Multiple Affinity States for Noncompetitive Blockers Revealed by [^{3}H]Phencyclidine Binding to Acetylcholine Receptor Rich Membrane Fragments from *Torpedo marmorata*†

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ABSTRACT: Phencyclidine, a potent noncompetitive blocker of the permeability response to acetylcholine, binds selectively (below 20 μM concentration) to the high-affinity *allosteric* site of the membrane-bound acetylcholine receptor from *Torpedo marmorata* electric organ [Heidmann, T., Oswald, R. E., & Changeux, J.-P. (1983) *Biochemistry* (preceding paper in this issue)]. The kinetics of [^{3}H]phencyclidine interaction with this particular site (present as a single copy per receptor light form) were investigated by a manual filtration technique and fluorescence stopped-flow measurements in the presence, and in the absence, of cholinergic ligands specific for the acetylcholine receptor site. When the membrane fragments were *equilibrated* with the agonist carbamoylcholine *prior* to the addition of [^{3}H]phencyclidine, the association and dissociation rate constants of [^{3}H]phencyclidine increased respectively from $(1.22 \pm 0.05) \times 10^3$ M^{-1} s^{-1} to $(7.2 \pm 0.5) \times 10^3$ M^{-1} s^{-1} and from $(7.5 \pm 0.1) \times 10^{-3}$ s^{-1} to $(9.8 \pm 0.7) \times 10^{-3}$ s^{-1}. On the other hand, preincubation with erabutoxin decreased both rates by a factor of about 2 from their value measured in the absence of effector. When carbamoylcholine was mixed with acetylcholine receptor rich membranes *simultaneously* with [^{3}H]phencyclidine, the association rate of [^{3}H]phencyclidine increased 10^3–10^4-fold and the dissociation rate 4–10-fold. These effects were not observed with the competitive antagonist *d*-tubocurarine. The data are interpreted in terms of a *four*-state model of the allosteric transitions of the acetylcholine receptor where D is a high-affinity slowly desensitized state, I an intermediate rapidly desensitized state, and A an active state, the only one where the ion channel is open. Rate constants for the interaction of [^{3}H]phencyclidine with these different conformations of the acetylcholine receptor are estimated. The results suggest that, in addition to its stabilization of the D state reported in the previous paper, phencyclidine binds with the fastest rate to the A state where the channel is open and accelerates a transition toward the I state.

The noncompetitive blockers (NCB)[1] of the permeability response to acetylcholine (AcCh) bind to, at least, three categories of "saturable" sites present on acetylcholine receptor (AcChR) rich membranes prepared from *Torpedo marmorata* electric organ (Heidmann et al., 1983). These sites are respectively (1) a high-affinity allosteric site (K_d = 0.2–5 μM) present as a unique copy per AcChR light form, (2) a large number of low-affinity allosteric sites (K_D greater than 10 μM) most likely located at the receptor protein–lipid interface, and (3) the AcCh binding sites themselves (K_D = 20–300 μM). Binding of NCBs to any of these sites was shown to elicit a slow stabilization of a "desensitized" conformation of the AcChR where the ion channel is closed and which exhibits a high affinity for cholinergic agonists [reference in the previous paper by Heidmann et al. (1983)].

Although this effect could account for the blocking of the response to AcCh under steady-state conditions, electrophysiological investigations have been primarily focused on a *rapid blocking* of the response interpreted, in most cases, as a direct and diffusion-controlled interaction of the NCBs with the ion channel itself (Adams, 1977; Neher & Steinbach, 1978). A large number of electrophysiological observations based on "noise" analysis, relaxation kinetics after voltage-jump, or single-channel recordings [reviewed in Adams (1981)] led to the suggestion that NCBs could block the permeability response to AcCh by entering and plugging the ion channel, thus sterically inhibiting ion translocation. A mechanism by which the *rapid blocking* would be mediated by an allosteric stabilization or an acceleration of a transition toward a closed channel conformation of the AcChR would be compatible with the electrophysiological data [see, e.g., Neher & Steinbach (1978), Katz & Miledi (1980), and Oswald et al. (1982)], although it would impose severe constraints on the time course and voltage dependence of this transition.

In the case of phencyclidine and histrionicotoxin, which interact only marginally with the low-affinity NCB sites and with the AcCh binding sites, the site relevant for the fast blocking of the response should be the high-affinity allosteric site for NCBs whose equilibrium binding properties and structural features have been analyzed extensively [see, e.g., Oswald & Changeux (1981a,b) and Heidmann et al. (1983)]. For instance, photoaffinity labeling studies with a covalent NCB, 5-azido[^{3}H]trimethisoquin (Oswald et al., 1980; Waksman et al., 1980; Oswald & Changeux, 1981b), or with [^{3}H]chlorpromazine (Oswald & Changeux, 1981a; Heidmann et al., 1983) have shown that the high-affinity site for NCBs resides at least partially on the δ subunit and is most likely located at the center of the AcChR molecule, in close vicinity

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[1] Abbreviations: AcCh, acetylcholine; AcChR, acetylcholine receptor; H_{12}HTX, perhydrohistrionicotoxin; Dns-C_6-Cho, 6-[5-(dimethylamino)naphthalenesulfonamido]hexanoic acid β-(methobromide) ethyl ester; NCB, noncompetitive blocker; PMSF, phenylmethanesulfonyl fluoride; α-[^{125}I]BGT, ^{125}I-labeled α-bungarotoxin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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to all five chains of the receptor molecule.

For further analysis of the mechanism of the noncompetitive blockade which accompanies the binding to the high-affinity NCB site, phencyclidine and its radioactive derivative, [^{3}H]phencyclidine, were selected as markers of the high-affinity site since, below 20 μM, essentially all of the binding can be attributed to this site. In this paper, we report *kinetic* measurements on the interaction of [^{3}H]phencyclidine with the membrane-bound AcChR by using a manual filtration technique and fluorescence stopped-flow recording. The data are interpreted in terms of a *four-state* model of the allosteric transitions of the AcChR developed by Heidmann & Changeux (1980) and Neubig & Cohen (1980).

Materials and Methods

Chemicals. Phencyclidine was a gift of Professors M. Sokolovsky and M. Lazdunski; erabutoxin b was provided by Dr. Tamiya; Dns-C_6-Cho was a gift of Dr. G. Waksman. [^{3}H]Phencyclidine and α-[^{125}I]BGT were purchased from New England Nuclear. Aprotinine, *d*-tubocurarine chloride, pepstatin, and PMSF were products of Sigma Chemical Co.; carbamoylcholine chloride was obtained from K & K Laboratories. Live *Torpedo marmorata* were provided by the Biological Station of Arcachon, France.

Preparation of AcChR-Rich Membranes. Membranes enriched in AcChR were purified from freshly dissected *Torpedo marmorata* electroplaque in a buffer designed to inhibit endogenous proteolytic activity (buffer A: 50 mM Tris-HCl, pH 7.5 at 20 °C, 3 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 5 units/mL aprotinine, and 5 μg/mL pepstatin; Saitoh et al., 1980). The membranes were stored until use in liquid nitrogen at a concentration of 20–25 μM in α-[^{125}I]BGT sites. The binding of α-[^{125}I]BGT was measured by the DE81 filter disk assay (Maelicke et al., 1977; Schmidt & Raftery, 1973).

Binding of [^{3}H]Phencyclidine. [^{3}H]Phencyclidine binding was measured by a modification of the procedure described by Eldefrawi et al. (1980a,b). Aliquots of 60 μL were applied to prewetted Whatman GF/B filter disks under negative pressure. The filters were washed immediately with 3 mL of Torpedo physiological solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7.0). The filters were then dried, and the radioactivity was measured by scintillation counting in a toluene/PPO/POPOP solution. Binding to the filter in the absence of tissue was less than 5% of the total signal, and the inclusion of 200 μM unlabeled phencyclidine decreased this by 50%, i.e., approximately 2.5% of the total signal. Nonspecific binding in the presence of tissue and 200 μM unlabeled phencyclidine was approximately 2.5% of the total signal.

Binding measurements were made by using AcChR-rich membranes at a concentration of 0.5–2 μM in α-bungarotoxin sites under the following conditions: (A) *Prior incubation with erabutoxin b*: Membranes were incubated with 10^{-5} M erabutoxin b for 30 min prior to the addition of [^{3}H]phencyclidine. (B) *No effector*: Membranes were not treated with any cholinergic effectors prior to the addition of a solution of [^{3}H]-phencyclidine which also did not contain cholinergic effectors. (C) *Prior addition*: Membranes were incubated with a given concentration of carbamoylcholine (except for one case in which *d*-tubocurarine was used) for at least 10 min prior to the addition of [^{3}H]phencyclidine. (D) *Simultaneous addition*: Membranes were not exposed to cholinergic effectors prior to the addition of a solution of [^{3}H]phencyclidine containing cholinergic effectors (in most cases, carbamoylcholine).

Conditions A–C will be considered "equilibrium" protocols as the AcChR is equilibrated with cholinergic effectors before NCB addition. Condition D will be considered a "transient" condition as receptor conformations not present in significant proportions at equilibrium will be present at the same time as [^{3}H]phencyclidine. All assays were performed in *Torpedo* physiological solution at 20 °C with polycarbonate test tubes.

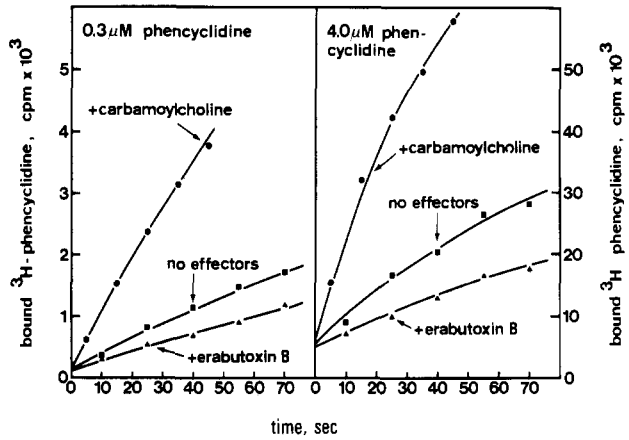


FIGURE 1: Kinetics of [^{3}H]phencyclidine association to AcChR-rich membranes from *Torpedo marmorata* electric organ: *effect of prior addition* of carbamoylcholine and erabutoxin b. Binding was measured at two concentrations of [^{3}H]phencyclidine (0.3 and 4 μM) under three conditions: (1) after preincubation and in the presence of 0.1 mM carbamoylcholine, (2) in the absence of cholinergic effectors, and (3) after preincubation and in the presence of 0.01 mM erabutoxin b. The curve drawn through the data points was calculated from the integrated form of the bimolecular rate equation (Maelicke et al., 1977; Oswald & Freeman, 1979), $k_1 = (1/t_i)\,[RP_\infty/(RP_\infty^2 - R_0P_0)]\{\ln [(RP_\infty - RP_i)/(R_0P_0 - RP_\infty RP_i)] + \ln [R_0P_0/(RP_\infty)]\}$, where k_1 is the bimolecular association rate constant, RP_i is the amount of binding at time t_i, RP_∞ is the amount of binding at equilibrium, R_0 is the total number of binding sites, and P_0 is the total amount of phencyclidine.

Stopped-Flow Experiments. Rapid kinetic experiments were carried out as described previously (Heidmann & Changeux, 1979) with a Gibson-Durrum stopped-flow rapid-mixing apparatus equipped for fluorescence detection. Phencyclidine and AcChR-rich membranes (0.1 μM in α-bungarotoxin sites) were preincubated, and the fluorescent agonist, Dns-C_6-Cho, was added under rapid-mixing conditions.

Quantitative Analysis of the Data. Association rate constants were calculated by a linear least-squares fit of the data to the integrated bimolecular rate equation (Maelicke et al., 1977; Oswald & Freeman, 1979). Dissociation rates were calculated by a linear least-squares fit to an exponential decay equation. Stopped-flow traces were best fitted by using a nonlinear iterative regression program (least-squares criteria) as previously described (Heidmann & Changeux, 1979, 1980).

Results

Equilibrium Conditions. The association and dissociation rates of [^{3}H]phencyclidine binding to the membrane-bound AcChR were measured under the following conditions: (1) after prior addition of carbamoylcholine (see Materials and Methods); (2) in the absence of effectors; and (3) after prior incubation with erabutoxin b.

(*A*) *Association.* Figure 1 shows that, qualitatively, preincubation of the membrane fragments with carbamoylcholine increased the rate of association of [^{3}H]phencyclidine while prior addition of erabutoxin b had the opposite action. In order to analyze quantitatively these effects, we measured the initial rates of [^{3}H]phencyclidine binding, but only at low ligand concentrations because of the temporal limitation of the manual filtration technique. The assumption was made that the initial binding event was bimolecular, and the asso-

Table I: Association and Dissociation Rate Constants of [^{3}H]Phencyclidine for the Membrane-Bound AcChR after Preequilibration with 0.1 mM Carbamoylcholine or 0.01 mM Erabutoxin b or in the Absence of Cholinergic Ligand

	k_1 (10^3 M^{-1} s^{-1})	SEM[a]	k_{-1} (10^{-3} s^{-1})	SEM	K_D[b] (μM)	K_D[c] (μM)
erabutoxin B	0.62	0.06	3.3	0.4	5.3	5.1
no effectors	1.22	0.05	7.5	0.1	6.1	3.6
carbamoylcholine	7.2	0.5	9.8	0.7	1.4	0.8

[a] Standard error of the mean. [b] $K_D = k_{-1}/k_1$. [c] K_D was taken from equilibrium measurements in Heidmann et al. (1983).

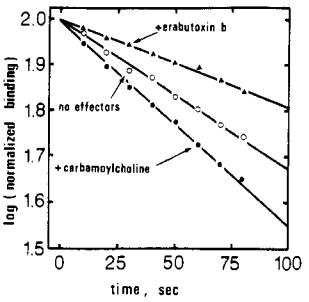


FIGURE 2: Dissociation of [^{3}H]phencyclidine from AcChR-rich membranes under the three conditions described in the legend to Figure 1. Membranes were incubated with or without cholinergic effectors for 10 min followed by addition of [^{3}H]phencyclidine to a final concentration of 0.8 μM. After a 30-min incubation, 1 μL of nonradioactive phencyclidine was added to a final concentration of 200 μM. An aliquot was assayed as described under Materials and Methods simultaneously with the addition of nonradioactive phencyclidine and at 10-s intervals thereafter. The data were normalized by dividing by the binding observed at zero time and multiplying by 100. The lines drawn through the points were generated from a linear least-squares fit to a first-order exponential decay.

ciation *rate*, k_1, was calculated from the equation given in the legend of Figure 1 [see also Maelicke et al. (1977)]. The experimental data could be adequately fitted by this equation (see Figure 1), and the value of k_1 was constant in the explored [^{3}H]phencyclidine concentration range (0.3–6 μM) with a concentration of α-[^{125}I]BGT sites of 0.5 μM. The average k_1 values obtained under these conditions are presented in Table I. Clearly, preincubation with carbamoylcholine quantitatively increased the association rate constant of [^{3}H]phencyclidine; the opposite effect was observed after equilibration of the membranes with erabutoxin b.

(*B*) *Dissociation.* The dissociation of AcChR–[^{3}H]phencyclidine complexes was measured after the addition of a 200-fold excess of unlabeled phencyclidine to a preequilibrated mixture of AcChR and [^{3}H]phencyclidine and in the presence or in the absence of cholinergic effector. As shown in Figure 2, the dissociation of previously formed AcChR–[^{3}H]phencyclidine complexes followed an exponential decay, and its rate was increased by prior addition of carbamoylcholine and retarded by erabutoxin b. The values of the dissociation rate constant, k_{-1}, calculated by assuming a first-order exponential decay, are given in Table I. The value of k_{-1} was constant for all initial concentrations of [^{3}H]phencyclidine tested (0.6–5 μM) and did not vary with the time elapsed between the addition of [^{3}H]phencyclidine and the addition of unlabeled phencyclidine.

The data for the dissociation rate can be used to estimate the error caused by washing the filters. The time between the onset of filtration and the end of the wash was reproducibly less than 2 s. As the dissociation followed exponential decay kinetics with half-lives between 1.1 and 3 min, the filtration process resulted in the underestimation of the actual binding by no more than 1–2%.

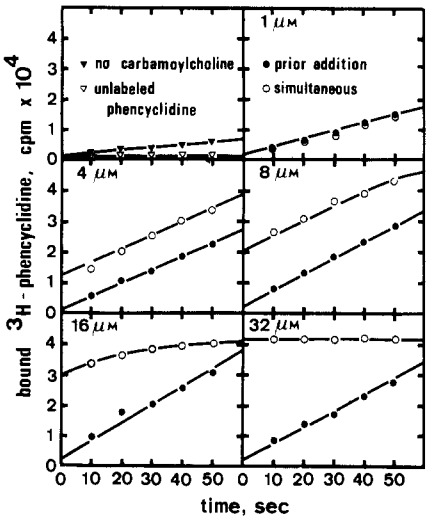


FIGURE 3: Rapid binding of [^{3}H]phencyclidine to AcChR-rich membranes upon "simultaneous" and "prior" addition of varying concentrations of carbamoylcholine. *Prior addition*: carbamoylcholine (concentrations indicated in the figure) was added 10 min prior to the addition of [^{3}H]phencyclidine. *Simultaneous addition*: carbamoylcholine was added simultaneously with the addition of [^{3}H]-phencyclidine. The [^{3}H]phencyclidine concentration was 0.8 μM in all cases.

Transient Processes. Carbamoylcholine and [^{3}H]phencyclidine were added *simultaneously* to AcChR-rich membrane fragments in order to study the *transient* conformations of the AcChR. Such a procedure allowed [^{3}H]phencyclidine to interact directly with conformational states occurring immediately after mixing with the agonist.

(*A*) *Association.* As shown in Figure 3, when the carbamoylcholine concentration was increased starting from 1 μM, an initial rapid-binding event was observed that could not be resolved by manual filtration techniques. Following this rapid event for carbamoylcholine concentrations smaller than 20 μM, a second slower rate could be observed which was related to that observed with the *prior addition* protocol. The data shown in Figure 3 were obtained by using 0.8 μM [^{3}H]phencyclidine, that is, the same concentration as the equilibrium dissociation constant of [^{3}H]phencyclidine for the AcChR in the presence of a saturating concentration of carbamoylcholine (Oswald & Changeux, 1981b; Heidmann et al., 1983). In addition to triggering the rapid-binding event, carbamoylcholine decreased the equilibrium dissociation constant of [^{3}H]phencyclidine for the AcChR (Eldefrawi et al., 1980a; Oswald & Changeux, 1981b; Heidmann et al., 1983) and caused an apparent increase in the association rate constant (see previous section) in a concentration-dependent manner. Thus, the concentration dependence of three processes could be inferred from the data in Figure 3: (1) final equilibrium level of binding; (2) association rate constant for the *prior addition* condition; and (3) amplitude of the unresolved rapid-binding event obtained by extrapolation of the second slower rate back to time zero. As shown in Figure 4, both the final equilibrium level of binding and the association rate constant for the *prior addition* condition had the same carbamoylcholine dependence, with the half-maximal effect at 1 μM carbamoylcholine. The rapid-binding event measured by an extrapolated amplitude reached half of its maximal value at a carbamoylcholine concentration of 10 μM.

As shown in Figure 5, the rapid event was not observed when the competitive antagonist *d*-tubocurarine replaced carba-

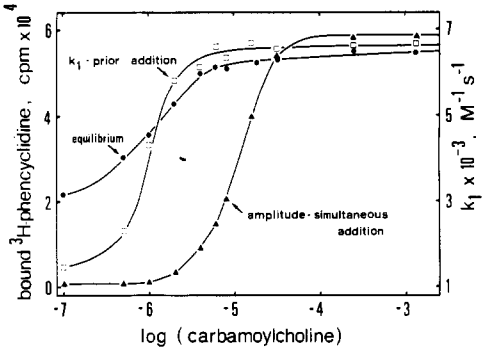


FIGURE 4: Effect of carbamoylcholine concentration on several parameters of [^{3}H]phencyclidine binding to AcChR-rich membrane fragments. The parameters were as follows: (1) k_1—*prior addition*, the bimolecular association rate constant measured under the prior addition protocol (see Materials and Methods); (2) *equilibrium*, the amount of binding measured after equilibrium was attained with 0.8 μM [^{3}H]phencyclidine; (3) *amplitude—simultaneous addition*, the amplitude of the rapid binding observed when carbamoylcholine and [^{3}H]phencyclidine were added simultaneously. The [^{3}H]phencyclidine concentration was 0.8 μM in all cases.

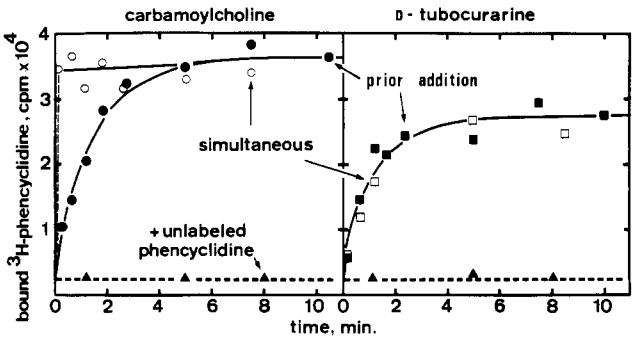


FIGURE 5: Binding of [^{3}H]phencyclidine to AcChR-rich membranes as a function of time. Either the simultaneous addition or the prior addition protocol (see Materials and Methods) was used with either 0.1 mM carbamoylcholine or 0.1 mM *d*-tubocurarine. The [^{3}H]-phencyclidine concentration was 0.8 μM in all cases.

moylcholine. *d*-Tubocurarine did, however, increase both the equilibrium dissociation constant and the association rate constant, possibly by stabilizing the same equilibrium state as carbamoylcholine.

(*B*) *Dissociation.* A transient event was also observed by following the dissociation rate after the simultaneous addition of carbamoylcholine and [^{3}H]phencyclidine. In such an experiment, saturating concentrations of carbamoylcholine and [^{3}H]phencyclidine were mixed with AcChR-rich membranes. After a variable interval of time, a small volume of unlabeled phencyclidine (1–2 μL; 200-fold excess over [^{3}H]phencyclidine) was added, and, at the same time, an aliquot of the original suspension was assayed. At various times after the addition of unlabeled phencyclidine, aliquots were sampled. Figure 6 shows the results of an experiment in which the time between the addition of carbamoylcholine and [^{3}H]phencyclidine and the addition of unlabeled phencyclidine varied. The most salient features of the results were that (1) the dissociation rate was biphasic and more rapid immediately after the addition of carbamoylcholine than later and (2) the rather slow linear dissociation rate noticed by using the *prior addition* protocol ($t_{1/2}$ = 1.1 min) was observed when the time between carbamoylcholine addition and unlabeled phencyclidine addition increased.

The rapidly dissociating component had an apparent half-time of dissociation of approximately 10 s. However, this value was considered as a lower limit on the real value of the $t_{1/2}$ of dissociation because the 2 s required for filtration and

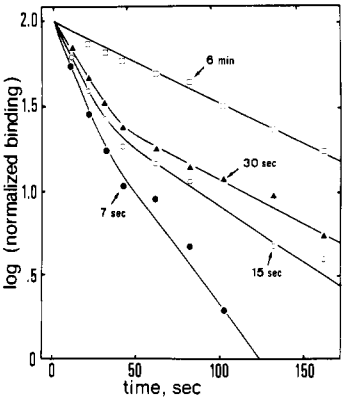


FIGURE 6: Dissociation of [^{3}H]phencyclidine from AcChR-rich membranes by using the simultaneous addition protocol (see Materials and Methods). [^{3}H]Phencyclidine (5 μM) and carbamoylcholine (0.1 mM) were added simultaneously; then at the times indicated on the figure (7 s, 15 s, 30 s, or 6 min), 1 μL of nonradioactive phencyclidine was added to a final concentration of 200 μM. An aliquot was assayed simultaneously with the addition of nonradioactive phencyclidine, and the data were normalized as described in the legend to Figure 2. The amount of binding at time zero was the same in all cases, and this value corresponded to the number of binding sites present for phencyclidine. Therefore, these dissociation curves are representative of all of the binding sites present rather than of simply a slowly dissociating population of sites.

washing of the filter undoubtedly led to the dissociation of a significant proportion of the AcChR–phencyclidine complexes. The more slowly dissociating component was similar to that observed after equilibration. Thus, again, a rapid and transient event was observed immediately following carbamoylcholine addition.

Stopped-Flow Kinetic Data. The rapid kinetics of the interaction of the fluorescent agonist Dns-C_6-Cho with the AcChR can be decomposed in at least three different relaxation processes referred to as "rapid", "intermediate", and "slow" (Heidmann & Changeux, 1979, 1980) and corresponding respectively to the diffusion-controlled binding of the agonist to a fraction of receptors already in a high-affinity desensitized (D) state, to the transient population of the remaining sites in an intermediate (I) state of the receptor in the 10–100-ms time range, and to the final stabilization of the D state in the time range of seconds.

As shown in Figure 7, the apparent rate constant (k_{app}) of the intermediate relaxation process increased with agonist concentration in a sigmoidal manner and leveled off at Dns-C_6-Cho concentrations higher than approximately 200 μM.

When phencyclidine was equilibrated with the AcChR-rich membrane fragments *before* being rapidly mixed with the fluorescent agonist, significant changes in the kinetics of Dns-C_6-Cho binding took place. Several of them concerned the stabilization of the D state of the AcChR and have been described and discussed in the preceding paper (Heidmann et al., 1983). We shall thus limit ourselves, in this paper, to the effects of phencyclidine on the *intermediate* relaxation process. As shown in Figure 7, preincubation of the membranes with saturating concentrations of phencyclidine (25 μM) resulted in an at least a 2-fold increase of the rate constant, k_{app}, for the intermediate relaxation process. The variation of k_{app} as a function of Dns-C_6-Cho concentration was, however, closely related to that measured in the absence of phencyclidine, with a sigmoidal shape and a leveling off at high agonist concentration.

The acceleration of the intermediate relaxation process by phencyclidine was analyzed further as a function of phencyclidine concentration under two conditions of Dns-C_6-Cho

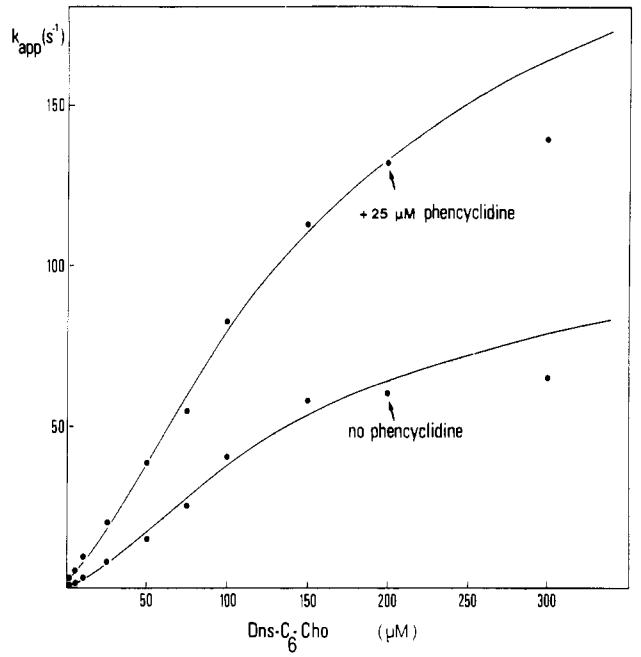


FIGURE 7: Apparent rate constant, k_{app}, of the state transition of the AcChR toward the intermediate state in the absence and presence of phencyclidine. AcChR-rich membrane fragments in *Torpedo* physiological solution (two membrane preparations tested, approximately 0.2 μM α-toxin binding sites) supplemented or unsupplemented with 25 μM phencyclidine were rapidly mixed in the stopped-flow apparatus with Dns-C_6-Cho solutions (concentrations indicated in the figure) in the same media. The rate constants of the intermediate relaxation processes [see Heidmann & Changeux (1979, 1980)] were determined by least-squares fit computer regressions and are plotted as a function of Dns-C_6-Cho concentration. The solid lines are the best fit of the data according to $k_{app} = (k_{-2} + k_{+2}[\text{Dns-}C_6\text{-Cho}]^2)/([(\text{Dns-}C_6\text{-Cho}] + K)^2]$ (see text) with $K = 88$ μM, $k_2 = 130$ s^{-1}, and $k_{-2} = 0.4$ s^{-1} in the absence of phencyclidine and $K = 92$ μM, $k_2 = 270$ s^{-1}, and $k_{-2} = 1.8$ s^{-1} in the presence of 25 μM phencyclidine.

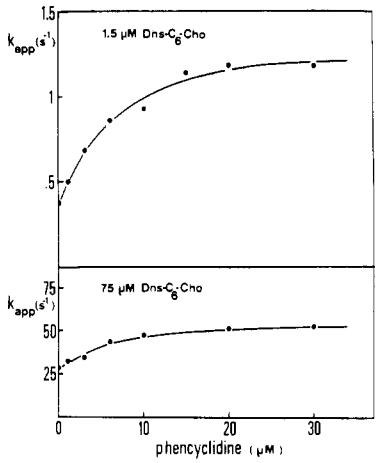


FIGURE 8: Concentration-dependent acceleration of the state transition of the AcChR toward the intermediate state as a function of phencyclidine concentration. 1:1 rapid mixing of suspensions of AcChR-rich membranes in *Torpedo* physiological solutions supplemented with the indicated concentrations of phencyclidine with solutions of 3 μM (upper panel) or 150 μM (lower panel) Dns-C_6-Cho in the same medium; the rate constants of the intermediate relaxation process were determined as above.

concentration: (a) 1.5 μM Dns-C_6-Cho and (b) 75 μM Dns-C_6-Cho. AcChR-rich membrane fragments were first preincubated with increasing concentrations of phencyclidine and then rapidly mixed with the indicated concentrations of Dns-C_6-Cho. The variations of k_{app} for the intermediate relaxation process under these conditions are represented in Figure 8.

Figure 8 shows that (1) the apparent dissociation constant for the accelerating effect of phencyclidine was equal to approximately 5 μM, a value consistent with the calculated equilibrium dissociation constant for the binding of phencyclidine to the AcChR in the absence of agonist (see preceding sections and Table I), and (2) the total extent of the k_{app} increase was more pronounced in the presence of low (approximately 3.2-fold) than in the presence of high (approximately 1.8-fold) concentrations of Dns-C_6-Cho, as observed previously with prilocaine [see Heidmann & Changeux (1979)].

Interpretation of the Data in Terms of a Four-State Model of the AcChR

In this section, the kinetic data obtained with [^{3}H]phencyclidine and Dns-C_6-Cho are compared with the predictions of the *four-state* model of AcChR allosteric transitions presented by Heidmann & Changeux (1980) and Neubig & Cohen (1980).

Equilibrium Conditions. Two main features of the data obtained under equilibrium conditions will be considered: (1) the absolute magnitude of the association rate constants of [^{3}H]phencyclidine; (2) comparison between the three equilibrium protocols, preequilibration with carbamoylcholine, preequilibration with erabutoxin b, and no effector.

(*A*) *Absolute Magnitude of Association Rate Constants.* The absolute magnitudes of the association rates are approximately 10^5-fold slower than expected for a diffusion-controlled interaction between a small ligand and a soluble globular protein (Eigen, 1974) and are, for instance, approximately 10^4–10^5-fold slower than the interaction of Dns-C_6-Cho with the membrane-bound AcChR (Heidmann & Changeux, 1979). Potential explanations for the slow binding rate of phencyclidine to the high-affinity allosteric site of the AcChR are the following:

(1) The binding site is in a sterically unfavorable region of the molecule such that the access of [^{3}H]phencyclidine is limited. This could arise if the binding site was in a crevice of the receptor or in contact with the lipid phase of the membrane, requiring phencyclidine to partition into the membrane prior to binding. In the first case, a tortuosity factor would limit diffusion, and in the second, a combination of partitioning of phencyclidine into the lipid phase and the increased viscosity of lipids relative to an aqueous solution would change the expected diffusion rate and effective concentration of phencyclidine.

(2) The binding of phencyclidine occurs only to a conformational state X that represents a minor fraction of the total AcChR population in the absence of phencyclidine but can be interconverted to the other states of the AcChR in the presence of phencyclidine. Accordingly, since phencyclidine association is found to be approximately 10^4-fold slower than diffusion-controlled association and since the dissociation constant of phencyclidine, at equilibrium, is in the micromolar range (Table I), the equilibrium dissociation constant for the binding of phencyclidine to the hypothetical state X would have to be in the 0.1 nM range. Dissociation constants of this magnitude have not been observed under any condition with phencyclidine, thus making this explanation unlikely.

(3) The kinetics of phencyclidine association are rate limited by a slow isomerization of the AcChR. Accordingly, the variation of the rate of phencyclidine association with phencyclidine concentration should be hyperbolic and level off for concentrations in the range of its equilibrium dissociation constant. No deviations from linearity were observed, however,

in the concentration range studied (up to 6 μM); neither was any biphasicity for association kinetics observed, which suggests either that hypothesis 3 is uncorrect or that the equilibrium dissociation constant of the AcChR in its native state is much higher than 6 μM, and most likely in the hundreds of micromolar range. Again, equilibrium dissociation constants of this magnitude have not been observed under any conditions for the binding of phencyclidine to its high-affinity site, which renders this third hypothesis unlikely.

This suggests that the binding kinetics measured in the "equilibrium" protocols reflect binding to the predominant receptor states existing under these conditions (R and D states). Thus, the first hypothesis of a limited access of phencyclidine to its site appears the most plausible one.

(*B*) *Comparison of Equilibrium Protocols.* The relative magnitudes of the changes in association and dissociation rates under different conditions of preincubation can be used, in a first approximation, to estimate the kinetic parameters of the interaction of phencyclidine with the R and D states.

(1) *D State.* Preincubation with carbamoylcholine (10^{-4} M) converts >99% of the AcChR molecules to the D state [reviewed in Heidmann & Changeux (1978, 1979)]. Thus, the values given in Table I for the desensitized condition can be considered as the kinetic parameters for the D state. As mentioned above, this interpretation is based on the assumption that neither a very high affinity receptor conformation present in small amounts nor an interconversion from a very low affinity state upon phencyclidine addition is responsible for the binding (hypotheses 2 and 3 above).

(2) *R State.* Estimation of the kinetic parameters for the R state is more difficult because no *unambiguous* treatment is available for converting 100% of the AcChR molecules to the R state. It has been suggested (Grünhagen & Changeux, 1976; Oswald & Changeux, 1981b) that erabutoxin b stabilizes the R state, but no direct method is available for estimating the isomerization constant between the R and D states in the presence of erabutoxin b. If nevertheless the assumption is made that essentially 100% of the AcChR molecules are in the R state in the presence of erabutoxin b, the kinetic values obtained in the presence of erabutoxin and given in Table I might be considered as those for the R state. This assumption can be tested to a limited extent by considering the kinetic parameters in the absence of effectors (see no effectors in Table I). Stopped-flow kinetic data (Heidmann et al., 1983) indicate that in the absence of effector, 20% of the AcChR molecules are in the D state and 80% in the R state, whereas at equilibrium in the presence of micromolar concentrations of phencyclidine, 50% is in the D state (the half-maximal effect occurs at 5 μM phencyclidine). If one assumes that the binding is more rapid than the isomerization, the initial association rate of binding should be simply proportional to a pseudo rate constant, $\bar{k}$, given by the following equation:

$$\bar{k} = f_R k_{1R} + f_D k_{1D} \quad (1)$$

where f_R and f_D are the fractions of AcChR in the R and D states, respectively, and k_{1R} and k_{1D} are the association rate constants for the R and D states, respectively. When the values obtained by preincubation with carbamoylcholine for k_{1D} and those obtained with erabutoxin b for k_{1R} are used, $\bar{k}$ is 1.9×10^3 M^{-1} s^{-1}, which is similar to the k_1 value derived in the absence of effectors, i.e., 1.2×10^3 M^{-1} s^{-1}. [The small deviation could be due to the forced fit of the binding data in the absence of effectors in terms of binding to a homogeneous class of sites and also to an overestimate of k_{1R}, since it was strongly suggested in the preceding paper (Heidmann et al., 1983) that in the presence of erabutoxin b there was still approximately 10% of the AcChR in the D conformation.]

In the case of the dissociation rate, a vast excess of unlabeled phencyclidine (200 μM) was added to previously equilibrated [^{3}H]phencyclidine–AcChR complexes. The AcChR would be expected to be initially 50% in the R state and 50% in the D state [see Heidmann et al. (1983)]. Again, eq 1 can be used except that the kinetic constants become dissociation rate constants, and f_R and f_D are both 0.5. This yields a value of $\bar{k}$ of 0.0066 s^{-1}, which is again close to the observed value of 0.0075 s^{-1}. Thus, the data are consistent with the notion that erabutoxin b stabilizes the R state, but the kinetic constants obtained in its presence must be considered as upper limits since the value of the isomerization constant L between R and D states should be such that 10% of D-state receptors are still present in the presence of erabutoxin b [see Heidmann et al. (1983)]. This interpretation is based on the assumption that erabutoxin b does not block sterically the NCB high-affinity site but rather alters its affinity in an allosteric manner which is consistent with the observation that the number of high-affinity sites for phencyclidine is the same both in the presence of either erabutoxin b and/or carbamoylcholine and in the absence of effector (Oswald & Changeux, 1981b; Heidmann et al., 1983).

(*C*) *Conclusions.* The interpretation of the equilibrium data in terms of the four-state model of the AcChR leads to the following conclusions: (1) the binding of [^{3}H]phencyclidine under the conditions of the equilibrium protocols plausibly represents an interaction with a sterically hindered site, and (2) both the association and dissociation rates for [^{3}H]phencyclidine are more rapid in the D state than in the R state. As shown in Table I, the equilibrium dissociation constants calculated from the rate constants are consistent with the dissociation constants determined in the previous paper with equilibrium binding measurements (Heidmann et al., 1983) for the "erabutoxin b" and "prior addition of carbamoylcholine" protocols. On the other hand, the calculated equilibrium dissociation constant for the "no effector" condition is significantly higher than that measured in the equilibrium studies. This is consistent with the interpretation mentioned above that in the "no effector" condition the AcChR is present as a mixture of both the R state and D state and that the percentages of R and D states are not the same in association and dissociation rate measurements.

Transient Conditions. The occurrence of a transient state (or states) which follows the rapid mixing with agonists and exhibits greatly enhanced rate constants for [^{3}H]phencyclidine can be readily interpreted in terms of the four-state model of Heidmann & Changeux (1980) and Neubig & Cohen (1980).

(*A*) *Association.* First of all, rapid-binding events take place after mixing with the agonist carbamoylcholine but not upon mixing with the competitive antagonist *d*-tubocurarine, supporting the view that they are linked to the pharmacological action of these compounds and thus possibly to the active A state of the AcChR where the ion channel is open. In addition, the concentration dependence of the effect of carbamoylcholine supports the interpretation that the rapid-binding events are associated with a state(s) other than those present in significant proportions at equilibrium. The data in Figure 4 show that 10-fold *less* carbamoylcholine is required to reach half of the maximal rate of [^{3}H]phencyclidine association when carbamoylcholine is preequilibrated with the membrane fragments than when it is rapidly mixed with them. A lower limit of 4×10^6 M^{-1} s^{-1} can be placed on the association rate constant of this rapid event since the kinetic process can reach more than 90% completion when binding is measured 5 s after the

addition of carbamoylcholine and [^{3}H]phencyclidine.

(*B*) *Dissociation.* The rapidly dissociating event can be more readily assigned to a dissociation from the I state rather than from the A state since (1) the slow dissociation rate ($t_{1/2}$ >10 s) is not consistent with the lifetime of the receptor in the A state and (2) the conversion of the rapidly dissociating state to the slowly dissociating state occurs in the time range of tens of seconds, which is the same as that for the transition of the I state to the D state (Heidmann & Changeux, 1979, 1980).

(*C*) *Stopped-Flow Kinetic Data.* As previously shown and analyzed by Heidmann & Changeux (1980), k_{app} for the intermediate relaxation process increases in a sigmoidal fashion with Dns-C_6-Cho concentration, and this concentration dependence can be fitted by the four-state model by assuming that (1) it concerns the transition toward the I state, (2) there are at least two AcCh binding sites per AcChR molecule, and (3) the binding processes are rapid compared to isomerization.

Scheme I

$$R \overset{K/2}{\leftrightarrow} RL \overset{2K}{\leftrightarrow} RL_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} IL_2$$

Accordingly, k_{app} is given by the following equation:

$$k_{app} = \left(\frac{[L]}{[L] + K}\right)^2 k_2 + k_{-2} \qquad (2)$$

The values for the intrinsic equilibrium dissociation constants (K) and for k_2 and k_{-2} can be calculated from the data in Figure 7 and are found equal to 88 ± 10 μM, 130 ± 25 s^{-1}, and 0.4 ± 0.1 s^{-1}, respectively.

Preincubation with phencyclidine results in an increase of k_{app}, but the variation of k_{app} with Dns-C_6-Cho concentration still follows a sigmoidal shape and levels off at high agonist concentration. The values of k_{app} measured in the presence of phencyclidine yield, according to eq 2, a value for the intrinsic equilibrium dissociation constant K equal to 92 ± 10 μM and k_2 and k_{-2} values equal to 270 ± 50 and 1.8 ± 0.5 s^{-1}, respectively. As expected for a model with preexisting states, preincubation with saturating concentrations of phencyclidine therefore does not modify the intrinsic binding constant K for Dns-C_6-Cho but only modulates the rates of the state transitions of the AcChR. Scheme I should therefore be rewritten as

Scheme II

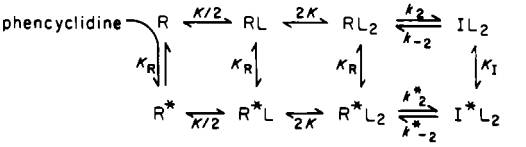
$$R^* \overset{K/2}{\leftrightarrow} R^*L \overset{2K}{\leftrightarrow} R^*L_2 \underset{k^*_{-2}}{\overset{k^*_2}{\rightleftharpoons}} I^*L_2$$

where the asterisk simply indicates that the NCB binding site is occupied by phencyclidine.

In Figure 8, the effect of phencyclidine concentration on k_{app} was measured under two conditions: (1) a low Dns-C_6-Cho concentration (1.5 μM) such that $k_{app} \simeq k_{-2}$, and (2) a high Dns-C_6-Cho concentration (75 μM) such that k_{-2} is negligible and k_{app} is proportional to k_2 (see eq 2). As noted under Results, the general conclusions were that the apparent dissociation constant for phencyclidine was 5 μM in both cases and that k_{app} increased by approximately 2–3-fold, depending on Dns-C_6-Cho concentration.

A general representation of the state transitions of the AcChR between the R and I conformations for intermediate phencyclidine concentrations should be that shown in Scheme III (see Schemes I and II) where K_R and K_I are the equilibrium dissociation constants for the binding of phencyclidine to the NCB binding site in the R and I conformations, respectively,

Scheme III



of the AcChR. A thermodynamic requirement in Scheme III is the following:

$$K_I \frac{k_2}{k_{-2}} = K_R \frac{k^*_2}{k^*_{-2}}$$

The ratio of K_I to K_R can be calculated from the data of Figure 8 (and also Figure 7) in that k^*_{-2}/k_{-2} is equal to $k_{app}^{\infty}/k_{app}^{0}$ in the top panel of Figure 8 and k^*_2/k_2 is equal to $k_{app}^{\infty}/k_{app}^{0}$ in the bottom panel of Figure 8. It can be therefore shown that

$$K_I/K_R \simeq 0.6$$

Given that K_R is equal to 5 μM, the value of K_I would thus be 3 μM. As the dissociation rate constant from the I state was found to be less that 0.069 s^{-1} ($t_{1/2}$ greater than 10 s), the association rate constant for the I state (i.e., $k_1 = k_{-1}/K_I$) must be less than 2 × 10^4 M^{-1} s^{-1}. The association rate constant for the rapid-binding event for [^{3}H]phencyclidine in the simultaneous addition protocol was found to be greater than 4 × 10^6 M^{-1} s^{-1}, i.e., at least 100-fold greater than the predicted value for association to the I state. This suggests that the rapid-binding event cannot be to the I state but is to the A state. In such a model, phencyclidine would bind to the receptor in the A state and both accelerate and promote the transition to the I state ($k^*_i > k_i$, $K_I < K_R$).

(*D*) *Conclusions.* The analysis in terms of the four-state model of the transient binding of [^{3}H]phencyclidine following mixing with the agonist carbamoylcholine and of the effect of phencyclidine on the intermediate relaxation associated with the rapid binding of the cholinergic agonist Dns-C_6-Cho suggests that (1) phencyclidine interacts with states different from the resting R and desensitized D states, which occur transiently upon mixing with the agonist carbamoylcholine, and (2) phencyclidine binds with the fastest rate to the A state and accelerates the transition to the I state.

Discussion

In agreement with the results of Eldefrawi et al. (1980b) and Aronstam et al. (1981) on perhydrohistrionicotoxin, we have shown in this paper and in the previous one (Heidmann et al., 1983) that [^{3}H]phencyclidine binding is, under equilibrium conditions, enhanced by the agonist carbamoylcholine and decreased by the antagonist erabutoxin b. Moreover, a dramatic enhancement of the association rate of [^{3}H]phencyclidine binding occurs transiently upon rapid mixing with the agonist carbamoylcholine but not with the competitive antagonist *d*-tubocurarine. Analysis of the data in terms of the four-state model of the AcChR suggests that this transient binding takes place primarily at the level of the A state but that acceleration of the transition to the I state also occurs.

Under conditions where the ion channel is closed, i.e., in the resting state in the absence of agonist and in the desensitized state after equilibration with carbamoylcholine, the absolute values of the association rates of [^{3}H]phencyclidine are, for instance, 10^4–10^5-fold lower than the values reported for the diffusion-controlled binding of the fluorescent agonist Dns-C_6-Cho to the AcChR sites (Heidmann & Changeux, 1979).

The most likely interpretation of these results is that the binding rates are limited by a conformation of the AcChR which introduces a severe restriction of diffusion in the region of the binding site. On the other hand, under conditions where the ion channel is expected to *open* at least transiently, i.e., after simultaneous mixing of the membranes with the agonist carbamoylcholine, the association rate of [^{3}H]phencyclidine binding becomes extremely *fast* ($>10^6$ M^{-1} s^{-1}) and approaches values predicted for a diffusion-controlled interaction of a water-soluble molecule with a freely accessible site [see Eigen (1974) and also see Adams (1977) and Neher & Steinbach (1978) for the rate of channel blockade by NCBs]. Thus, a *correlation* exists between the absolute values of the rates of phencyclidine binding and the state of opening of the ion channel.

Desensitization of the permeability response to AcCh occurs as a two-step process (Feltz & Trautmann, 1980, 1982; Sakmann et al., 1980; Walker et al., 1981). The stabilization of the high-affinity (D) state of the receptor by agonists has been assigned to the slow desensitization process (Weber et al., 1975; Heidmann & Changeux, 1979; Boyd & Cohen, 1980; Neubig et al., 1982) and the stabilization of a state of intermediate affinity, I state, to the fast desensitization process (Neubig & Cohen, 1980; Heidman & Changeux, 1980). The analysis of the *dissociation* of phencyclidine transiently following the mixing with carbamoylcholine and the stopped-flow data concerning the effect of phencyclidine on Dns-C_6-Cho fluorescence are consistent with such a two-step process. The rapid dissociation of [^{3}H]phencyclidine ($t_{1/2} \sim 15$ s) observed immediately after being mixed with carbamoylcholine becomes biphasic with rapid ($t_{1/2} \sim 15$ s) and slow ($t_{1/2} \sim 1$ min) dissociation rates when the time between the addition of carbamoylcholine and of unlabeled phencyclidine increases. Finally, after complete equilibration with carbamoylcholine, only a slow dissociation rate is found. The most plausible interpretation of these results jointly with the stopped-flow data is that the rapid dissociation of [^{3}H]phencyclidine is from the intermediate I state, which should be populated in a fraction of a second upon agonist addition (Heidmann & Changeux, 1980, and present data), and that the transition to the slowly dissociating state corresponds to the transition from the I to the D states, which occurs in the second-minute time range (Heidmann & Changeux, 1979).

A large set of electrophysiological experiments including "noise" analysis, voltage-jump relaxations, and single-channel recordings [reviewed in Adams (1981)] have led to the suggestion that NCBs could transiently enter and plug the open ion channel and, thereby, block ion translocation. The voltage dependence of the reaction rates for blockade of ion translocation suggested that the binding site for NCBs is located approximately one-half to three-quarters of the distance across the membrane (Adams, 1977; Neher & Steinbach, 1978). The present and the preceding (Heidmann et al., 1983) papers are compatible with this hypothesis in two ways: (1) The high-affinity allosteric site for NCBs is located close to all four chains of the AcChR, most likely in the central hydrophilic depression of the molecule (Cartaud et al., 1978; Klymkowsky & Stroud, 1979; Kistler et al., 1982); (2) the very slow association rates for phencyclidine when the majority of the channel is closed and the greater than 1000-fold increase in association rate in the presence of agonists are consistent with the hypothesis that the site is in the lumen of the ion channel and that opening the channel by the agonist transiently removes the diffusional restriction imposed by the closed channel, thus increasing the association rate.

Due to the rapid association of phencyclidine with the AcChR in the A conformation, other mechanisms compatible with the rapid blockade of the physiological response as observed in vivo with most NCBs also appear plausible. Yet, binding of NCBs to the A conformation not only might result in the steric blockade of the open channel but also might regulate the rate of the state transitions of the AcChR to *closed* channel conformations. This is precisely what is observed in the presence of phencyclidine for the transition of the AcChR to the I state, with an at least 2-fold increase of the rate of the transition; a similar increase was observed in vitro for the corresponding rapid inactivation of the permeability response (Karpen et al., 1982). This increase is, however, too small to exclude the "steric blockade" model as the most plausible one: (a) a significant decrease of the amplitude of the permeability response would indeed require that the rate of the transition to I in the presence of NCBs (approximately 100–200 s^{-1}; see Results) is much higher than the rate for channel closure in the absence of effector (i.e., 250–1000 s^{-1}; Adams, 1981), which is not the case, and (b) the voltage dependence of the blockade observed with charged NCBs but not neutral ones [see, e.g., Adams (1981)] strongly suggests that the rate-limiting step in the blockade of ion translocation is not a conformational transition of the AcChR molecule. However, it cannot be excluded that under appropriate experimental conditions, limited alterations in channel *kinetics* could be mediated via an allosteric interaction of NCBs with the A conformation of the AcChR.

In conclusion, in this paper and in the preceding one (Heidmann et al., 1983), evidence is presented that NCBs might interact with the AcChR (1) through multiple sites and (2) for given sites through multiple AcChR conformations. With phencyclidine in the low concentration range, binding takes place primarily at the level of the high-affinity NCB site which seems to be associated with the ion channel. At this level, phencyclidine can (1) stabilize, to various extents and in the second-minute time range, the high-affinity desensitized (D) state of the receptor prior to agonist addition and therefore regulate the fraction of AcChRs accessible for activation and (2) bind rapidly to the AcChR when in its open-channel conformation and at this level both block *sterically* ion translocation and accelerate *allosterically* some of the state transitions of the AcChR. These two classes of effects might be responsible for the complex inhibition of the permeability response elicited by NCBs when applied in bath and for the apparent lack of correlation between the effects of NCBs on (a) channel kinetics and (b) steady-state conductances or end-plate current peak amplitudes (Adams, 1977; Masukawa & Albuquerque, 1978; Koblin & Lester, 1979; Tiedt et al., 1979; Albuquerque et al., 1980a,b; Schofield et al., 1981; Spivak et al., 1982).

Added in Proof

Recent studies (R. E. Oswald, M. Bamberger, and J. Mc Laughlin, unpublished results) in which the concentrations of α-BGT sites and [^{3}H]PCP were varied over a larger concentration range indicate that the pseudo-first-order association rate is a hyperbolic function of PCP concentration and a linear function of α-BGT site concentration, in the absence of carbamoylcholine and following prior addition of carbamoylcholine. This suggests that the binding mechanism under these conditions may be more complex than that discussed here.

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References

Adams, P. R. (1977) *J. Physiol. (London) 268*, 291–318.
Adams, P. R. (1981) *J. Membr. Biol. 58*, 161–174.
Albuquerque, E. X., Tsai, M.-C., Aronstam, R., Witkop, B., Eldefrawi, A. T., & Eldefrawi, M. E. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1224–1228.
Albuquerque, E. X., Tsai, M.-C., Aronstam, R., Eldefrawi, A. T., & Eldefrawi, M. E. (1980b) *Mol. Pharmacol. 18*, 159–178.
Aronstam, R., Eldefrawi, A. T., Pessah, I. N., Daly, J. D., Albuquerque, E. X., & Eldefrawi, M. E. (1981) *J. Biol. Chem. 256*, 2843–2850.
Boyd, N. D., & Cohen, J. B. (1980) *Biochemistry 19*, 5344–5368.
Cartaud, J., Benedetti, L., Sobel, A., & Changeux, J.-P. (1978) *J. Cell Sci. 29*, 313–337.
Eigen, M. (1974) in *Quantum Statistical Mechanisms in the Natural Sciences* (Mintz, S. L., & Widmayer, S. M., Eds.) pp 37–61, Plenum Press, New York.
Eldefrawi, M. E., Eldefrawi, A. T., Aronstam, R. S., Maleque, M. A., Warnick, J. E., & Albuquerque, E. X. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7458–7462.
Eldefrawi, M. E., Aronstam, R. S., Bakry, N., Eldefrawi, A. T., & Albuquerque, E. X. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2309–2313.
Feltz, A., & Trautmann, A. (1980) *J. Physiol. (London) 299*, 533–552.
Feltz, A., & Trautmann, A. (1982) *J. Physiol. (London) 322*, 257–272.
Grünhagen, H. H., & Changeux, J.-P. (1976) *J. Mol. Biol. 106*, 497–535.
Heidmann, T., & Changeux, J.-P. (1978) *Annu. Rev. Biochem. 47*, 371–411.
Heidmann, T., & Changeux, J.-P. (1979) *Eur. J. Biochem. 94*, 255–279, 281–296.
Heidmann, T., & Changeux, J.-P. (1980) *Biochem. Biophys. Res. Commun. 97*, 889–896.
Heidmann, T., Oswald, R. E., & Changeux, J.-P. (1983) *Biochemistry* (preceding paper in this issue).
Karpen, J. W., Aoshima, H., Abood, L. G., & Hess, G. P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2509–2513.
Katz, B., & Miledi, R. (1980) *INSERM Symp. No. 13*, 171–178.
Kistler, J., Stroud, R. M., Klymkowsky, M. W., Lalancette, R., & Fairclough, R. H. (1982) *Biophys. J. 37*, 371–383.
Klymkowksy, M. W., & Stroud, R. M. (1979) *J. Mol. Biol. 128*, 319–334.
Koblin, D. K., & Lester, H. A. (1979) *Mol. Pharmacol. 15*, 559–580.
Maelicke, A., Fulpius, B. W., Klett, R. P., & Reich, E. (1977) *J. Biol. Chem. 252*, 4811–4830.
Masukawa, L. M., & Albuquerque, E. X. (1978) *J. Gen. Physiol. 72*, 351–367.
Neher, E., & Steinbach, J. H. (1978) *J. Physiol. (London) 277*, 153–176.
Neubig, R. R., & Cohen, J. B. (1980) *Biochemistry 19*, 2770–2779.
Neubig, R. R., Boyd, N. D., & Cohen, J. B. (1982) *Biochemistry 21*, 3460–3467.
Oswald, R., & Freeman, J. A. (1979) *J. Biol. Chem. 254*, 3419–3426.
Oswald, R., & Changeux, J.-P. (1981a) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3925–3929.
Oswald, R., & Changeux, J.-P. (1981b) *Biochemistry 20*, 7166–7174.
Oswald, R., Sobel, A., Waksman, G., Roques, B., & Changeux, J.-P. (1980) *FEBS Lett. 111*, 29–34.
Oswald, R., Heidmann, T., & Changeux, J.-P. (1982) in *Protides of the Biological Fluids* (Peeters, H., Ed.) Pergamon Press, Oxford and New York.
Saitoh, T., Oswald, R., Wennogle, L., & Changeux, J.-P. (1980) *FEBS Lett. 116*, 30–36.
Sakmann, B., Patlak, J., & Neher, E. (1980) *Nature (London) 286*, 71–73.
Schmidt, J., & Raftery, M. A. (1973) *Anal. Biochem. 52*, 349–354.
Schofield, G. G., Witkop, B., Warnick, J. E., & Albuquerque, E. X. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5240–5244.
Spivak, G. E., Maleque, M. A., Oliveira, A. C., Masukawa, L. M., Tokuyama, T., Daly, J. W., & Albuquerque, E. X. (1982) *Mol. Pharmacol. 21*, 351–361.
Tiedt, T. N., Albuquerque, E. X., Bakry, N. M., Eldefrawi, M. E., & Eldefrawi, A. T. (1979) *Mol. Pharmacol. 16*, 909.
Waksman, G., Oswald, R., Changeux, J.-P., & Roques, B. (1980) *FEBS Lett. 111*, 23–28.
Walker, J. W., McNamee, M. G., Pasquale, E., Cash, D. J., & Hess, G. P. (1981) *Biochem. Biophys. Res. Commun. 100*, 86–90.
Weber, M., David-Pfeuty, T., & Changeux, J.-P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3443–3447.