of parameters of individual curves see the legend to Figure 3. Linear regression analysis indicated significant correlation between k_{obs_1} and k_{obs_2} ($r = 0.96$, $p < 0.02$), k_{obs_1} and K_d ($r = 0.95$, $p = 0.05$) and k_{obs_2} and K_d ($r = 0.95$, $p = 0.05$).

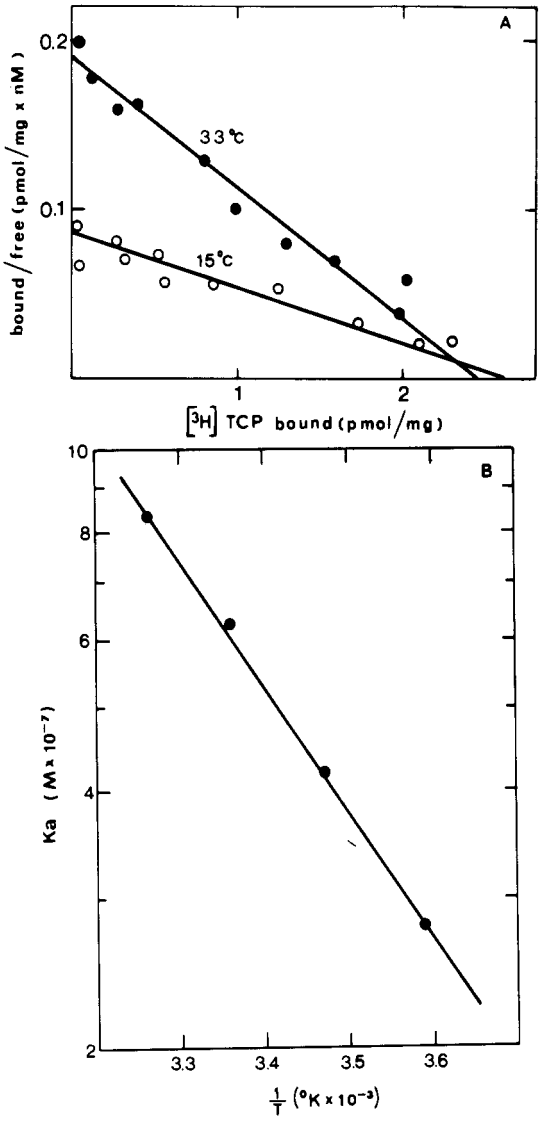

FIGURE 1: (A) Representative Scatchard plots of [^{3}H]TCP binding to the NMDA receptor. Binding was determined after incubation for 24 h as described under Methods in the presence of 1 μM glutamate and 1 μM glycine, at 15 or 33 °C. (B) van't Hoff plot. K_d values were estimated from the linear Scatchard plots of binding of [^{3}H]TCP to the NMDA receptor at 5, 15, 25, and 33 °C. Each point represents the means of three separate measurements at each temperature.

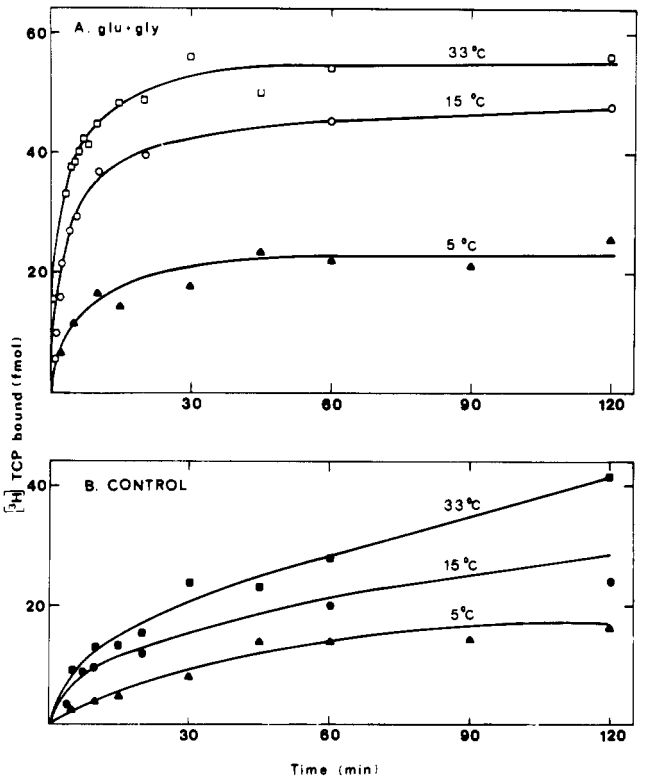

FIGURE 2: Time courses of induced (A) and basal (B) binding of [^{3}H]TCP to the NMDA receptor determined at various temperatures. Binding of [^{3}H]TCP (5 nM) as a function of time was determined at 5, 15, and 33 °C, in the presence (A, Glu + Gly) and in the absence (B, control) of 1 μM Glu and 1 μM Gly. Values represent the means obtained from a single experiment performed in triplicate by using the same batch of membrane preparation.

2-fold were found between preparations in reaction half-times of the basal association or dissociation reactions; however, preparations with "fast" and with "slow" basal association binding rates also showed correspondingly fast and slow basal dissociation rates. Moreover, whatever the basal binding rates and at all temperatures under study, glutamate and glycine accelerated the rates of [^{3}H]TCP binding. Figure 2 shows the results of a typical experiment, where association rates of 5 nM [^{3}H]TCP were obtained at 5, 15, and 33 °C. As shown, raising of the temperature caused a gradual increase in both the basal and the glutamate- and glycine-induced binding of [^{3}H]TCP, but especially in the latter. The half-times of the induced association reactions decreased with increasing temperature (20, 10, and 4 min at 5, 15, and 33 °C, respectively), whereas those of the basal association reactions showed almost no temperature dependency.

The decrease with increasing temperature in the association half-times of the induced binding, as well as the gradual decrease with increasing temperature in the level of [^{3}H]TCP binding as equilibrium was approached (Figure 2), is consistent with the observed temperature-related increase in affinity (Table I). These results do not, however, indicate whether

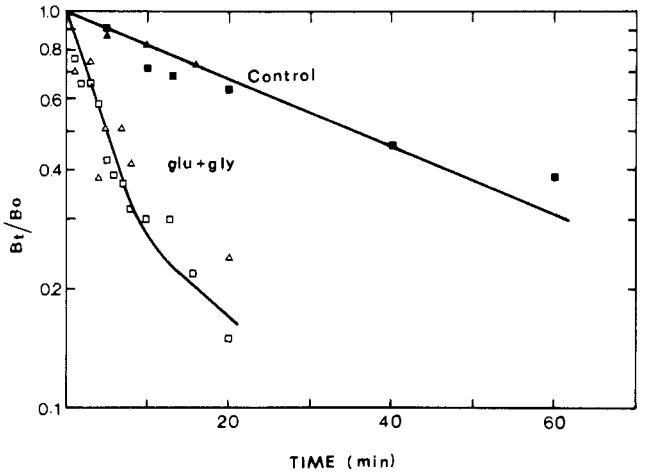

FIGURE 3: Time courses of dissociation of [^{3}H]TCP–receptor complexes. Samples were incubated at 5 (squares) or at 33 °C (triangles) with 20 nM [^{3}H]TCP for 1 h. Dissociation reactions were initiated by the addition of 100 μM PCP with (open symbols) and without (solid symbols) 1 μM glutamate plus 1 μM glycine. Values represent the means obtained from a single experiment performed in triplicate by using the same batch of membranes. Results are expressed in the form of first-order plots, where B_0 and B_t are the amounts of [^{3}H]TCP binding at zero time and at time t, respectively.

Table II: Analysis of the Potentiating Effect of Glycine on Glutamate-Induced [^{3}H]TCP Association Kinetics[a]

assay conditions	α	k_{obs1} (min^{-1})	k_{obs2} (min^{-1})
25 °C, Glu	0.21 ± 0.09	0.65 ± 0.03	0.028 ± 0.004
25 °C, Glu + Gly	0.65 ± 0.07	0.60 ± 0.05	0.041 ± 0.006
33 °C, Glu	0.34 ± 0.15	0.91 ± 0.06	0.100 ± 0.004
33 °C, Glu + Gly	0.60 ± 0.13	0.88 ± 0.11	0.060 ± 0.010

[a] Association kinetics were determined with 5 nM [^{3}H]TCP (see the legend to Figure 2) in the presence of either 1 μM Glu or 1 μM Glu + 1 μM Gly, at the indicated temperatures. Data were analyzed as described in Table I. Values of the time constants and α represent the means ± SD of at least three separate kinetic experiments.

temperature affected the apparent association and/or dissociation time constants. Examination of the dissociation rates of [^{3}H]TCP–receptor complexes at 5 and 33 °C (Figure 3) revealed no differences in either the basal or the induced rates at the two temperatures; in both cases glutamate and glycine induced a marked decrease in the dissociation half-times (from 70–90 min to 4–10 min). We thus conclude that temperature affects mainly the apparent association time constants of [^{3}H]TCP binding to the activated NMDA-receptor channel, thereby increasing its affinity.

The lack of temperature dependency of the basal association and dissociation half-times of [^{3}H]TCP binding (Figures 2 and 3) is consistent with the suggestion that basal [^{3}H]TCP binding reflects mostly diffusion into and out of the channel, since at the biological range of temperatures such processes are hardly affected by temperature variations. Accordingly, we analyzed the data on basal and induced [^{3}H]TCP binding to the receptor, assuming that diffusion of the ligand into the channel is followed by the binding reaction (Kloog et al., 1988a,b); diffusion would then be rate-limiting under conditions where NMDA-receptor channels are not activated by glutamate and glycine (basal binding). The analysis (Figure 4; Table II) revealed a good fit to monoexponential kinetics of basal [^{3}H]TCP binding at all temperatures under study, as predicted by the model. The time constants thus obtained did not vary as a function of temperature (Table II) and were similar to the basal dissociation time constants (5.5–11.5 × 10^{-3} min^{-1}), also as predicted by the model.

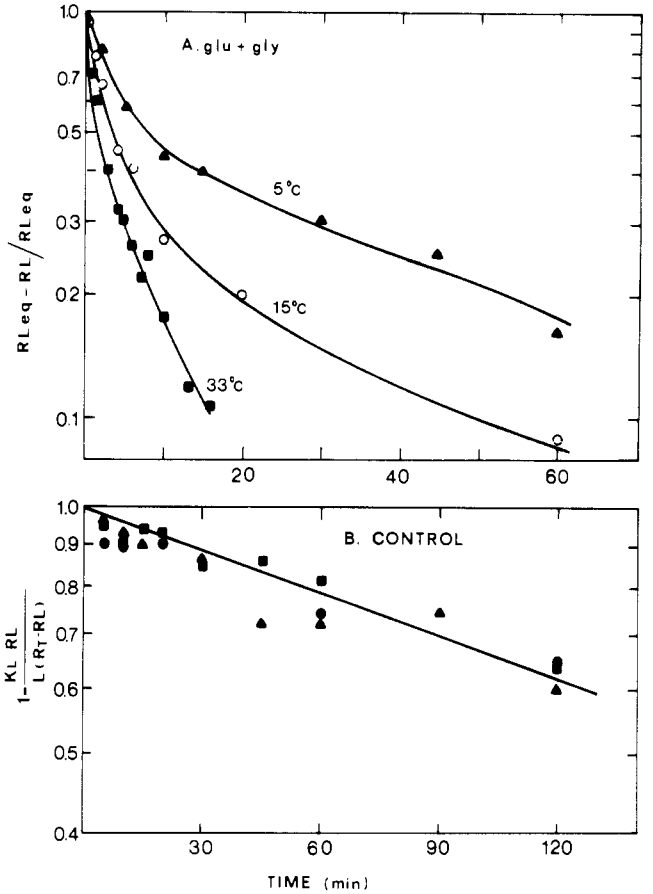

FIGURE 4: Kinetic analysis of the association of 5 nM [^{3}H]TCP with the NMDA receptor at various temperatures. The kinetic data in Figure 2 were analyzed according to the access-limited model of ligand binding as described under Methods. (Upper panel, Glu + Gly) Analysis of [^{3}H]TCP binding to the NMDA receptor in the presence of 1 μM glu and 1 μM gly at 5 (▲), 15 (○), and 33 °C (■) according to eq 1. The curves deviate significantly from a single exponent (eq 1) and show a better fit to the sum of two exponential terms [residual sums of squares for the fit to the sum of two exponents were in all cases significantly lower ($p < 0.002$, F test) than the residual sum of squares for the fit to a single exponent]. The parameters ± SD of the curves are as follows: at 33 °C, $\alpha = 0.63 \pm 0.12$, $k_{obs1} = 0.71 \pm 0.21$, $k_{obs2} = 0.07 \pm 0.03$; at 15 °C, $\alpha = 0.67 \pm 0.04$, $k_{obs1} = 0.32 \pm 0.03$, $k_{obs2} = 0.022 \pm 0.005$; at 5 °C, $\alpha = 0.52 \pm 0.04$, $k_{obs1} = 0.24 \pm 0.04$, $k_{obs2} = 0.016 \pm 0.002$. (Lower panel, control) Analysis of basal [^{3}H]TCP binding to the NMDA receptor at 5 (▲), 15 (●), and 33 °C (■) according to eq 2.

According to the two-step model, under ideal conditions [^{3}H]TCP association with the receptor induced by glutamate and glycine would proceed without access limitations, thus following bimolecular kinetics. The nonlinear first-order plots of the induced association of [^{3}H]TCP observed at all temperatures studied (Figure 4) could suggest (a) deviation from the proposed mechanism, (b) apparent site heterogeneity which was not detected at equilibrium (see Figure 1), or (c) nonuniformity of the NMDA-receptor channel opening. Three sets of independent observations led us to select the last-mentioned possibility as the most likely: (1) The association kinetics were consistent with the sum of two exponential terms specifying fast and slow binding components with apparent time constants of k_{obs1} and k_{obs2}, respectively (Table I). Both time constants increased with temperature, and the increase of each one was correlated significantly with the corresponding decrease in K_d (Table I). The relative proportions of the two components did not change with temperature (Table I). (2) At 25 °C glutamate alone induced a dose-dependent increase in k_{obs1} and k_{obs2} with similar ED_{50} values (i.e., the concen-

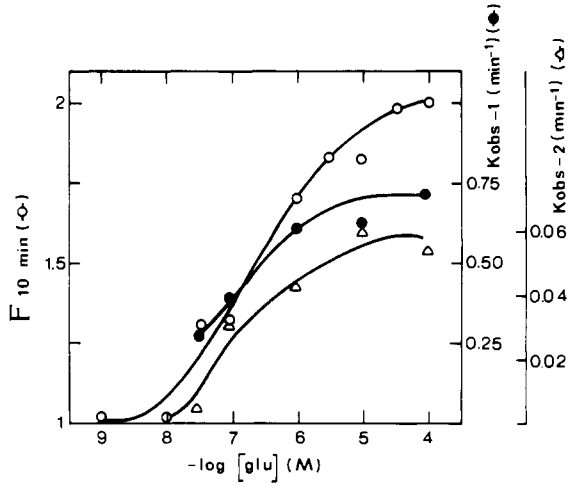

FIGURE 5: Dose–response curves for the glutamate-induced association of [^{3}H]TCP with the NMDA receptor. The rates of association of [^{3}H]TCP (5 nM) with its sites were determined in the presence of various concentrations of glutamate, as described in the legend to Figure 2. Each association curve was fitted to the sum of two exponential terms as described under Methods and in the legend to Figure 4. The association time constants for the apparent fast (k_{obs1}, ●) and slow (k_{obs2}, △) components of association thus evaluated are plotted as a function of glutamate concentrations. The corresponding values of α were 0.25 ± 0.03, 0.32 ± 0.06, 0.27 ± 0.05, 0.31 ± 0.03, 0.26 ± 0.05, and 0.25 ± 0.03 for the association curves evaluated in the presence of 0.05, 0.1, 1.0, 10, and 100 μM glutamate, respectively. Also shown are the results of a separate experiment where basal and glutamate-induced [^{3}H]TCP binding to the NMDA receptor were determined at 10 min; data are expressed as the fold increase (F, 10 min) in binding (○).

tration yielding 50% of maximal effect) for the increase of both time constants (0.1 and 0.25 μM, respectively) (Figure 5). The increase in [^{3}H]TCP binding (induced over basal) determined at 10 min yielded the same ED_{50} value (Figure 5). There was no change in relative proportions of the two associating components as a function of glutamate concentration (see the legend to Figure 5). (3) The potentiating effect of glycine on the glutamate-induced acceleration of [^{3}H]TCP binding to the receptor is not manifested by a change in k_{obs1} or k_{obs2} but rather by an increase in the proportion of the fast association component (Table II).

Discussion

The results of this study show that the affinity of [^{3}H]TCP toward the activated NMDA-receptor channel is increased with temperature, mostly through a temperature-related effect on the apparent association time constants. Temperature had only an insignificant effect on the association and dissociation time constants of [^{3}H]TCP binding to the nonactivated receptor, consistent with the notion of a diffusion-limited movement of the ligand into and out of the channel. Previous studies have shown a temperature-related increase in the affinity of ligands for the PCP receptor in unwashed membranes (Sorensen & Blaustein, 1988); our results are in line with these findings as unwashed preparations are enriched in glutamate and glycine (Loo et al., 1986; Foster & Wong, 1987; Kornhuber et al., 1989).

Reduction of the content of glutamate and glycine by repeated washing of the brain membranes enabled us to distinguish between the effects of temperature on basal and on induced [^{3}H]TCP binding (Figures 2 and 3). Whether the brain membranes were washed (Figure 1) or not (Sorensen & Blaustein, 1988), alterations in the temperature did not lead to a change in the density of [^{3}H]TCP-binding sites, nor did it induce site heterogeneity. The high-affinity [^{3}H]TCP-binding sites associated with the NMDA receptor thus appear to be homogeneous. It is important to note that earlier studies have demonstrated the existence in rat brain membranes of low-affinity [^{3}H]TCP-binding sites (K_d = 100–150 nM) (Haring et al., 1987; Vignon et al., 1986), which are not sensitive to haloperidol but are clearly distinct from the high-affinity [^{3}H]TCP receptors [i.e., they differ in their sensitivities to mono- and divalent cations and in their regional distributions (Haring et al., 1987)]. It is not known whether or not the low-affinity sites are associated with NMDA receptors. The high-affinity [^{3}H]TCP- or [^{3}H]MK-801-binding sites (K_d = 3–15 nM), however, are clearly associated with the NMDA receptors, as shown by their sensitivity to NMDA-receptor agonists, antagonists, and modulators (Loo et al., 1986; Fagg, 1987; Foster & Wong, 1987; Johnson et al., 1988; Kloog et al., 1988a; Javitt & Zukin, 1989a). Also, kinetically derived K_d values for [^{3}H]TCP binding to the high-affinity sites, determined by varying the concentrations of either the ligand (Kloog et al., 1988a,b) or NMDA (Bonhaus & McNamara, 1988), showed good agreement with the K_d values determined at equilibrium in the presence of agonist and glycine. Thus, deviation of the agonist-induced [^{3}H]TCP kinetics from the ordinary bimolecular reaction scheme (Figures 3 and 4) must not be confused with the existence of site heterogeneity due to the presence of receptors other than the NMDA receptor which bind PCP-like drugs with relatively low affinity.

Previous studies have pointed to nonlinearity of the ln transformation of [^{3}H]MK-801 (Javitt & Zukin, 1989a) and of [^{3}H]TCP (Bonhaus & McNamara, 1988) binding kinetics. It was suggested that this would correlate with [^{3}H]MK-801 site heterogeneity, in line with the observed nonlinear Scatchard plots (Javitt & Zukin, 1989b; Loo et al., 1987). Our own studies with [^{3}H]TCP (Kloog et al., 1988a) and [^{3}H]-MK-801 (Kloog et al., 1988b), as well as those of Bonhaus and McNamara (1988) with [^{3}H]TCP, revealed no evidence of site heterogeneity associated with the NMDA receptor, most probably because in both cases the ligand concentrations used for kinetic and equilibrium experiments were close to the K_d of the high-affinity sites; it was thus possible to compare their kinetic and equilibrium data without the interference of other, low-affinity sites.

Previous findings and the present results appear to support the concept of a hidden receptor site for PCP-like drugs within the NMDA-receptor channel. We earlier proposed a two-step model ($L_o \rightleftharpoons L_c + R \rightleftharpoons RL$) to describe the binding of such ligands to the receptor channel. Bonhaus and McNamara (1988) adopted the "guarded receptor" model for transiently accessible sites (Starmer & Grant, 1985) to describe [^{3}H]TCP binding. The two models are similar in that they both posit limitation of ligand access to the nonactivated channel and activation-dependent ingress and egress of the ligand. As predicted by these models, and consistent with the electrophysiologically determined open channel block (Anis et al., 1983; Honey et al., 1985; Huettner & Bean, 1988), NMDA-receptor agonists accelerate the association and dissociation rates of the PCP-like drugs without changing their site density or K_d values (Kloog et al., 1988a,b; Bonhaus & McNamara, 1988; present study).

The results of this study suggest that the nonlinearity of the ln transformation of [^{3}H]TCP kinetics reflects nonuniformity in the opening of the NMDA-receptor channels, since temperature as well as glutamate affected both the fast and the slow time constants of association of [^{3}H]TCP to the receptor channel (Table I; Figure 5).

The fact that the fast and slow components retained the same relative proportions when exposed to changes in either temperature or glutamate concentration suggests that they already existed in the washed membrane preparations. We cannot infer from this, however, that such nonuniformity of NMDA-receptor channel opening, as reflected by [^{3}H]TCP binding, exists also in intact cells. It is interesting to note that Mody et al. (1988) described two components of NMDA-gated channels, one of which requires a supply of high-energy phosphates and the other requiring no such supply. In another study, Mayer et al. (1989) showed that Ca^{2+} influx through NMDA receptors appears to trigger secondary mechanisms which lead to glycine-resistant desensitization. Thus, not only endogenous glutamate and glycine but also endogenous metabolites (or metabolic pathways) may be affecting NMDA-gating mechanisms and resulting in the apparent observed nonuniformity of channel opening. If this is the case, then such mechanisms are likely also to involve the glycine site, since glycine apparently induced conversion from the slow to the fast association component without altering the time constants of [^{3}H]TCP binding (Table II). This effect of glycine, which plausibly explains its potentiating effects on glutamate-induced [^{3}H]TCP and [^{3}H]MK-801 binding, is certainly consistent with its observed allosteric effects on NMDA-gated ionic currents (Johnson & Ascher, 1987; Mayer et al., 1989; Kleckner & Dingledine, 1988; Kushner et al., 1988). An increase in frequency of ion-channel opening (Johnson & Ascher, 1987) or a decrease in receptor desensitization (Mayer et al., 1989) should show up in our binding experiments as an apparent increase in the number of channels accessible to [^{3}H]TCP.

Two more points call for comment. First, the dose–response curves for glutamate (Figure 5) demonstrate that the potencies of glutamate, as estimated by its enhancement of [^{3}H]TCP binding from initial rates (k_{obs1}) or from the approach to equilibrium (k_{obs2}) or from any intermediate time point (e.g., 10 min, Figure 5), are all very similar. These results, which are consistent with use dependency, explain the generally good agreement in the estimated potencies of NMDA-receptor agonists determined by assays using [^{3}H]TCP or [^{3}H]MK-801, despite the variety of incubation times used by different groups. Second, a comparison between the temperature dependency of [^{3}H]TCP binding and of NMDA-receptor channel blocking (Davies et al., 1988) reveals significant differences as well as significant similarities. Davis et al. (1988) observed a decrease with increasing temperature in the NMDA use-dependent block by MK-801 or PCP-like drugs and suggested that at higher temperatures MK-801 may gain access to the channel even in the absence of agonists (Davies et al., 1988). In our experiments on association kinetics we found no such reduction in the ratios of induced to basal association rates with increasing temperature. The reason for these differences is not known. Nonetheless, the absence of temperature-related changes in basal or induced dissociation rates of [^{3}H]TCP–receptor complexes is in line with the use-dependent recovery of MK-801 block observed both at high and at low temperatures (Davies et al., 1988).

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Registry No. TCP, 21500-98-1; L-Glu, 56-86-0; Gly, 56-40-6.

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Multinuclear Magnetic Resonance Studies of the 2Fe·2S* Ferredoxin from *Anabaena* Species Strain PCC 7120. 1. Sequence-Specific Hydrogen-1 Resonance Assignments and Secondary Structure in Solution of the Oxidized Form†

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ABSTRACT: Complete sequence-specific assignments were determined for the diamagnetic ^1H resonances from *Anabaena* 7120 ferredoxin (M_r = 11 000). A novel assignment procedure was followed whose first step was the identification of the ^{13}C spin systems of the amino acids by a $^{13}C\{^{13}C\}$ double quantum correlation experiment [Oh, B.-H., Westler, M. W., Darba, P., & Markley, J. L. (1988) *Science 240*, 908–911]. Then, the ^1H spin systems of the amino acids were identified from the ^{13}C spin systems by means of direct and relayed $^1H\{^{13}C\}$ single-bond correlations [Oh, B.-H., Westler, W. M., & Markley, J. L. (1989) *J. Am. Chem. Soc. 111*, 3083–3085]. The sequential resonance assignments were based mainly on conventional interresidue $^1H^{\alpha}_i$–$^1H^N_{i+1}$ NOE connectivities. Resonances from 18 residues were not resolved in two-dimensional ^1H NMR spectra. When these residues were mapped onto the X-ray crystal structure of the homologous ferredoxin from *Spirulina platensis* [Fukuyama, K., Hase, T., Matsumoto, S., Tsukihara, T., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., & Matsubara, H. (1980) *Nature 286*, 522–524], it was found that they correspond to amino acids close to the paramagnetic 2Fe·2S* cluster. Cross peaks in two-dimensional homonuclear ^1H NMR spectra were not observed for any protons closer than about 7.8 Å to both iron atoms. Secondary structural features identified in solution include two antiparallel β-sheets, one parallel β-sheet, and one α-helix.

Plant-type ferredoxins (M_r = 11 000) belong to a class of iron–sulfur proteins that are present in virtually all organisms. Iron–sulfur proteins can be classified according to the number of iron atoms and acid-labile sulfur atoms contained in the clusters. Plant-type ferredoxins contain a 2Fe·2S* cluster ligated to the protein by four cysteine residues. Plant-type ferredoxins undergo a one-electron redox reaction with a redox potential of around −420 mV at pH 7 (Cammack et al., 1977). In the oxidized form, both iron atoms are formally Fe(III). In the reduced form, one of the iron atoms remains Fe(III) and the other becomes Fe(II). The physiological function of ferredoxin in plants and cyanobacteria is to serve as a terminal electron acceptor from photosystem I (Trebst & Avron, 1977) and subsequently to donate electrons in several reactions: reduction of $NADP^+$ to NADPH (Masaki et al., 1982), reduction of nitrite to ammonia (Ida, 1977), sulfur assimilation (Aketagawa & Tamura, 1980), and glutamate synthesis (Lea & Miflin, 1974). Plant-type ferredoxins have been sequenced from nearly 30 plants and cyanobacteria (Tsukihara et al., 1986). Crystal structures have been determined for two cyanobacterial ferredoxins (Fukuyama et al., 1980; Tsukihara et al., 1981; Tsutusi et al., 1983).

NMR[1] spectroscopy has emerged as a powerful tool for the determining solution structures of biological macromolecules. It is the only method that provides full three-dimensional structures of biomolecules such as proteins and nucleic acids in solution. NMR spectroscopy can provide, in addition, information about dynamics, kinetics, and enzyme mechanisms. The quality and content of information from NMR spectroscopy are heavily dependent on the extent of resonance assignments. Early NMR studies of 2Fe·2S* ferredoxins

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