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Kinetic Characterization of the Phencyclidine–*N*-Methyl-D-aspartate Receptor Interaction: Evidence for a Steric Blockade of the Channel†

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ABSTRACT: The nature of the interactions between the *N*-methyl-D-aspartate (NMDA) and the phencyclidine (PCP) receptors was studied in membranes obtained from rat cerebral cortex and washed repeatedly to remove endogenous excitatory amino acids. Binding of [^{3}H]-*N*-[1-(2-thienyl)cyclohexyl]piperidine ([^{3}H]TCP) to its receptor sites in these membranes proceeded slowly and did not reach equilibrium even after incubation for 4 h at 25 °C. The dissociation rate of [^{3}H]TCP–receptor complexes was also slow ($t_{1/2}$ = 128–165 min). Both association and dissociation followed first-order reaction kinetics, with similar time constants (0.0054 min^{-1}). Addition of glutamate and glycine to the washed membranes was immediately followed by a marked increase in the rates of both association of [^{3}H]TCP with the receptors and its dissociation from them ($t_{1/2}$ = 8 min). Association now followed second-order reaction kinetics. Accelerated association of [^{3}H]TCP with its binding sites could also be induced by NMDA or by glutamate alone, and glycine enhanced the effect. All effects of glutamate and glycine on [^{3}H]TCP binding kinetics were blocked by the competitive NMDA receptor antagonist AP-5 [D-(−)-2-amino-5-phosphovaleric acid]. [^{3}H]TCP–receptor interactions at equilibrium were not altered by AP-5 or by glutamate and glycine. The binding data were fitted to a model in which interactions of [^{3}H]TCP with the receptor involve a two-step process: the outside ligand must cross a barrier (presumably a closed NMDA receptor channel in the absence of agonists). Once agonists are added, this limitation is removed (presumably because the channel is open). The excellent agreement between the kinetic and equilibrium binding parameters with the predictions of our model, as well as with previous electrophysiological data on the mode of noncompetitive blocking of the NMDA receptor channel by PCP-like drugs, suggests that these drugs are steric blockers of the channel and prefer its open state.

Receptors for the dissociative anesthetic phencyclidine (PCP)[1] in the rat brain (Zukin & Zukin, 1979; Vincent et al., 1979; Zukin et al., 1983; Quirion et al., 1981; Hampton et al., 1982; Vignon et al., 1986; Haring et al., 1986) were shown to be associated with the behavioral effects of PCP-like drugs (Contreras et al., 1986; Domino & Luby, 1981), which include PCP analogues and σ-opioids.

Anis et al. (1983) showed that PCP and ketamine are selective blockers of the *N*-methyl-D-aspartate (NMDA) type of excitatory amino acid receptors. Subsequent studies demonstrated the voltage dependence of the NMDA receptor channel block by PCP-like drugs (Honey et al., 1985; Macdonald et al., 1986) as well as the noncompetitive nature of this block (Berry et al., 1984; Fagg et al., 1986; Snell & Johnson, 1986; Jones et al., 1987). These findings, as well as the similar regional distributions of the PCP receptors and the NMDA receptors (Maragos et al., 1986; Cotman et al., 1987), suggest that the two receptors may well represent a single receptor channel complex. This suggestion is further supported by radiation inactivation experiments (Honore et al., 1987), as well as by binding (Loo et al., 1986) and photoaffinity labeling experiments (Haring et al., 1987).

Previous reports have shown that the agonist-induced increase in binding of [^{3}H]TCP or of [^{3}H]MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine

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[1] Abbreviations: PCP, phencyclidine; [^{3}H]TCP, [^{3}H]-*N*-[1-(2-thienyl)cyclohexyl]piperidine; NMDA, *N*-methyl-D-aspartate; AP-5, D-(−)-2-amino-5-phosphovaleric acid.

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maleate] is associated with the noncompetitive blocking of the NMDA receptor channel (Loo et al., 1986; Foster & Wong, 1987; Johnson et al., 1987). However, the mechanism of these interactions has remained obscure. The kinetic and equilibrium binding experiments with [^{3}H]TCP described in this study point to a nonallosteric interaction between NMDA receptor agonist binding sites and the [^{3}H]TCP binding sites and are consistent with a mechanism involving steric blockade of the NMDA receptor channel by PCP-like drugs.

Experimental Procedures

Materials. [^{3}H]TCP (40 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. 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 (Johnson et al., 1987; Foster & Wong, 1987). Briefly, the washed crude membranes were prepared by homogenizing the tissue in 30 volumes of ice-cold double-distilled water. The homogenate was incubated for 30 min at 37 °C and then centrifuged at 48000*g* for 20 min at 4 °C. The pellet was rehomogenized in the original volume of water, and the incubation and centrifugation procedures were repeated. The resulting pellet was resuspended in the original volume of 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 2 mM ethylenediaminetetraacetic acid (EDTA) and then again centrifuged at 48000*g* for 20 min. This washing step was repeated 3 times. The last resuspended membrane preparation was frozen under liquid nitrogen and stored at −70 °C. On the day of the experiment the membrane preparation was thawed at room temperature and washed twice as described above. The final pellet was resuspended in 20 volumes of 20 mM HEPES buffer, pH 7.4, and 50-μL aliquots of this preparation were used for binding assays.

(B) Binding Assay. The binding assay was performed essentially as described previously (Haring et al., 1986, 1987), except that the buffer used was 20 mM HEPES, pH 7.4. The reaction mixture consisted of 50 μL of the membrane preparation (100–120 μg of protein) and 150 μL of 20 mM Hepes buffer containing [^{3}H]TCP or [^{3}H]TCP, the desired NMDA receptor ligands, and 10 μM $MgCl_2$. Usually we used 1 μM L-glutamate together with 1 μM glycine, but sometimes 1 μM L-glutamate alone or 100 μM NMDA was used. Blocking of NMDA receptor sites was achieved by the use of 100 μM AP-5. Reactions were initiated by the addition of the membrane preparation to the cocktail of ligands, and mixtures were incubated at 25 °C for the desired period of time. 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 detailed previously (Haring et al., 1986, 1987).

Results

The experiments described here were performed with lysed and repeatedly washed cerebral cortex membranes (for details, see Methods); the washing was necessary in order to reduce endogenous glutamate to a minimum. Membranes were incubated at 25 °C in 20 mM Hepes buffer, pH 7.4, containing [^{3}H]TCP, with or without exogenous agonists. In order to achieve the maximal effects of NMDA receptor ligands on [^{3}H]TCP binding, we used 1 μM glutamate together with 1 μM glycine and 10 μM Mg^{2+} (Johnson et al., 1987). Mg^{2+} was always included in assays where NMDA receptor ligands were present. Nonspecific binding in the presence and in the absence of these ligands was similar. Figure 1 demonstrates typical time courses for the association between [^{3}H]TCP (1 and 5 nM) and the PCP binding sites in the washed membranes, with and without exogenous glutamate and glycine. Several important features of [^{3}H]TCP binding are apparent: (a) The association rate of [^{3}H]TCP in the absence of exogenous agonists was so slow that within the first 1 and 5 min (respectively for 5 and 1 nM [^{3}H]TCP) no specific binding could be detected (Figure 1, inset), and even after incubation for 4 h the reaction failed to reach equilibrium. (b) On the addition of exogenous agonists the rate of [^{3}H]TCP binding to the receptor was markedly increased; even at 1 nM [^{3}H]-TCP the reaction approached equilibrium after only 1 h of incubation (Figure 1). (c) The effects of glutamate and glycine on [^{3}H]TCP binding to the receptor appear to be instantaneous; specific binding of [^{3}H]TCP was already observed within the first 10–20 s (5 nM) and 1–2 min (1 nM) (Figure 1 inset). (d) The increase in [^{3}H]TCP binding induced by the exogenous agonists is time dependent; immediately after onset of the binding reactions it is very high (7–9-fold) and then declines (Figures 1 and 2).

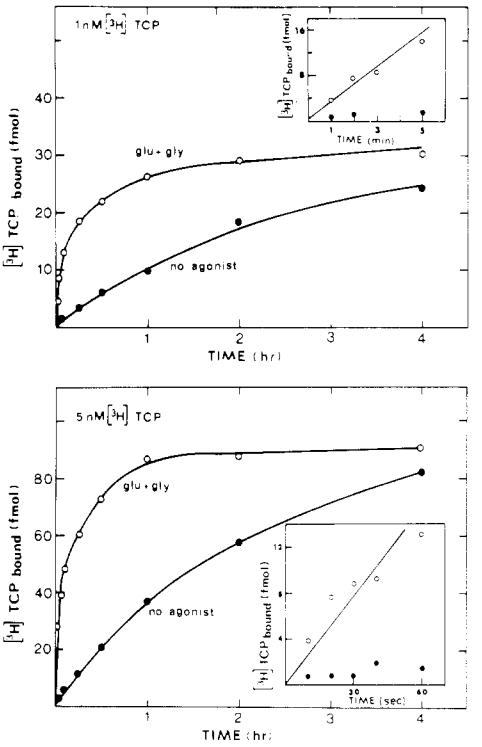

FIGURE 1: Time course of association of [^{3}H]TCP with the PCP receptors in the absence and in the presence of glutamate and glycine. Binding of 1 nM (upper) or of 5 nM (lower) [^{3}H]TCP to the PCP receptors was measured as a function of time at 25 °C, as detailed under Methods. Binding was determined in the absence (no agonist, ●) and in the presence (Glu + Gly, ○) of 1 μM glutamate and 1 μM glycine. Insets: Initial rates of the binding reactions. Binding in the absence of agonists during the short incubation times was close to the background; thus no lines are drawn between data points. The data shown are from one of two experiments that gave similar results.

The data shown in Figure 2 suggest that acceleration of [^{3}H]TCP binding to the receptor induced by glutamate and glycine is mediated by the NMDA receptors; the effect can

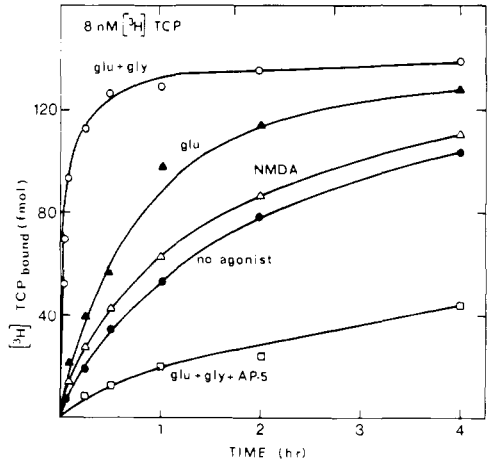

FIGURE 2: NMDA receptor specificity of the increase in the rate of [^{3}H]TCP association with its receptors. The time course of association of 8 nM [^{3}H]TCP with its receptors was determined as described in Figure 1 in the absence (●) and in the presence of 100 μM NMDA (△), 1 μM glutamate (▲), 1 μM glutamate + 1 μM glycine (○), or 1 μM glutamate + 1 μM glycine + 100 μM AP-5 (□). The data shown are from one of three experiments that gave similar results.

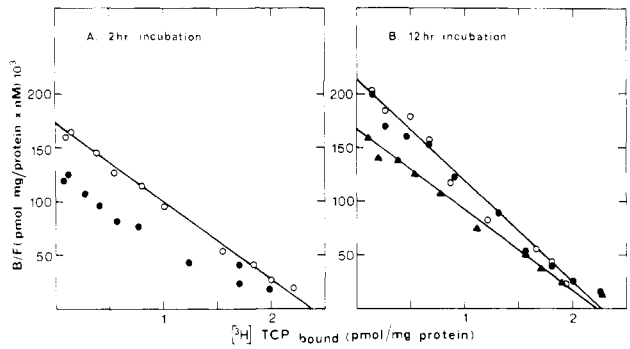

FIGURE 3: Scatchard plots of [^{3}H]TCP binding to PCP receptors. Binding of [^{3}H]TCP as a function of its concentration (0.7–150 nM) was determined at 25 °C in the absence (●) and in the presence of 1 μM glutamate + 1 μM glycine (○) or 1 μM glutamate + 1 μM glycine + 100 μM AP-5 (▲). (A) Samples were incubated for 2 h. No line is drawn between data points determined in the absence of agonists, as the reaction did not reach equilibrium. (B) Samples were incubated for 12 h.

be induced by glutamate alone, and the presence of NMDA itself (100 μM), which is far less potent than glutamate (Watkins & Olverman, 1987), resulted in a smaller increase in the rate of [^{3}H]TCP binding to the receptor. The addition of glycine to glutamate enhanced the effect, and the selective NMDA receptor antagonist AP-5 (Watkins & Olverman, 1987; Fagg & Matus, 1984) abolished it.

Figure 3 demonstrates the binding of [^{3}H]TCP as a function of its concentration after incubation for 2 h and for 12 h, in the absence and in the presence of the agonists. The results show that the binding characteristics of [^{3}H]TCP in the presence of glutamate and glycine are very similar after 2 and after 12 h of incubation (K_d = 13 nM and 10 nM and B_{max} = 2.38 and 2.28 pmol/mg of protein, respectively). It thus appears that [^{3}H]TCP binding to the receptor in the presence of these agonists had reached equilibrium after 2 h. In their absence an incubation time of 2 h was not enough (Figure 3A), but after 12 h [^{3}H]TCP binding to the receptor had reached equilibrium (K_d = 10 nM; B_{max} = 2.28 pmol/mg of protein; Figure 3B). Also, the apparent inhibition of [^{3}H]TCP binding to the receptor by AP-5 [seen as a reduction in the rate of ligand–receptor association beyond that of the control in the presence (Figure 2) and in the absence (not shown) of the exogenous agonists] was almost undetectable after 12 h (K_d for [^{3}H]TCP binding was 13 nM and B_{max} was 2.20 pmol/mg of protein; Figure 3B).

Since the presence of glutamate and glycine did not alter the equilibrium binding of [^{3}H]TCP but did accelerate the rate of ligand–receptor association, it would appear that the mechanism by which equilibrium is reached in the absence of these agonists differs from that by which it is reached in their presence. This is already apparent from a comparison of the half-times of the association binding reactions (Figures 1 and 2): for the binding of 1, 5, and 8 nM [^{3}H]TCP the half-times were 10–12, 4–5, and 1.5–2.5 min, respectively, in the presence of the agonists and 90–100, 80–90, and 90–100 min in their absence. Thus, the rate of [^{3}H]TCP binding to the receptor appears to depend on the ligand concentration in the presence of the exogenous agonists, but not in their absence.

A simple model that could account for both the equilibrium and the kinetic data on [^{3}H]TCP binding (eq 1) assumes that the binding of the ligand (L) to the receptor involves two processes: diffusion of the ligand from the outside (L_o) into the interior (L_c) of the NMDA receptor channel and, once inside, binding of the ligand to its receptor sites (R). Such a model was originally formulated by Furchgott (1955) to account for the existence of barriers in the way of a drug to its receptors. Assuming first-order diffusion kinetics, the process of binding of [^{3}H]TCP to the receptor is represented by

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

where k_a and k_b are the first-order rate constants for the forward and backward diffusion reactions, respectively, k_1 is the second-order rate constant for the formation of the receptor–ligand (RL) complex, and k_{-1} is the first-order rate constant describing the dissociation of the RL complex. This model cannot be solved numerically, but one can describe two extreme cases (Furchgott, 1955): (1) When the diffusion process is very fast relative to the binding reaction (i.e., when [^{3}H]TCP binds in the presence of glutamate and glycine to a presumably open state of the NMDA receptor channel), then $[L_c] = k_a/k_b[L_o]$, and the time course of receptor occupation is

$$\frac{[RL]_{eq} - [RL]}{[RL]_{eq}} = e^{-[k_1(k_a/k_b)[L]+k_{-1}]t} \qquad (2)$$

where [RL] and $[RL]_{eq}$ are the receptor–ligand complexes at time t and at equilibrium, respectively, and $[L] = [L_o]$ under conditions where there is only a small depletion of the added ligand ($[L_o] \gg [RL]_{eq}$). (2) When the binding reaction is very fast relative to the diffusion process (i.e., when [^{3}H]TCP binds to its receptor sites in the absence of glutamate and glycine), then $k_1[L_c][R] = k_{-1}[RL]$, and the rate of the association reaction is (Furchgott, 1955)

$$1 - \frac{(k_{-1}/k_1)(k_b/k_a)[RL]}{[L]([R_T] - [RL])} = e^{-k_b t} \qquad (3)$$

where $[R_T]$ represents the concentration of receptor sites. In either case (barrier limited and barrier unlimited), the overall equilibrium binding constant (K_d) is given by $(k_{-1}/k_1)(k_b/k_a)$.

The above cases appear to be consistent with the time course of association of [^{3}H]TCP with its receptor sites in the presence and in the absence of glutamate and glycine: binding of [^{3}H]TCP to the receptor in the presence of the agonists followed a pseudo-first-order reaction scheme (Figure 4A), and binding in the absence of the agonists followed a first-order kinetic pattern as predicted from eq 3 (Figure 4B). The rate

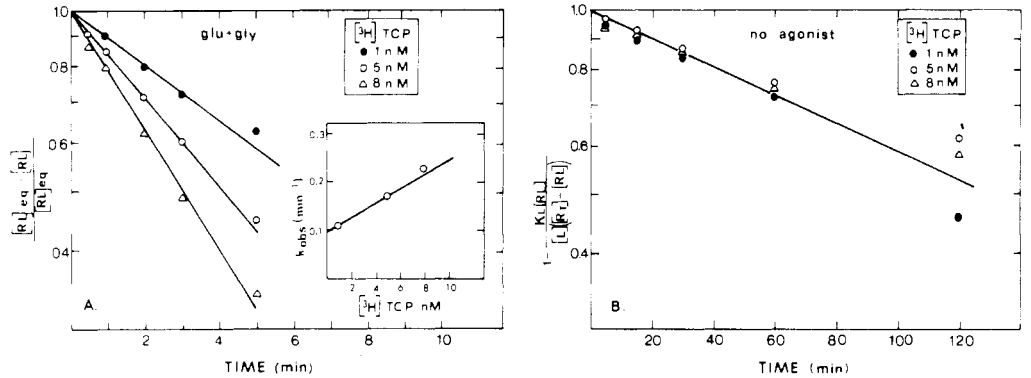

FIGURE 4: Kinetic analysis of the association reactions of [^{3}H]TCP with its receptors. (A) Pseudo-first-order plots of the association reaction of [^{3}H]TCP with its receptors in the presence of 1 μM glutamate and 1 μM glycine (Figures 1 and 2) according to eq 2. Inset: Observed time constants (k_{obsd}) plotted as a function of [^{3}H]TCP concentration. (B) Data presented in Figures 1 and 2 for the association reaction in the absence of agonists, replotted according to eq 3. The lack of dependence on the [^{3}H]TCP concentration and the linearity are consistent with a first-order reaction according to eq 3.

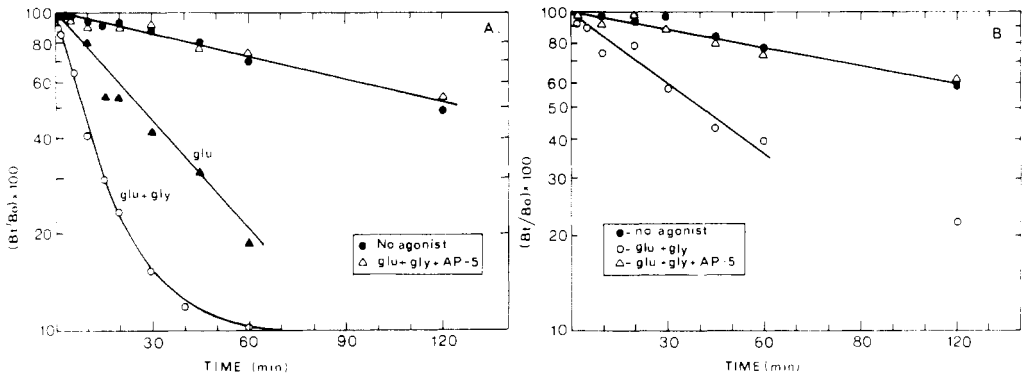

FIGURE 5: First-order plots of the dissociation of [^{3}H]TCP–receptor complexes. (A) Samples were incubated at 25 °C with 25 nM [^{3}H]TCP for 1 h. The dissociation reaction was initiated by the addition of 100 μM unlabeled PCP. Data show the dissociation reaction without added agonists (●) and in the presence of 1 μM glutamate (▲), 1 μM glutamate + 1 μM glycine (○), or 1 μM glutamate + 1 μM glycine + 100 μM AP-5 (△). Reactions were terminated either immediately (zero time) or at the indicated times. B_0 = amount of [^{3}H]TCP bound at zero time. B_t = amount of [^{3}H]TCP bound at time t. (B) The dissociation reactions were measured as detailed in (A), except that [^{3}H]TCP–receptor complexes were preformed in the absence (●) or in the presence of glutamate and glycine (○) or glutamate, glycine, and AP-5 (△). The data shown are from one of three experiments that gave similar results.

constants derived from the kinetic analysis are summarized in Table I. As shown, the ratio between the dissociation rate constant (k_{-1}) and the apparent association rate constant [$k_1(k_a/k_b)$] determined in the presence of glutamate and glycine is 7×10^{-9} M, which is very close to the K_d of [^{3}H]TCP determined at equilibrium (10×10^{-9} M). It is important to note that, in the absence of glutamate and glycine and according to eq 3, the on-rate of [^{3}H]TCP binding to the receptor will depend only on the backward diffusion rate constant (k_b). If this were the case, then the apparent first-order rate constant determined from the association kinetics should be equal to that determined from dissociation kinetics. As shown in Figure 5, in the absence of glutamate and glycine the dissociation reaction proceeded very slowly, and the apparent first-order rate constant of 4.8×10^{-3} min^{-1} (Table I) is very close to that determined from the association kinetics (5.4×10^{-3} min^{-1}).

Figure 5A demonstrates that the addition of glutamate to [^{3}H]TCP–receptor complexes that were preformed in the absence of the agonist resulted in a marked increase in the ligand–receptor dissociation rate (the half-times were 128 and 26 min, respectively, for the reactions in the absence and in the presence of glutamate). When glutamate and glycine were added together, the dissociation rate was further increased (half-time = 8–9 min; Figure 5A) and the first-order rate constant (k_{-1}) calculated from the initial dissociation rate was 0.08 min^{-1}, which is close to the value determined from the association kinetics (Table I). The agonist-induced acceleration in the dissociation rate of [^{3}H]TCP–receptor complexes was completely blocked by AP-5 (Figure 5). It is interesting to note that when [^{3}H]TCP–receptor complexes were preformed in the presence of glutamate and glycine, their dissociation rates were significantly lower ($t_{1/2}$ = 40 min; Figure 5B) than those obtained when the agonists were added only at zero dissociation time ($t_{1/2}$ = 8–9 min; Figure 5A).

Table I: Kinetic and Equilibrium Constants for [^{3}H]TCP Binding[a]

binding constant	value determined	method of determination
$k_1(k_a/k_b)$ (M^{-1} min^{-1})	1.4×10^7 (25)	association (Glu + Gly)
k_{-1} (min^{-1})	0.10 (28)	association (Glu + Gly)
k_{-1} (min^{-1})	0.08 (30)	dissociation (Glu + Gly)
k_b (min^{-1})	5.4×10^{-3} (22)	association (no agonist)
k_b (min^{-1})	4.8×10^{-3} (28)	dissociation (no agonist)
$(k_{-1}/k_1)(k_b/k_a)$ (M)	7×10^{-9} (33)	association (Glu + Gly)
K_d (M)	10×10^{-9} (35)	equilibrium (Glu + Gly)
K_d (M)	10×10^{-9} (35)	equilibrium (no agonist)

[a] The kinetic binding constants were calculated from the data presented in Figures 4 and 5 and the equilibrium binding constants (K_d) from the Scatchard plots shown in Figure 3B. The percentage difference between values determined in these experiments and replicate experiments is given in parentheses.

Discussion

The main finding of this work is that glutamate and glycine do not alter the equilibrium binding of [^{3}H]TCP to the receptor but rather that they alter the kinetics of the binding reaction. This finding has important implications for the mechanism of action of PCP-like drugs on the NMDA receptor channel; in particular, it indicates that in their effects

on [^{3}H]TCP binding the agonist glutamate (Watkins & Olverman, 1987; Fagg et al., 1986) and its effector glycine (Johnson & Ascher, 1987; Ascher & Novak, 1987) do not operate via an allosteric mechanism (no change in K_d and no change in B_{max}; Figure 3). This conclusion is well in line with the lack of effect of PCP–receptor ligands on [^{3}H]glutamate binding to the NMDA receptors (Foster & Wong, 1987; Monahan & Michel, 1987).

The kinetic data described here provide further evidence for the nonallosteric interactions between glutamate–receptor sites and [^{3}H]TCP–receptor sites and are compatible with the observed mode of NMDA receptor ion-channel blockade by PCP-like drugs: (1) The blocking effect of these drugs on the ion channel (Honey et al., 1985; Macdonald et al., 1986), like the rate of [^{3}H]TCP binding (Figures 1 and 2), is increased in the presence of agonists. (2) Repeated application of agonist accelerates not only recovery after blocking (Macdonald et al., 1986) but also the dissociation rate of [^{3}H]TCP–receptor complexes (Figure 5). (3) Glycine potentiates the NMDA receptor response to agonists (Ascher & Novak, 1987; Johnson & Ascher, 1987) and enhances the glutamate-induced increase in [^{3}H]TCP binding to the receptor (Johnson et al., 1987); similarly, in the present study it enhanced the glutamate-induced acceleration in the rate of [^{3}H]TCP association with (Figure 2) and dissociation from (Figure 5) the receptors. It should be noted that the glycine effect on NMDA receptors is strychnine-insensitive and is therefore not mediated via the glycine receptors (Ascher & Novak, 1987; Johnson et al., 1987). (4) The mechanism by which [^{3}H]TCP appears to bind to the receptors, in the presence and in the absence of agonists (eq 1), is strongly reminiscent of accelerated binding to the open state and delayed binding to the closed state of the NMDA receptor channel, as suggested by above electrophysiological data.

Our model [^{3}H]TCP binding to the NMDA receptor channel (eq 1) is supported by both the kinetic and the equilibrium binding data obtained under two extreme conditions, i.e., in the absence and and in the presence of the exogenous agonists glutamate and glycine. The principal requirements of such a model are fulfilled, namely, first-order kinetics for both association (Figure 4) and dissociation (Figure 5) binding reactions in the absence of agonists, pseudo-first-order kinetics of [^{3}H]TCP association with the receptors in the presence of the agonists (Figure 4), and similarity between the equilibrium binding constants (K_d) for [^{3}H]TCP in the presence and in the absence of agonists (Figure 3). These data, as well as the close agreement between the K_d values for [^{3}H]TCP determined kinetically and at equilibrium (Table I), suggest that the two sets of conditions in our experiments allow the binding process to occur in an almost ideal barrier-limited milieu (no added agonists) on the one hand and a barrier-unlimited environment (in the presence of glutamate and glycine) on the other. We know, however, that in spite of repeated washings cortical membranes nevertheless remain contaminated with some endogenous glutamate (or aspartate) (Loo et al., 1986; Foster & Wong, 1987). The existence of some free glutamate in our preparations may have led us to overestimate the backward diffusion time constant (k_b; eq 1). We can, however, assert that even when the glutamate sites are blocked (by AP-5), [^{3}H]TCP is able to reach its own sites (Figure 2) and dissociate from them (Figure 5). Thus, PCP-like drugs appear to bind (albeit very slowly) to the NMDA receptor channel even in the closed state.

In addition to the implicit assumption that in well-washed membranes with no added agonists the NMDA receptor channels are closed, we have also assumed that in the presence of saturating concentrations of glutamate and glycine there are no barrier limitations and thus the rate-limiting step of the binding reaction is the association of [^{3}H]TCP with its receptor sites (R + L → RL; eq 1). This assumption is validated by the good agreement between the dissociation rate constant (k_{-1}) determined from the initial on-rate kinetics of [^{3}H]TCP binding in the presence of glutamate and glycine and the k_{-1} determined by dissociation experiments (Table I). Two independent sets of experimental results show, however, that this no-barrier assumption is not valid under conditions in which the glutamate and the glycine sites are occupied for relatively long periods (>20 min): (1) As shown in Figure 5, the accelerated dissociation of about 80% of the [^{3}H]-TCP–receptor complexes, which occurs upon the addition of glutamate and glycine, was completed within the first 20 min in a simple exponential kinetic (k_{-1} = 0.08 min^{-1}). Thereafter, however, the acceleration effect declined, as shown by the reduced dissociation rate of [^{3}H]TCP–receptor complexes. (2) When [^{3}H]TCP–receptor complexes were preformed in the presence of exogenous agonists during incubation, their subsequent dissociation rate was again found to be slower ($t_{1/2}$ = 40 min; Figure 5) than when the agonists were added at zero dissociation time ($t_{1/2}$ = 8.3 min; Figure 5). Taken together, these data suggest that the glutamate and/or the glycine sites undergo changes with time, which in turn are reflected in the kinetics of [^{3}H]TCP binding.

Finally, the kinetic data described here together with previous electrophysiological (Barry et al., 1984; Macdonald et al., 1986) and neurochemical data (Jones et al., 1987) suggest that PCP-like ligands are steric blockers of the NMDA receptor channel. This type of interaction is analogous to the interaction of picratoxin with the GABA-ergic system (Tallman & Gallager, 1985) and the interactions of several noncompetitive blockers of the nicotinic cholinergic receptor with its ligand-operated cation channel (Guy & Hucho, 1987). In view of the 8-fold difference between the K_d values for [^{3}H]MK-801 observed in washed and in unwashed cortical membranes (Foster & Wong, 1987), one cannot rule out the possibility that noncompetitive channel blockers other than [^{3}H]TCP may act simultaneously as steric blockers and as allosteric effectors.

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Transbilayer Movement of Phosphatidylserine in Erythrocytes: Inhibition of Transport and Preferential Labeling of a 31 000-Dalton Protein by Sulfhydryl Reactive Reagents†

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ABSTRACT: A series of labeled thiolation reagents were synthesized on the basis of the parent structure pyridyldithioethylamine (PDA). These compounds specifically and reversibly inhibit the active intrabilayer transport of phosphatidylserine (PS) in human red blood cells. The binding of PDA to cells can be quantified since the thiol–disulfide exchange reaction yields a chromophore. In addition, the presence of a primary amine makes it amenable to derivatization with a variety of compounds. An iodinated derivative of PDA preferentially labeled a 31 000-dalton red blood cell peptide. The labeled component, which may represent the PS transporter, comigrated with integral membrane protein band 7.

The asymmetric distribution of phosphatidylserine (PS)[1] in the membrane of normal red blood cells (RBC) is unique since it is the only phospholipid that resides exclusively in the cells' inner leaflet (Verkleij et al., 1973; Gordesky et al., 1975). That maintenance of this asymmetry is an important component of homeostasis is suggested by the fact that the exposure of PS in the cells' outer leaflet has dramatic pathophysiological consequences. The translocation of endogenous PS from the inner to outer leaflet in sickled RBC, for example, or the exposure of exogenously inserted fluorescent PS analogues in RBC results in their recognition by cells of the reticuloendothelial system (RES) (Tanaka & Schroit, 1983; Schwartz et al., 1985; Schroit et al., 1985). These results have suggested that the appearance of PS on the cells' outer leaflet may play a specific role as a recognition moiety signaling RBC removal by the RES.

Although the translocation of PS seems to be an important factor of macrophage recognition in the maintenance of ho-

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[1] Abbreviations: [^{125}I]B/H, 3-(3-[^{125}I]iodo-4-hydroxyphenyl)-propionate (monoiodinated Bolton–Hunter reagent); 2-TP, 2-thiopyridone; DTT, dithiothreitol; DOPC, dioleoylphosphatidylcholine; HEPES–saline, 145 mM NaCl, 5 mM KCl, 20 mM HEPES, and 10 mM glucose; NBD-PS, 1-oleoyl-2-[[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]caproyl]phosphatidylserine; PDA, pyridyldithioethylamine; RBC, human red blood cells; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicles; TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid; Tnp, trinitrophenyl; DMSO, dimethyl sulfoxide; kDa, kilodalton(s).

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