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Mechanism of Phencyclidine Binding to the Acetylcholine Receptor from *Torpedo* Electroplaque

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SUMMARY

The mechanism of phencyclidine binding to *Torpedo* acetylcholine receptor-rich membranes was investigated. The rate of [^{3}H]phencyclidine association is 10^3- to 10^4-fold more rapid when phencyclidine and carbamoylcholine are added simultaneously to acetylcholine receptor-rich membranes than when phencyclidine is added to membranes previously equilibrated with carbamoylcholine or membranes in the absence of carbamoylcholine. The mechanism of binding under conditions in which the slower rate was observed was studied with thermodynamic, viscosity, and kinetic experiments. Association and dissociation rates were highly dependent on temperature with activation energies of 26–30 kcal/mole. Viscosity had no effect on the association rate but increased the dissociation rate. These studies suggest that the binding is not diffusion-controlled but rather is limited by a significant energy barrier. The association rate was determined as a function of the concentration of acetylcholine receptor-rich membranes and the concentration of phencyclidine. In the presence of carbamoylcholine, the association rate was highly dependent upon the concentration of acetylcholine receptor but virtually insensitive to the concentration of phencyclidine. In the absence of carbamoylcholine, the association rate seemed to be a hyperbolic function of both the phencyclidine and the acetylcholine receptor concentration. The minimal model capable of explaining the data is a mechanism by which phencyclidine binds to two conformations of the acetylcholine receptor, one conformation having a higher affinity and constituting a lower percentage of receptors and the other having a lower affinity and constituting a higher percentage. The data are consistent with the possibility that the high-affinity conformation is the open-channel state of the acetylcholine receptor.

INTRODUCTION

The nicotinic AcChR[1] from the electric organ of *Torpedo* consists of four different subunits of apparent molecular weights: 40,000 (α), 50,000 (β), 60,000 (γ) and 66,000 (δ) (for review see refs. 1 and 2). These components exist in a 250,000 M_r complex with a stoichiometry of $\alpha_2\beta\gamma\delta$ (for review see refs. 1 and 2). Detergent solubilization, purification, and reconstitution into artificial lipid membranes have demonstrated that this complex alone is capable of AcCh-mediated ion flux. AcCh binds to a site located, at least in part, on each α subunit (1). The portion of the molecule forming the ion channel has not been definitively characterized. Electron microscopic observations of negatively stained AcChR preparations have disclosed a hydrophilic pit in the center of the multisubunit receptor rosette (2) that has been proposed as a possible site for an ion channel. A potential approach to characterizing the ion channel is the correlation of pharmacological, affinity labeling, and electrophysiological experiments on a series of compounds known collectively as noncompetitive blockers. These agents, which include aminated local anesthetics, histrionicotoxin, quinacrine, phenothiazines, and phencyclidine, bind to a high-affinity site on the AcChR that is distinct from the AcCh binding site (3–7) and is present in a stoichiometry of two AcCh binding sites per one high-affinity site for noncompetitive blockers (7). For most noncompetitive blockers (notably phencyclidine and chlorpromazine), the equilibrium affinity of this site is regulated by agonists and some antagonists, inasmuch as the affinity can be increased by the addition of agonists and antagonists relative to the absence of allosteric effectors. In addition, these agents seem to bind to variable numbers of low-affinity, lipid-associated sites that are capable of producing cooperative effects on the conformational transitions of the AcChR (7, 8). Electrophysiological observations,

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[1] The abbreviations used are: AcCh, acetylcholine; AcChR, acetylcholine receptor; EPC, end-plate current; PCP, phencyclidine; αBgt, α-bungarotoxin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid.

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including EPC analysis (9), noise analysis (10), voltage-jump analysis (11, 12), and single-channel recording (13), have led to the suggestion that noncompetitive blockers interact preferentially with the open-ion channel of the AcChR but may also have some interaction with the AcChR in closed-channel states. Multiple modes of action, including interactions with high- and low-affinity sites which either sterically block the ion channel or modify allosterically the conformation of the AcChR have been proposed (12, 14). The site of interaction of noncompetitive blockers with the AcChR has been investigated using affinity labeling techniques, either with a radioactive azido derivative of a local anesthetic (15) or by UV irradiation of radioactive noncompetitive blockers in the presence of AcChR-rich membranes (7, 16). In most cases, the δ subunit was labeled with the specificity characteristic of the reversible binding of noncompetitive blockers. One exception was [^{3}H]chlorpromazine, which labeled all four types of AcChR subunits with the specificity of the high-affinity site for noncompetitive blockers (16). More detailed studies (7) have demonstrated that the labeling of the four subunits is a result of binding to one high-affinity binding site on the AcChR, most likely the same binding site as that for histrionicotoxin and PCP. This suggests that at least four, and possibly all five, subunits may contribute to a single common binding site, possibly in the hydrophilic pit of the 250,000 M_r oligomer.

One apparent contradiction between the binding and affinity labeling studies and the electrophysiological studies is that the former are performed, in general, under conditions where most or all of the AcChR molecules are in the closed-channel conformation owing to absence of agonist or to desensitization by agonist (17, 18), whereas the latter can observe only open-ion channels. Binding studies (19, 20) and affinity labeling studies[2] performed under conditions in which a significant percentage of open channels is present transiently (i.e., *simultaneous addition* of agonist and noncompetitive blocker) led to a drastic increase in the association rate over conditions in which an extremely low percentage of open channels is present as a result of desensitization (i.e., *prior addition* of agonist before addition of noncompetitive blocker). Little change in the equilibrium parameters or affinity labeling pattern is observed between simultaneous and prior addition of agonist. The mechanism by which noncompetitive blockers interact at their high-affinity binding site with the AcChR following desensitization by agonist (or in the absence of agonist) is not clear.

The purpose of the studies described in this communication was to investigate the binding mechanism of noncompetitive blockers to their high-affinity site, using thermodynamic and kinetic techniques. The noncompetitive blocker, PCP, was used in all cases because of its high specificity for the high-affinity binding site and negligible interaction with low-affinity sites at concentrations below 10 μM (7). Thermodynamic and viscosity studies were performed to determine whether the interaction of PCP with the AcChR is diffusion-controlled—that is, whether restricted diffusion in the approach to the binding site results in a slow rate of binding or whether isomerization of the AcChR or PCP molecules limits the rate. Kinetic studies were designed to examine the reaction mechanism for PCP interaction with the AcChR.

[2] R. E. Oswald, unpublished observations.

MATERIALS AND METHODS

Preparation of membranes. AcChR-rich membranes were prepared from freshly dissected electroplaque from *Torpedo californica* and *Torpedo marmorata* as described previously (21). The membranes were stored in liquid nitrogen at a concentration of 10–20 μM (expressed in α[^{125}I]Bgt sites). Preparations ranged from 1 to 2 nmoles of α[^{125}I]Bgt sites per milligram of protein. *T. californica* was used exclusively in all experiments except the temperature experiments. For the temperature experiments, both *T. californica* and *T. marmorata* were used and gave identical results.

Binding assays. The binding of [^{3}H]PCP to AcChR-rich membrane fragments was performed using a modification (19) of the filter assay described by Eldefrawi *et al.* (20). Nonspecific binding, measured in the presence of a 200-fold excess of nonradioactive PCP, ranged between 5% and 20% of the total [^{3}H]PCP binding, depending on the concentrations of [^{3}H]PCP and AcChR-rich membrane fragments. In all cases, the concentration of [^{3}H]PCP was sufficiently low (<8 μM) so that binding to low-affinity sites was negligible, and only binding to the high-affinity site was observed. Except where noted otherwise, the buffer used was 50 mM MOPS-NaOH (pH 7.5)/1 mM EGTA. MOPS-NaOH was chosen because of its insensitivity to changes in temperature ($\Delta pK_a/^\circ = -0.006$). EGTA was included because calcium noncompetitively decreases the affinity of PCP for the AcChR (22) and activates a calcium-dependent protease in these membrane fragments (21).

The concentration of α[^{125}I]Bgt sites was measured with the DE81 filter disc assay (23) using 1% (w/v) Triton X-100 in 20 mM MOPS-NaOH (pH 7.5) as the buffer.

Data analysis. Equilibrium association (K_A) and dissociation (K_D) constants were calculated using a linear least-squares fit to the Scatchard equation. Pseudo-first order association rate constants (k_{obs}) and dissociation constants (k_{-1}) were calculated by a linear least-squares fit to the semilog transform of the data. Association rates were calculated, in most cases, from the initial 50% of the reaction, and, in all cases, less than 15% of the reactant in excess was bound at equilibrium. Kinetic data were used only under conditions in which less than 5% of the reactant in excess was bound and in which the data could be described by a single exponential ($r < -0.9$). Numerical integrations indicated that the deviations from pseudo-first order behavior were insignificant. More complicated mechanisms were modeled using the Runge-Kutta fourth-order method to solve numerically the differential equations.

Materials. PCP hydrochloride was obtained from U. S. Pharmacopeial Convention, Inc. (Rockville, Md.). [^{3}H]PCP (48 Ci/mmole) and α[^{125}I]Bgt (70–140 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). The radiochemical purity of [^{3}H]PCP was checked periodically by thin-layer chromatography on silica gel [1-butanol/acetic acid/water (25:4:10)]. Live *T. californica* were purchased from Pacific Bio-Marine (Venice, Calif.). Live *T. marmorata* were provided by the biological station of Arcachon, France.

RESULTS

Effect of temperature. The temperature dependence of the kinetic and equilibrium parameters of [^{3}H]PCP binding to AcChR-rich membranes was investigated in the presence and absence of carbamoylcholine. The rationale for these experiments was that a small temperature dependence would be expected for a diffusion-controlled reaction, whereas a significant temperature dependence

would be expected in cases where an energy barrier limits binding.

Although diffusion-controlled reactions typically have rate constants (approximately 10^9 M^{-1} sec^{-1}) several orders of magnitude greater than those observed for PCP binding to AcChR-rich membranes (19), orientational constraints (24) can decrease the rate constant of a diffusion-controlled reaction by several orders of magnitude. A *restricted* diffusion-controlled mechanism could arise, for example, from a binding site situated in a crevice of the AcChR protein such that a precise orientation would be required for entering the crevice, and diffusion would be restricted within the crevice.

Kinetic conditions. The apparent association rate of [^{3}H]PCP with AcChR-rich membrane fragments was determined at various temperatures between 0° and 37°. Figure 1 shows the Arrhenius plots of the temperature dependence of the apparent association rate for [^{3}H]PCP binding in the presence and absence of 0.2 mM carbamoylcholine. The activation energy (E_a), calculated from the slope of the Arrhenius plot, was 26.0 kcal/mole in the presence of carbamoylcholine and 29.7 kcal/mole in its absence. The magnitude of this temperature effect can be illustrated better by the Q_{10} values between 20° and 30°, which were 4.3 in the presence of carbamoylcholine and 6.4 in its absence.

Figure 2 shows the Arrhenius plots for the dissociation of [^{3}H]PCP-AcChR complexes (measured by the addition of excess unlabeled PCP to previously equilibrated [^{3}H]PCP-AcChR complexes). An extreme temperature dependence was also observed for dissociation rates, with activation energies of 29.7 kcal/mole in the presence of carbamoylcholine and 26.4 kcal/mole in its absence. Q_{10} values between 20° and 30° were 5.4 in the presence of carbamoylcholine and 4.5 in its absence.

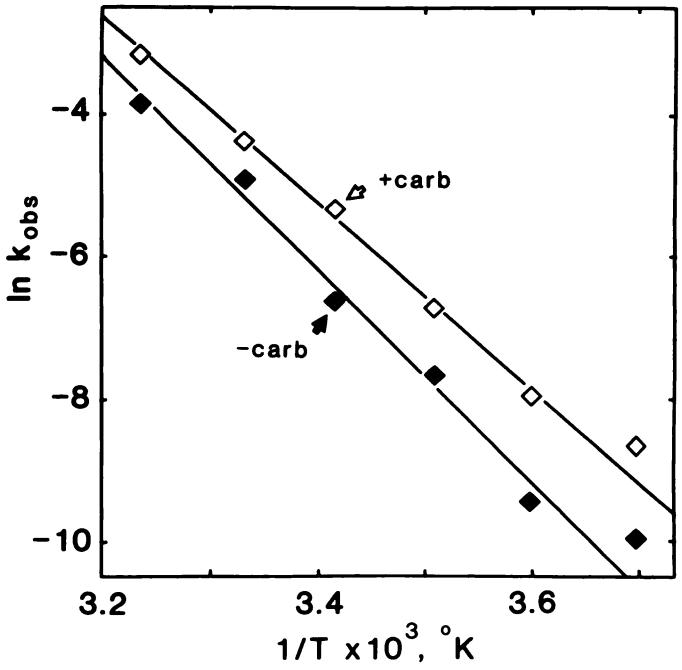

FIG. 1. *Arrhenius plot of the pseudo-first order association rate constants for the binding of [^{3}H]PCP to AcChR-rich membranes*

The data are a composite of three independent experiments. The standard error of the mean in this and subsequent figures is shown only when it is larger than the size of the plotting symbol. The AcChR concentration was 50 nM (100 nM in αBgt sites), the [^{3}H]PCP concentration was 100 nM, and the carbamoylcholine concentration (when used) was 0.2 mM. (1/*T*, °K for 0° is 0.003663 and for 37° is 0.003226). Approximately 11% of the total [^{3}H]PCP was bound at equilibrium in the presence of carbamoylcholine and 3% in the absence of carbamoylcholine.

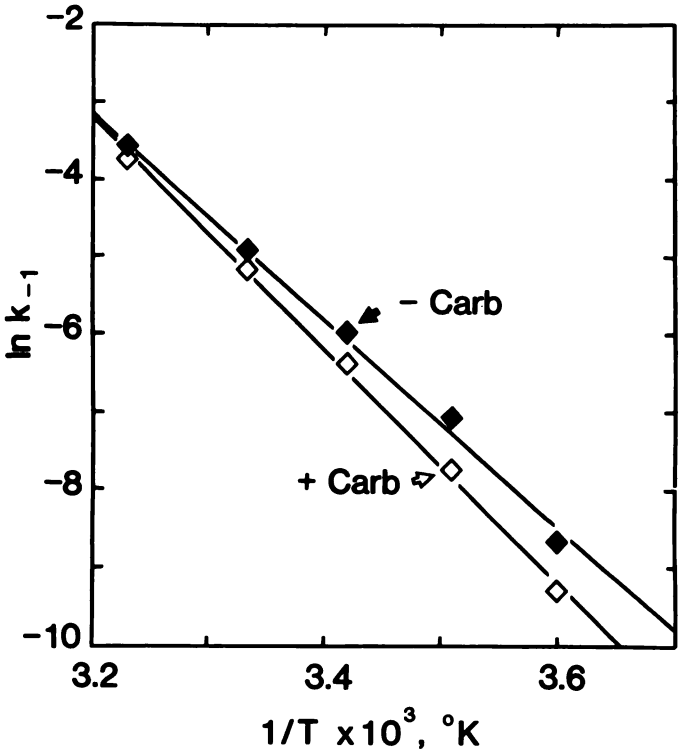

FIG. 2. *Arrhenius plot of the first-order dissociation rate constants for the decomposition of [^{3}H]PCP-AcChR complexes*

The data are a composite of three independent experiments. The AcChR concentration was 50 nM (100 nM in αBgt sites), the [^{3}H]PCP concentration was 100 nM, and the carbamoylcholine concentration (when used) was 0.2 mM. Dissociation was measured by the addition of nonradioactive PCP to 150 μM.

To demonstrate that these temperature effects were reversible and not due to an irreversible structural change in the AcChR, an experiment was performed in which the temperature was varied either up or down and the change in the rate of [^{3}H]PCP association in the presence of carbamoylcholine was observed. The association rate at 30° was approximately 10-fold greater than that at 10°. When AcChR-rich membranes were equilibrated with 0.2 mM carbamoylcholine at 10° and, immediately following the addition of [^{3}H]PCP, were transferred to a 30° water bath, the association rate was slow at first, corresponding to a temperature near 10°, gradually becoming more rapid as the solution warmed (Fig. 3). When equilibration was at 30° and a transfer to a 10° bath was made, the rate was fast at first and gradually slowed. The calculated curves (see the legend to Fig. 3) in each case had the same shape as the experimental points; however, some discrepancy was observed, presumably due to the heat capacity of the probe and the response time of the digital thermometer. These data demonstrate that the strong dependence of reaction rates

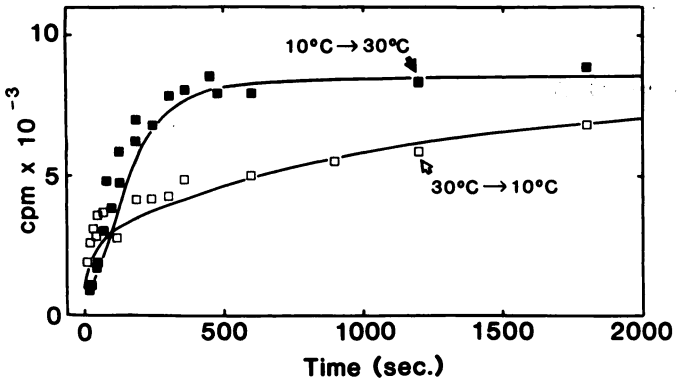

FIG. 3. *Association of [³H]PCP with AcChR-rich membranes during a temperature change*

Membranes were equilibrated in the presence of 0.2 mM carbamoylcholine at either 30° or 10°. [³H]PCP (100 nM) was then added, and the tube was transferred to a water bath of a different temperature (30° to 10°; 10° to 30°). The binding was then measured as a function of time. Because the equilibrium level of binding in the presence of carbamoylcholine is essentially constant between 0° and 37° (see Equilibrium conditions, under Results), the experiment can be modeled using the measured values of temperature as a function of time and calculating the new pseudo-first order association rate constant from the linear relationship between the logarithm of rate and the reciprocal of the absolute temperature. The expected curve can then be generated using a pseudo-first order equation in which the association rate "constant" is adjusted for changes in temperature at 0.1-sec intervals.

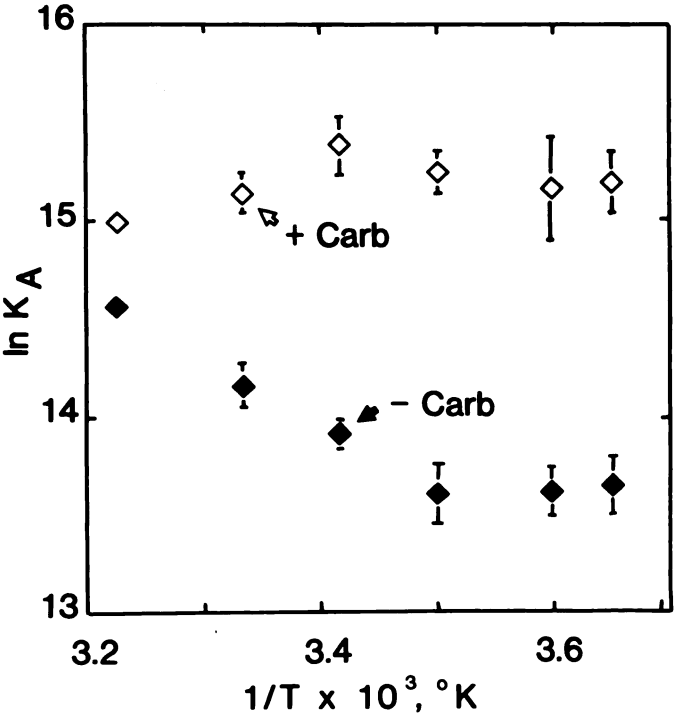

FIG. 4. *Van't Hoff plot of the equilibrium binding of [³H]PCP to AcChR-rich membranes*

Equilibrium association constants (K_A) were calculated using the Scatchard transformation. The time of incubation was sufficient for equilibration at all concentrations of PCP and varied between 20 min at 37° and 8 hr at 0°. The 0° condition was repeated with a 24-hr incubation and gave results identical with the 8-hr incubation. The AcChR concentration was 50 nM, the [³H]PCP concentration ranged between 50 nM and 8 μM, and the carbamoylcholine concentration (when used) was 0.2 mM. Nonspecific binding was assessed by the addition of 0.5 mM nonradioactive PCP. The data are a composite of three independent experiments, and the error bars represent the standard error of the mean.

on the temperature is entirely reversible and not due to an irreversible structural change of the AcChR.

Equilibrium conditions. Unlike the kinetic constants, the measured equilibrium binding constants had only a minor temperature dependence, as shown in the van't Hoff plots in Fig. 4. In the presence of carbamoylcholine, very little change with temperature was observed for the equilibrium association constant (K_A), although the affinity seemed to be consistently 10%–20% lower at 37°. In the absence of carbamoylcholine, the affinity decreased slightly with decreasing temperature. Similar results have been reported by Eldefrawi *et al.* (25). Because of the very slight temperature dependencies of the equilibrium constants, accurate values for the thermodynamic parameters were not possible to obtain; however, some conclusions can be drawn. The relatively constant K_A in the presence of carbamoylcholine and the decreasing K_A in the absence of carbamoylcholine with decreasing temperature suggest a zero or small positive $\Delta H°$; therefore, $\Delta S°$ is relatively large and positive to account for the negative $\Delta G°$. That is, the binding is largely entropy-driven.

Thus, the results of the thermodynamic studies demonstrate that a significant enthalpic barrier exists to association and dissociation but that the equilibrium affinity has only a minor enthalpic component. This is consistent with a binding reaction that is not diffusion-controlled (26).

Effect of pH. One possible explanation for the temperature dependence would be a temperature-dependent change in the ionization constant (pK_a) for an ionizable moiety that is involved in the binding reaction. For this reason, association rates were determined at various values of pH. As shown in Fig. 5A, the association rate increased with pH up to approximately pH 8.5; at pH values higher than 10, the association rate began to decrease. The equilibrium level of binding followed approximately the same pH dependence (Fig. 5B). This difference seems to be due to a change in the affinity for [³H]PCP rather than to a change in the number of binding sites (data not shown). The similar pH dependence for the association rate in the presence and absence of carbamoylcholine suggests that the effect is not due to a modification of the affinity for carbamoylcholine. The pH effect may be due to the state of ionization of PCP, because the pK_a of PCP is 8.3 (27), and its quaternary amine derivative (PCP methiodide) has an approximately 5-fold lower affinity for the AcChR (25). These results indicate that changes in pK_a most likely do not explain the thermodynamic data, because the magnitude of the pH effect was much smaller and in a direction opposite to that expected, were temperature modifying the pK_a.

Effect of solvent viscosity. The Smolochowski equation for a diffusion-controlled reaction suggests that a diffusion-controlled association rate should be inversely proportional to solvent viscosity. As an additional test of the notion that the association rate is not diffusion-

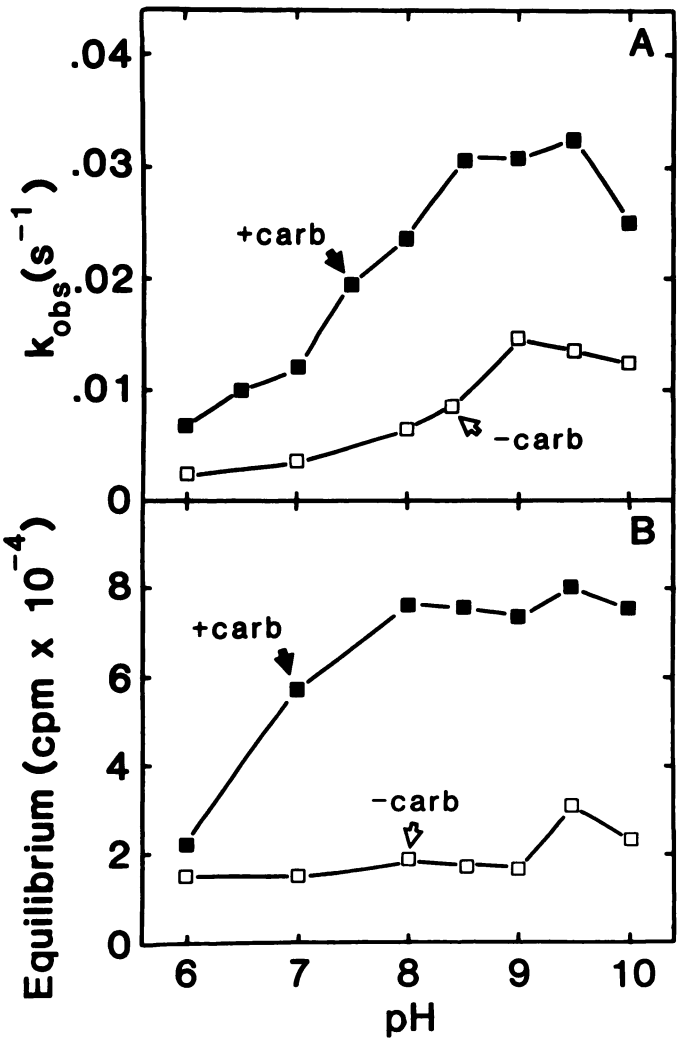

FIG. 5. *Binding of [^{3}H]PCP to AcChR-rich membranes as a function of pH of the medium at 24°*

A. Pseudo-first order association rate is shown as a function of pH in the presence and absence of 0.2 mM carbamoylcholine. The AcChR concentration was 50 nM and the [^{3}H]PCP concentration was 200 nM. B. Equilibrium binding of 200 nM [^{3}H]PCP with 50 nM AcChR as a function of pH. The buffer was 100 mM sodium phosphate in all cases. Approximately 9% of the total [^{3}H]PCP was bound at equilibrium in the presence of carbamoylcholine and less than 2% in the absence of carbamoylcholine.

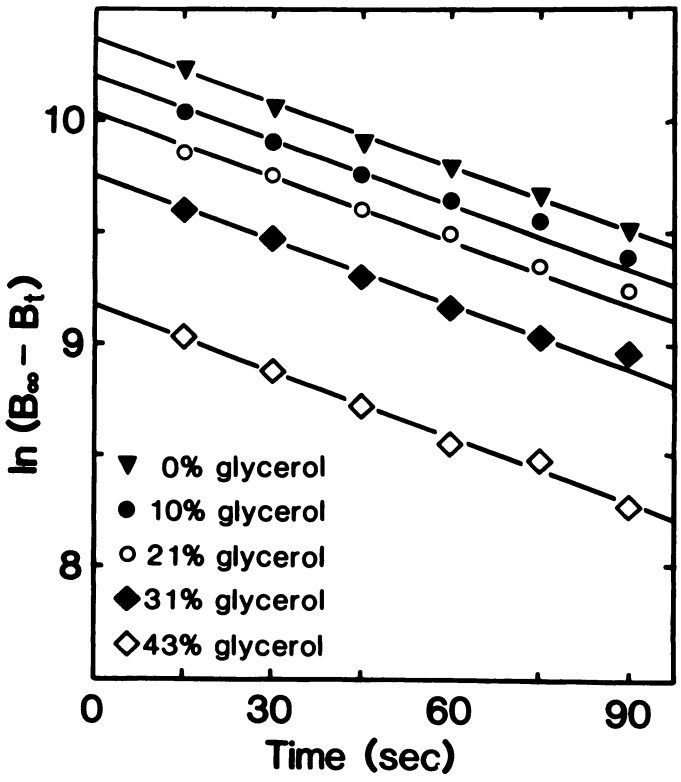

FIG. 6. *Association of [^{3}H]PCP with AcChR-rich membranes at 24° in the presence of varying concentrations of glycerol (in the presence of 0.2 mM carbamoylcholine)*

The initial concentration of [^{3}H]PCP was 300 nM and that of AcChR was 100 nM. The kinematic viscosities ranged between 1 and 5 centistokes for glycerol solutions between 0 and 50% (v/v). Viscosities were measured using an Ostwald viscometer at 24°. Similar results were observed when the viscosity was varied using sucrose. Approximately 15% of the total [^{3}H]PCP was bound at equilibrium.

controlled, the association rate was measured in solvents in which the viscosity was varied by the addition of glycerol or sucrose. As shown in Fig. 6, the pseudo-first order association rate constant (slope of the plot) did not vary; however, the equilibrium level of binding did seem to decrease with increasing viscosity. Equilibrium binding experiments confirmed that this decrease was due to a change in affinity rather than a change in the number of binding sites (data not shown). No change in association rate was observed in either glycerol or sucrose with viscosities as high as 5.2 centistokes. Because the association rate for a diffusion-controlled reaction is expected to decrease with increasing viscosity, these results are consistent with the notion that the association rate is not diffusion-controlled.

As shown in Fig. 7, this decrease in affinity seemed to be due to an *increase* in the dissociation rate with increasing viscosity. A diffusion-controlled dissociation rate is expected to *decrease* with increasing viscosity, suggesting that the dissociation rate was not limited by a restricted diffusion-controlled mechanism.

Effect of AcChR and PCP concentrations. The effect of AcChR-rich membrane and PCP concentration on the association rate of AcChR-PCP complexes was observed over a 60-fold range of concentration of PCP and a 120-fold concentration range of AcChR-rich membranes. The results observed in the presence of 0.2 mM carbamoylcholine are shown in Fig. 8. In the presence of 60 nM PCP binding sites (120 nM αBgt sites), the pseudo-first order association rate (k_{obs}) seemed to be a hyperbolic function of PCP concentration. The accuracy of data obtained at concentrations greater than 1 μM was limited by a relatively high level of nonspecifically bound radioactivity due to the low concentration of binding sites used in these experiments.

The variation of association rate with changes in the concentration of AcChR-rich membranes was markedly different from that observed with variations in the [^{3}H]PCP concentration. The association rate increased with increasing AcChR-rich membrane concentration (expressed in terms of the concentration of AcChR monomers). No evidence of a plateau was observed for concen-

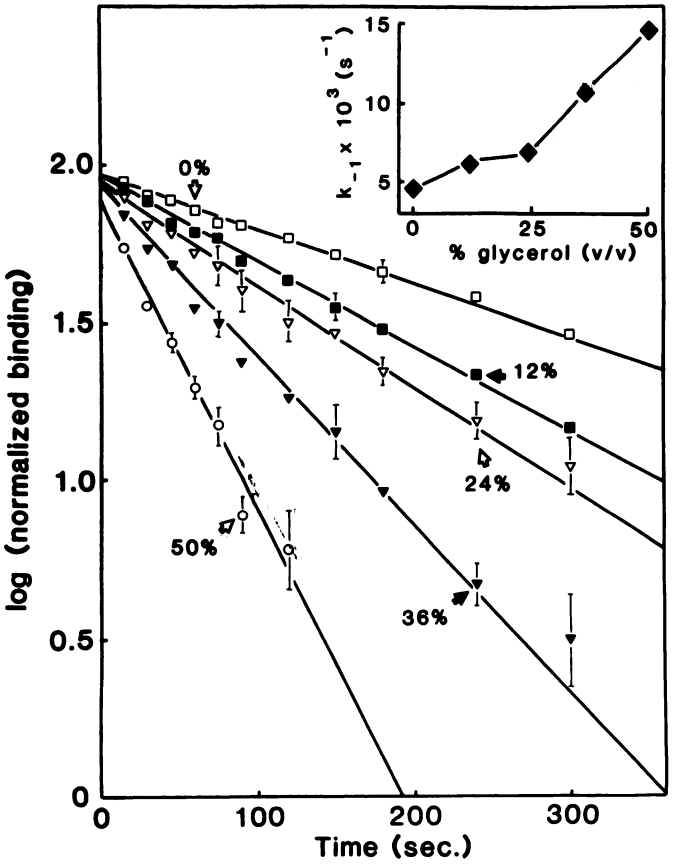

FIG. 7. *Dissociation of [^{3}H]PCP-AcChR complexes at 24° in the presence of varying concentrations of glycerol (in the presence of 0.2 mM carbamoylcholine)*

The initial concentration of [^{3}H]PCP was 200 nM and that of AcChR was 50 nM. Viscosities are given in the legend to Fig. 6. The error bars represent the standard error of the mean of these determinations.

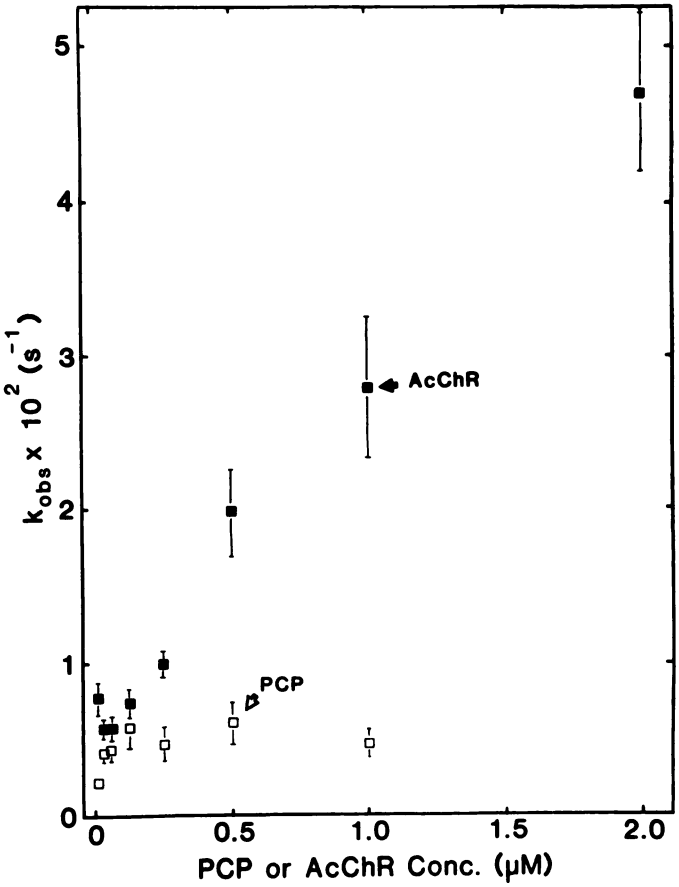

FIG. 8. *Pseudo-first order association rate constants (k_{obs}) as a function of either PCP or AcChR concentration in the presence of 0.2 mM carbamoylcholine at 24°*

The concentration of AcChR was 60 nM when the PCP concentration was varied, and the PCP concentration was 60 nM when the AcChR concentration was varied. In most cases, less than 50% of the course of the reaction was measured, and, in all cases, the results were checked graphically for first-order exponential behavior. Data sets with linear correlation coefficients less than −0.9 for a semilog transform of the data were discarded. Each point represents the mean of six to nine determinations, and the error bars show the standard error of the mean. Less than 15% of the total [^{3}H]PCP was bound at equilibrium in all cases.

trations of AcChR up to 2 μM. Above this concentration, the association rate became too rapid to measure accurately with manual techniques.

The association rate in the absence of carbamoylcholine also was determined as a function of AcChR and PCP concentrations (Fig. 9). In the absence of carbamoylcholine, the variation in the rate as a function of [^{3}H]PCP concentration was much more difficult to measure than the rate in the presence of carbamoylcholine, particularly at [^{3}H]PCP concentrations greater than 0.5 μM. The general shape, however, was hyperbolic. In the absence of carbamoylcholine, variation in k_{obs} as a function of AcChR concentration in the absence of carbamoylcholine seemed to be essentially the same as the variation as a function of PCP concentration.

The magnitude of the increase in apparent association rate in the presence of carbamoylcholine relative to the absence of carbamoylcholine was controlled almost exclusively by the concentration of AcChR. These data strongly suggest that a conformational change in the AcChR is associated with the binding of PCP in the presence of carbamoylcholine.

DISCUSSION

The analysis of noncompetitive blocker action on the AcChR has been pursued by direct binding studies and by studies which observe the modification of AcChR properties as a result of interaction with noncompetitive blockers (2). Direct binding studies have the potential of defining the stoichiometry and affinity of sites at which the noncompetitive blockers interact with the AcChR. This analysis is complicated by multiple binding sites and by the multiple conformational states available to the AcChR (7, 8, 19). However, a definition of the microscopic rate and equilibrium constants for each interaction of a noncompetitive blocker with each state of the AcChR should allow a correlation between binding and the effect of AcChR function.

All of the studies described in this communication deal with the interaction of PCP with the high-affinity binding site on the AcChR, inasmuch as the concentration of PCP was never greater than 8 μM (1 μM or less in kinetic studies). Thus, the parameters observed can be attributed to the properties of the high-affinity binding site, which

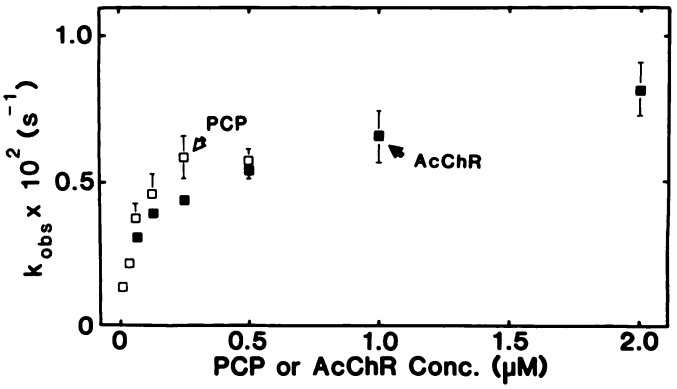

FIG. 9. *Pseudo-first order association rate constants as a function of either PCP or AcChR concentration in the absence of carbamoylcholine at 24°*

Conditions and analyses were the same as those described in the legend to Fig. 8. Less than 3% of the total [^{3}H]PCP was bound at equilibrium in all cases.

has been postulated to be associated with the ion channel (2). This site is regulated noncompetitively by cholinergic agonists and antagonists. Both agonists and antagonists (with the exception of α-neurotoxins) increase the apparent equilibrium affinity of noncompetitive blockers for the AcChR (5, 15, 20, 25). This is largely due to a 4- to 5-fold increase in the association rate (19, 20). When agonists, but not antagonists, are added simultaneously with the addition of radiolabeled noncompetitive blockers, both the association (19, 20) and dissociation (19) rates are increased by several orders of magnitude. The results of the electrophysiological and biochemical studies have led to the suggestion that the high-affinity binding site may be in or associated with the ion channel. If the binding site were in the lumen of the ion channel, binding in the absence of agonist or following desensitization would be expected to be restricted. The decreased association and dissociation rate constants observed under desensitizing conditions and in the absence of agonist suggest that a significant restriction to binding exists.

In this paper, the mechanism of this restriction was investigated. The thermodynamic and viscosity data argue strongly against a diffusion-limited mechanism, suggesting that a conformational change in the ligand or receptor may be responsible for the rate limitation. A simple bimolecular binding mechanism can be ruled out on the bases that (a) the k_{obs} is not a linear function of PCP concentration in the presence or absence of carbamoylcholine, and (b) the variation in k_{obs} (in the presence of carbamoylcholine) with AcChR concentration is not equivalent to the variation in k_{obs} with PCP concentration. Limitation of the binding by a conformational change in PCP or an ionization of PCP can be ruled out by the finding that, in the presence of carbamoylcholine, the association rate varies over a greater range with changes in AcChR concentration than with changes in PCP concentration.

A mechanism in which a slow isomerization of a PCP-AcChR complex follows a rapidly reversible and undetectable bimolecular interaction between PCP and AcChR is capable of explaining the data in the absence but not in the presence of carbamoylcholine. This mechanism is given by the following scheme:

$$R + P \underset{}{\overset{K_1}{\rightleftharpoons}} RP \overset{K_2}{\rightleftharpoons} R^*P \qquad (1)$$

where R is the AcChR concentration, P is the free PCP concentration, RP is the concentration of the rapidly reversible complex, R^*P is the concentration of the more stable complex, $K_1 = [R][P]/[RP]$, and $K_2 = [RP]/[R^*P]$. For this scheme, the value of k_{obs} should be a hyperbolic function of both PCP and AcChR concentration, and variation of the concentration of PCP should be equivalent to a variation in the concentration of AcChR. These conditions are fulfilled in the absence of carbamoylcholine, but the dramatic differences between the dependence of k_{obs} on AcChR concentration and on PCP concentration in the presence of carbamoylcholine rule out this mechanism (Fig. 8).

A mechanism by which a prior isomerization of the AcChR limits k_{obs} seems to be more consistent with the data. The simplest case is given by the following scheme:

$$\begin{array}{ccc} R & \overset{K_1}{\rightleftharpoons} & R^* \\ & & K_2 \downarrow \curvearrowright P \\ & & R^*P \end{array} \qquad (2)$$

where R is incapable of binding PCP, R^* is capable of binding PCP, $K_1 = [R]/[R^*]$, and $K_2 = [R^*][P]/[R^*P]$. The rate is limited by a conformational transition of the AcChR so that k_{obs} should increase with the concentration of AcChR (AcChR in excess). In the presence of carbamoylcholine (Fig. 8), k_{obs} does increase with increasing AcChR concentration. However, this mechanism cannot explain the variation in k_{obs} with changing PCP concentrations. Scheme 2 predicts that k_{obs} should be a decreasing function of PCP concentration, but k_{obs} was found to be a hyperbolic (increasing) function of PCP concentration. By introducing the condition that state R can bind PCP with low affinity, the data can be explained. A scheme for this mechanism is given by:

$$\begin{array}{ccc} R & \overset{K_1}{\rightleftharpoons} & R^* \\ P \curvearrowright \downarrow K_4 & & K_2 \downarrow \curvearrowright P \\ RP & \overset{K_3}{\rightleftharpoons} & R^*P \end{array} \qquad (3)$$

with the symbols having the same significance as in Scheme 2. The equilibrium between RP and R^*P may or may not be present. In this scheme, k_{obs} is an increasing or hyperbolic function of AcChR concentration but can be an increasing, decreasing, or biphasic function of PCP concentration, depending upon the microscopic kinetic constants.

The binding curve as a function of time at each concentration of PCP and AcChR can be simulated using the fourth-order Runge-Kutta technique to solve the differential equations implied by Scheme 3. The k_{obs} can then be calculated by choosing time points at which binding was measured in an actual experiment and using the calculated AcChR-PCP concentration to generate a pseudo-first order log-transform plot. The values of the

microscopic kinetic constants can then be adjusted to fit the k_{obs} as a function of PCP and AcChR concentration. This approach is useful for determining whether the models are consistent with the data but does not provide unique values for the kinetic constants. The major problem is that the time scale in which the measurements were made (seconds) allowed detection of only a single exponential process, whereas the mechanism implies a biphasic association. Binding measurements in the millisecond range would be required to assess unambiguously the microscopic kinetic constants. In general, however, the constants which fit the data most accurately in the presence of carbamoylcholine resulted in values of K_1 of greater than 50 but, in the absence of carbamoylcholine, required an increase to greater than 1000. This suggests that R^* represents less than 2% of the total AcChR population in the presence of carbamoylcholine and less than 0.01% in the absence of carbamoylcholine. The value of K_2 was in the nanomolar or subnanomolar range, suggesting a high-affinity interaction with the R^* state; and the value of K_4 was in the micromolar range, suggesting a lower-affinity interaction of PCP with the R state than with the R^* state. The exact nature of R and R^* is unclear; however, some assumptions can be made on the basis of the known conformational equilibria of the AcChR (2). *Torpedo* AcChR seems, in the absence of ligands, to exist *mainly* in equilibrium between a "resting" state (80%) and a "desensitized" state (20%; ref. 2). Following equilibration with saturating concentrations of cholinergic agonists, more than 99% of the AcChR is in the "desensitized" state. The apparent increase in the fraction of R^* following equilibration with agonist suggests that R^* is not the "resting" state. Likewise, because only a small fraction of AcChRs is in the R^* state following equilibration with agonists, R^* is presumably not the "desensitized" state.

The data are consistent with the notion that R^* represents an open-channel conformation of the AcChR, although binding to another conformation existing in a small fraction of AcChR, such as an "intermediate" form (17, 28, 29), cannot be ruled out on the basis of these data. Several lines of evidence suggest that the R^* state may be the open channel. For example, *d*-tubocurarine increases the binding rate relative to the unliganded receptor (19). This is consistent with the recent findings of Trautmann (30), which demonstrate that *d*-tubocurarine can open AcChR ion channels on primary cultured muscle cells. The values of K_2 (dissociation constant for R^*) which fit the data best were in the nanomolar range, suggesting that, if PCP were to act as a channel-blocking agent on open channels, it may do so at nanomolar concentrations. Recent single-channel studies of the mean channel open time of AcChRs from myoballs prepared from primary cultures of rat muscle by colchicine treatment indicate that PCP shortens mean channel open time from 6 msec to 0.9 msec with an IC_{50} of approximately 10 nM.[3]

The dramatic increase in association rate observed upon simultaneous addition of PCP and carbamoylcholine (19, 20; see Introduction) may reflect binding to an open-channel state and would correspond to a dramatic increase in the fraction of AcChR in the R^* state. Following desensitization, the proportion of AcChR in the R^* state would then decrease to less than 2% of the total AcChR. If the R^* state were the open-channel state, an equilibrium population of AcChR in the open-channel state should exist under desensitizing conditions. An equilibrium population of AcChR in the open-channel conformation seems to exist following desensitization of *Electrophorus electricus* and some skeletal muscle AcChRs (18). Although ion flux studies on the millisecond and second time scale (17) suggest that no such equilibrium population exists for *Torpedo* AcChR, the time scale (millisecond to second) under which these experiments were performed may have precluded the detection of a small fraction of open channels. Furthermore, the temperature sensitivity of k_{obs} for PCP binding may reflect a decrease in the proportion of R^* with decreasing temperature. The studies of Walker *et al.* (17) were performed at 0°, possibly decreasing the probability of observing an equilibrium population of open channels.

The mechanism (Scheme 3) which fits the data most closely is similar to that proposed by Adams (11) for the effect of procaine on the voltage-jump analysis of the end-plate conductance and by Albuquerque *et al.* (9) for the effect of PCP on the EPC. In the models of both Adams (11) and Albuquerque *et al.* (9), a state analogous to R^* in the present model is the open-channel state and is assumed to bind PCP (or procaine) preferentially. An additional interaction with a closed-channel state is required to explain the data adequately, although the exact nature (e.g., resting, desensitized) of the closed-channel state is unclear.

Thus, the binding of PCP to its high-affinity site seems to be highly dependent upon AcChR conformation, with the favored conformation being a state that is represented at equilibrium by only a small fraction of AcChR molecules and which may be the open-channel conformation of the AcChR.

[3] R. E. Oswald and D. Tank, unpublished observations.

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