Characterization of the Transient Agonist-Triggered State of the Acetylcholine Receptor Rapidly Labeled by the Noncompetitive Blocker [^{3}H]Chlorpromazine: Additional Evidence for the *Open Channel* Conformation†

Thierry Heidmann and Jean-Pierre Changeux*

Unité de Neurobiologie Moléculaire, Unité associée au CNRS UA 041149, Interactions Moléculaires et Cellulaires, Institut Pasteur, 75724 Paris Cédex 15, France

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ABSTRACT: The kinetics of covalent labeling of the α, β, γ, and δ chains of the acetylcholine receptor (AcChR) from *Torpedo marmorata* by the noncompetitive blocker [^{3}H]chlorpromazine ([^{3}H]CPZ) are investigated by using rapid mixing photolabeling techniques. In an initial study [Heidmann, T., & Changeux, J. P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1897–1901], it was shown that the rate of [^{3}H]CPZ labeling increases 100–1000-fold upon *simultaneous* addition of nicotinic agonists to the AcChR and that *prior* addition of these agonists abolishes the effect. The data were interpreted in terms of the rapid labeling of the transient *active state* of the AcChR where the ion channel is in its open configuration. This interpretation was recently challenged [Cox, R. N., Kaldany, R. R. J., Di Paola, M., & Karlin, A. (1985) *J. Biol. Chem. 260*, 7186–7193] on the ground of studies with a *different* noncompetitive blocker, [^{3}H]quinacrine azide, and the suggestion was made that this compound labels the *rapidly desensitized* closed channel conformation of the AcChR. In this paper it is shown that the rate of rapid labeling of the AcChR by [^{3}H]CPZ decreases to negligible values upon exposure of the AcChR to nicotinic agonists, in the 100–500-ms time range. The absolute values of the rate constants of this decrease (10–15 s^{-1} for saturating concentrations of acetylcholine and carbamoylcholine) and their variation with agonist concentration (apparent dissociation constants of 40 μM and 0.4 mM for acetylcholine and carbamoylcholine, respectively) are those expected for the rapid desensitization of the AcChR. In addition, it is shown that at variance with what is observed with [^{3}H]quinacrine azide, the rate of [^{3}H]CPZ rapid labeling increases *linearly* with CPZ concentration, without saturation. The data support the conclusion that, as previously suggested, the state of the AcChR that [^{3}H]CPZ rapidly labels is *not* the rapidly desensitized but the active conformation where the ion channel is open and to which [^{3}H]CPZ binds in a diffusion-controlled manner.

Extensive analysis, in parallel, of ion flux and nicotinic agonist binding to the acetylcholine receptor (AcChR)[1] by rapid-mixing techniques (Neubig et al., 1982; Heidmann et al., 1983a) has led to the proposal of a *minimum* four-state model [review Changeux et al. (1984)] for the allosteric transitions of this membrane-bound regulatory protein. According to this model, the nicotinic AcChR exists in *four* discrete interconvertible conformations, resting (R), active (A), intermediate (I), and desensitized (D), which differ by (a) their affinity for cholinergic agonists and other ligands of the AcChR and (b) the state of opening of the ion channel which is open only in the active (A) conformation. Rapid addition of high concentrations of agonist then results in the quantitative population of the active state, followed, if the agonist is maintained for a sufficient period of time, by the rapid and transient population of the I state (within 100 ms to 1 s) and the final stabilization of the D state (within seconds). These last two steps have been correlated with the two-step desensitization process, demonstrated in vivo by electrophysiological methods (Sakmann et al., 1980; Feltz & Trautmann, 1982) and in vitro by rapid ion flux measurements (Hess et al., 1982; Walker et al., 1982; Heidmann et al., 1983a).

The permeability response to AcCh is blocked by a group of compounds referred to as NCBs [review in Heidmann et al. (1983b), Boyd and Cohen (1984), and Changeux et al. (1984)], which provide potentially useful tools for the structural identification of the components of the ion channel (Adams, 1981; Giraudat et al., 1986). They bind to high-affinity sites on AcChR-rich membranes, which (a) are present as a single copy per AcChR light form, (b) are coupled in an allosteric manner to the AcCh binding sites, and (c) are possibly located in the axis of symmetry on the AcChR molecule, in close proximity to all five chains of the molecule. Indeed, UV irradiation of the AcChR in the presence of photoactivable NCBs, under equilibrium conditions, results depending on the species of *Torpedo* and on the structure of the compound in the covalent labeling of the α, β, γ, and/or δ chain of the receptor, suggesting that all of them contribute to this unique site (Oswald & Changeux, 1981a,b; Kaldany & Karlin, 1983; Muhn & Hucho, 1983; Heidmann et al., 1983b; Haring et al., 1983).

The kinetics of covalent attachment of photoactivable NCBs to the AcChR can be resolved by using a rapid mixing photolabeling apparatus (Karlin et al., 1983; Heidmann & Changeux, 1984; Muhn et al., 1984; Cox et al., 1985). Rapid addition of [^{3}H]chlorpromazine ([^{3}H]CPZ) to AcChR-rich membrane fragments followed by brief (<20 ms) UV irradiation of the mixture results in the simultaneous covalent

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[1] Abbreviations: AcCh, acetylcholine; AcChR, acetylcholine receptor; CPZ, chlorpromazine; QA, quinacrine azide; SDS, sodium dodecyl sulfate.

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labeling of all four chains of the AcChR, in a time-dependent manner (Heidmann & Changeux, 1984). The rate of labeling increases 10^2–10^3-fold under conditions of simultaneous addition of AcCh (or agonist) with CPZ, suggesting that *rapid* association takes place in the open-channel conformation (Heidmann & Changeux, 1984) [see also Eldefrawi et al. (1980), El-Fakahany et al. (1982), and Oswald et al. (1983)].

These results fit with the models proposed on the basis of electrophysiological recordings [Adams, 1977; Neher & Steinbach, 1978; review in Adams (1981)], which assume that some, though not necessarily all, NCBs block the permeability response by entering the open channel in a diffusion-controlled manner and thus inhibit ion translocation by steric hindrance [see Changeux et al. (1986)].

Such interpretation of the CPZ data was challenged by Cox et al. (1985) in a recent publication where the covalent labeling of the AcChR by an azido derivative of a NCB initially studied in our laboratory, quinacrine (Grünhagen & Changeux, 1976; Grünhagen et al., 1977), was analyzed under conditions similar to those used by us with CPZ. On the basis of the report that the rate of rapid labeling seems independent of the concentration of QA, Cox et al. (1985) suggested that QA and by extension CPZ label the intermediate, rapidly desensitized, I conformation of the AcChR rather than the active "open channel" conformation. In order to settle the issue we reinvestigated several critical points on the following basis. If rapid labeling by [^{3}H]CPZ occurs at the level of the A state and not of the I state, the decrease of the rate of [^{3}H]CPZ rapid labeling observed upon desensitization of the response (Heidmann & Changeux, 1984) should parallel the *rapid* desensitization process associated with the A → I transition and *not* the *slow* desensitization process associated with the I → D transition.

In order to test this prediction, we first measured the extent of rapid [^{3}H]CPZ labeling under conditions where rapid desensitization takes place with a significant amplitude, i.e., in the range of high agonist concentrations. The rapid mixing photolabeling apparatus previously developed was thus slightly modified to improve the time resolution and to allow variations of the desensitization period in the 100-ms time range. Second, we reinvestigated whether a rate-limiting step for [^{3}H]CPZ rapid labeling takes place in the domain of concentrations where it was reported for QA. In both cases, the results obtained confirm and further extend our previous data and lead to the proposal of a model that is consistent with our previous conclusion and also accounts for Cox et al. (1985) data with QA though not for their conclusions regarding CPZ.

Materials and Methods

Materials. AcChR-rich membranes were purified from freshly dissected *Torpedo marmorata* electric organ as described in Sobel et al. (1977) with protease inhibitors and chelating agents to limit proteolysis (Saitoh et al., 1979). Purified membranes were stored in liquid nitrogen and thawed at room temperature immediately before use. [^{3}H]CPZ (15–30 Ci/mmol) was from New England Nuclear, and carbamoylcholine and AcCh were from Sigma.

Rapid Mixing Photolabeling Apparatus. The rapid mixing photolabeling apparatus has been described in a previous publication (Heidmann & Changeux, 1984) and was only slightly modified, as follows, to improve time resolution and allow analysis of processes associated with the rapid desensitization of the AcChR. Basically, an additional delay line (delay 2) was introduced between mixing chamber M_2 and the irradiation chamber ("quartz tube", cf. Figure 1) by introducing a derivation within the original Durrum mixing chamber itself. A minimal dead volume of approximately 20 μL was thus attained, allowing time resolutions in the 10–20-ms range.

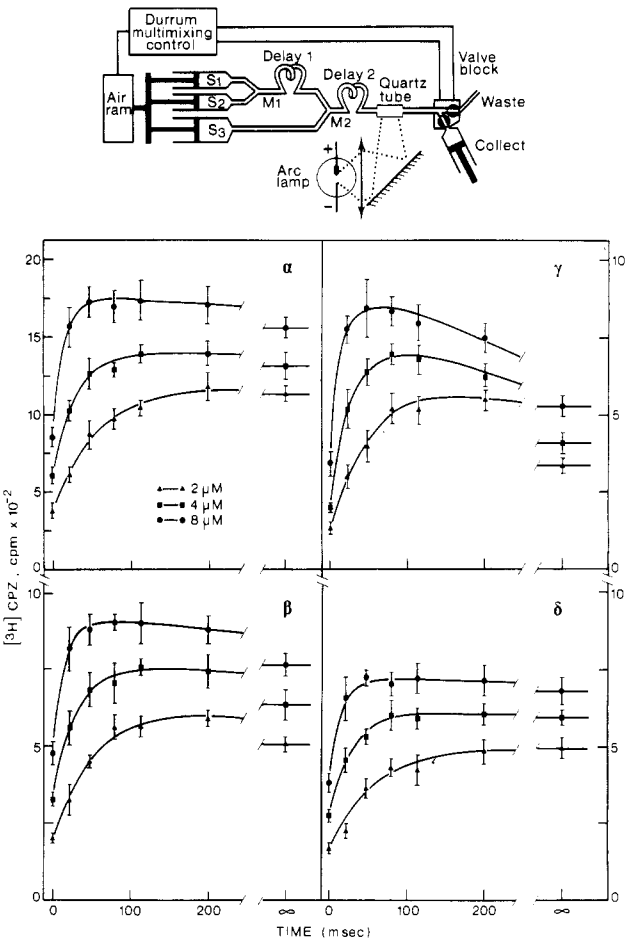

FIGURE 1: (Top) Schematic drawing of the rapid mixing photolabeling apparatus: S, drive syringes; M, mixing chambers; flow rate between M_2 and collect, 3 μL/ms. (Bottom) Kinetics of [^{3}H]CPZ incorporation in the α, β, γ, and δ polypeptide chains of the AcChR. AcChR-rich membrane fragments (0.2 μM α-toxin sites) were introduced in drive syringes S_1 and S_2, and [^{3}H]CPZ (twice the concentrations indicated in the figure, 1:10 isotopic dilution; ▲, 2 μM, ■, 4 μM ●, 8 μM) supplemented with 0.2 mM AcCh was introduced in syringe S_3. Delay time between mixing (in M_2) and photoirradiation was varied by changing the length of delay line 2. The zero-time values were determined by independent measurement of [^{3}H]CPZ incorporation in the presence of the highly selective noncompetitive blocker phencyclidine (100 μM) added to the membranes 10 min before rapid mixing. Each point is the mean of 3–5 determinations (standard errors indicated by the bars). A least-squares fit of the data in the 0–100-ms time range by single exponentials yields the following values for the covalent rapid labeling of the AcChR (sum of cpm in the four chains) by [^{3}H]CPZ: 18 ± 3, 37 ± 5, and 67 ± 15 s^{-1} for respectively 2, 4, and 8 μM CPZ.

Experiments were performed either in the *continuous* mode, in which case the delays were adjusted by varying the length of the delay lines, or in the *interrupt* mode, for delays longer than 400 ms.

All photolabeling experiments were performed at 20 °C in *Torpedo* saline solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM phosphate buffer, pH 7.4) supplemented with 50 μM *O,O*-diethyl *S*-[β-(diethylamino)-ethyl]phosphorothiolate (Tetram) to inhibit acetylcholinesterase activity. Reactants were mixed in mixing chambers 1 and/or 2 (see text and legends to figures) and irradiated while passing through the quartz tube (irradiation period <5 ms). The membranes were then collected in "collect syringe" and centrifuged for 10 min in an Eppendorf Minifuge. The

pellet was resuspended in sample loading buffer and submitted to SDS gel electrophoresis as described in Heidmann and Changeux (1984). Radioactivity was measured by cutting out appropriate sections of the dried gel.

Results

(1) Time Course of [^{3}H]CPZ Rapid Labeling in the High-Concentration Range of CPZ. In a previous publication (Heidmann & Changeux, 1984), [^{3}H]CPZ rapid labeling was kinetically resolved for CPZ concentrations up to 1 μM with a time resolution of 100 ms. Increasing the flow rate by a factor of 3 in the rapid mixing photolabeling apparatus and using a more appropriate delay line (D2, cf. Materials and Figure 1), we improved the time resolution to approximately 20 ms, and CPZ concentrations up to 5–10 μM were thus explored. The results obtained under these conditions are illustrated in Figure 1. They clearly indicate that when [^{3}H]CPZ and AcCh are added simultaneously, the rate of [^{3}H]CPZ rapid labeling increases with increasing CPZ concentration, as previously observed for CPZ concentrations in the 0–1 μM range. The time course of [^{3}H]CPZ rapid labeling can be reasonably well fitted in the 0–100-ms time range by a single exponential, and the derived rate constant increases linearly with CPZ concentration (in the accessible domain of concentrations) as expected for a simple bimolecular binding reaction with an *on* rate constant close to 10^7 M^{-1} s^{-1} (see values in legend to Figure 1).

(2) Decrease of the Initial Rate of [^{3}H]CPZ Rapid Labeling under Conditions of AcChR Rapid Desensitization. According to Figure 1, the extent of [^{3}H]CPZ incorporation measured 20 ms after [^{3}H]CPZ addition can be taken as a reliable estimate of the initial rate of the rapid association process, at least for CPZ concentrations below 5 μM. Accordingly, the extent of [^{3}H]CPZ labeling 20 ms after the rapid addition of 4 μM [^{3}H]CPZ to the membranes was quantitatively monitored under various conditions of AcChR desensitization. Typically, AcChR-rich membrane fragments were *first* rapidly mixed in mixing chamber 1 with varying concentrations of agonist and, *second*, after a delay that could be varied from approximately 50 ms to several seconds or minutes, were rapidly mixed in mixing chamber 2 with [^{3}H]CPZ and agonist. [^{3}H]CPZ binding to the AcChR was allowed to proceed for approximately 20 ms, and the mixture was then briefly UV irradiated (less than 5 ms) while passing through the quartz chamber. The extent of [^{3}H]CPZ incorporation was thus monitored as a function of the time of preincubation of the AcChR with the agonist with, then, a time resolution of approximately 20 ms.

The results obtained in the presence of 100 μM AcCh are shown in Figure 2A for each polypeptide chain of the AcChR. Clearly, preincubation of the AcChR for periods of time as short as 100 ms results in a significant decrease of the extent of [^{3}H]CPZ rapid labeling with an almost complete inhibition after 300–500 ms. Similar time courses were observed for each chain of the AcChR and were reasonably well fitted by a single exponential. Figure 2B illustrates the kinetics obtained at several AcCh concentrations in the case of the γ chain (for which the signal/noise ratio is maximum).

To further analyze this effect, the inactivation of [^{3}H]CPZ rapid labeling was monitored for varying concentrations of two distinct agonists, AcCh and carbamoylcholine. The data obtained are illustrated in Figure 3, which clearly shows that the rate constant for the inactivation of [^{3}H]CPZ rapid labeling increases with increasing agonist concentration in a dose-dependent manner with an apparent dissociation constant for the effect close to 40 μM for AcCh and 0.4 mM for carba-

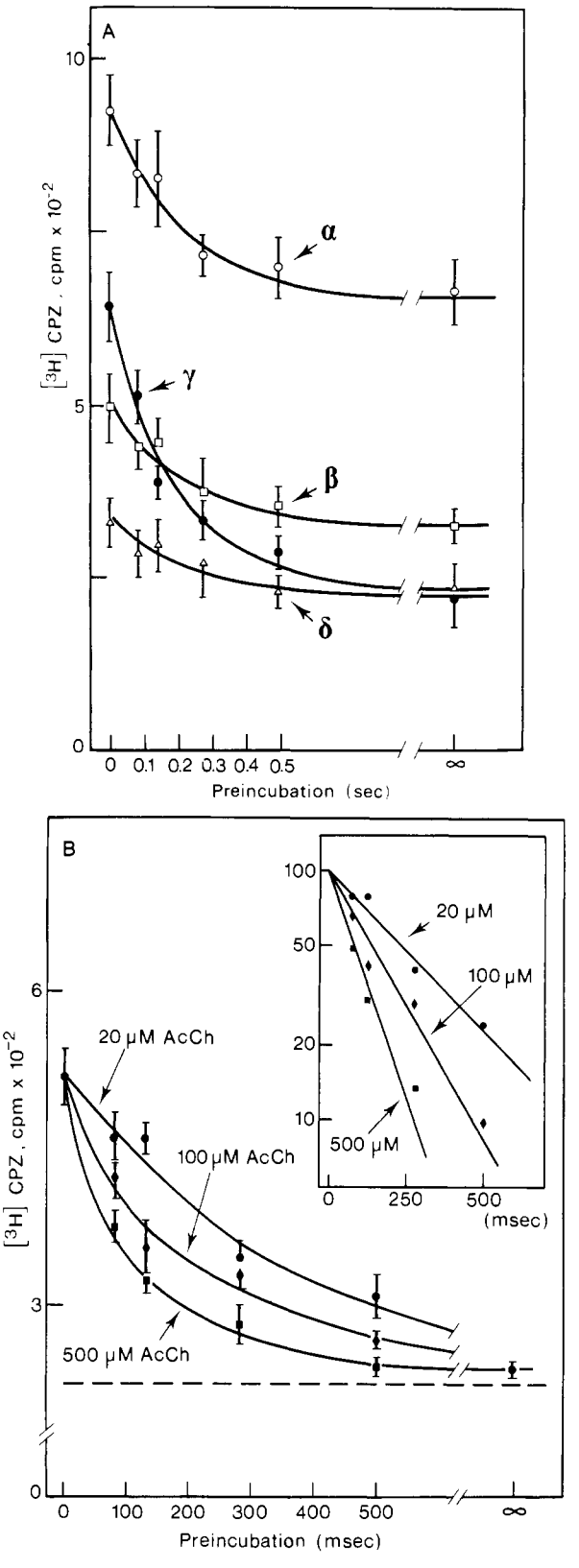

FIGURE 2: Effect of desensitization on [^{3}H]CPZ rapid labeling: S_1, AcChR-rich membranes (0.4 μM α-toxin sites); S_2, 200 μM AcCh or no effector for the zero-time determination; S_3, [^{3}H]CPZ (8 μM, 1:10 isotopic dilution) supplemented with 0.5 mM AcCh. The apparatus was used in the continuous mode. Preincubation was varied by increasing the length of delay line 1, and photoirradiation was achieved 20 ms after the rapid mixing in M_2 by adjusting the length of delay line 2. (A) Extent of [^{3}H]CPZ incorporation into each chain of the AcChR as a function of the duration of AcChR exposure to 100 μM AcCh. (B) Extent of [^{3}H]CPZ incorporation into the γ chain as a function of the duration of AcChR exposure to 20, 100, and 500 μM AcCh. (Inset) Semilog plot of the data.

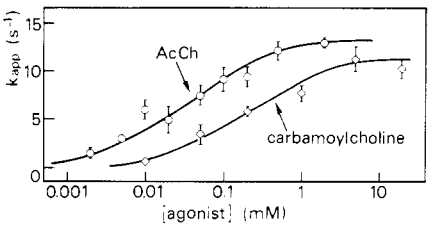

FIGURE 3: Rate of inactivation of [^{3}H]CPZ rapid association as a function of acetylcholine or carbamoylcholine concentration. Same experimental conditions as in Figure 2.

moylcholine and no evidence for sigmoidicity.

Discussion

Analysis of the CPZ Data. Extension of our previous analysis of the agonist-dependent rapid labeling of the AcChR by [^{3}H]CPZ to higher CPZ concentrations did not reveal any leveling off in the apparent rate constant of the rapid process, but rather a linear increase to values close to 100 s^{-1}. Again, the simplest interpretation of the data is that the rapid process is associated with a simple bimolecular binding reaction, close to diffusion-controlled, between [^{3}H]CPZ and a transient conformation of the receptor populated by the agonist. The characterization of this transient state can be further refined by taking into account the data presented above. Indeed, a close to 100% decrease of the initial rate of [^{3}H]CPZ rapid labeling takes place within a fraction of a second upon preincubation of the AcChR with agonist concentrations susceptible to elicit the rapid desensitization of the receptor. The half-life of this process, 10–15 s^{-1} for high concentrations of AcCh and carbamoylcholine, is closely related to the values reported in the literature for the rapid inactivation of the ion flux response as measured with *Torpedo californica* AcChR either in native membranes [maximum rate for carbamoylcholine, 2.2 s^{-1}, see Hess et al. (1982)] or in reconstituted vesicles [maximum rate for carbamoylcholine, 4.9 s^{-1}, see Walker et al. (1982)] and also with *Electrophorus electricus* AcChR (where slow desensitization is not observed), with maximum rates for AcCh and carbamoylcholine of respectively 9.5 and 11.2 s^{-1} [see Cash et al. (1981)]. In addition, the values obtained clearly differ from those measured for the *slow* inactivation process, which has been reported to plateau at a value of 0.7 s^{-1} in the case of AcCh [see Neubig et al. (1982)] or even less in the case of carbamoylcholine [0.1 s^{-1}, see Hess et al. (1982) and Walker et al. (1982)], i.e., they are at least 20-fold slower. The difference cannot even be due to CPZ per se, which is added at the end of the preincubation period for a short duration (20 ms) as compared with the measured time course of the inactivation process that develops in the 100–500-ms time range.

Analysis of the concentration-effect curves for the inactivation of [^{3}H]CPZ rapid labeling yields apparent dissociation constants closely related to those obtained for the variation of the rate of the AcChR *rapid desensitization*, with values in the *high* agonist concentration range of 0.4 mM for carbamoylcholine and 40 μM for AcCh when measured with [^{3}H]CPZ, 1.3–3 mM for carbamoylcholine when measured by following ion flux with *T. californica* AcChR [see Hess et al. (1982) and Walker et al (1982)], and 80 μM for AcCh for the unique rapid phase of desensitization of *E. electricus* AcChR (Cash et al., 1981). The slightly smaller values obtained with [^{3}H]CPZ (but within less than 1 order of magnitude) as well as the apparent lack of sigmoidicity of the curves in Figure 3 might be due to an underestimation of the k_{app} values at saturating concentrations of agonist, for which more than 50% of the kinetics has already proceeded before the first 50-ms measurement (see Figure 2).

In conclusion, a good quantitative correlation exists between inactivation of [^{3}H]CPZ rapid labeling and AcChR *rapid desensitization*, and in any case, the data presented above are *not* compatible with the notion that the inactivation of [^{3}H]CPZ rapid labeling results from the *slow* desensitization of the AcChR, i.e., the slow stabilization of the high-affinity D state.

Comparison with QA Data: A Plausible Common Interpretation of Both Sets of Data. Although the analysis of QA interaction reported by Cox et al. (1985) still appears less extensive than that provided for CPZ, a major difference noticed by Cox et al. (1985) between the two sets of data concerns the dependence of the rates of rapid labeling with NCB concentration. No difference was reported when the labeling of the AcChR by QA was measured at 2 and 3.5 μM (which, however, are possibly too close to each other to allow a difference to be clearly detected). On the other hand, with CPZ a linear increase was observed in the 0–1 μM range (Heidmann & Changeux, 1984) which further extends to concentrations up to 8 μM (at which the limit of the time resolution of the apparatus is reached).

A simple model that could account for both CPZ and QA data is based on the following assumptions. (1) The covalent labeling of any given site on the AcChR is a two-step process which includes, first, the *reversible binding of the ligand* to its site and, second, the *covalent reaction* of the bound ligand to a reactive amino acid side chain located within this site or in its vicinity [see Singer (1967) and Karlin (1969)]. (2) The kinetics of each of these two processes may vary with the conformation of the AcChR. In particular, the state which binds with the fastest rate might be distinct from that which shows the highest reactivity for the considered ligand.

In the case of QA, one may then assume that *reversible* binding takes place at the level of the A state but that the covalent reaction of the bound complex with the α and β chains occurs with a significant efficacy only with the I state. In the case of CPZ, on the other hand, binding *and* labeling would already occur with the A conformation. This interpretation is consistent with (1) the possible difference in the reactivity of [^{3}H]CPZ and [^{3}H]QA, since [^{3}H]QA labels only the α and β chains in *T. californica* AcChR whereas [^{3}H]CPZ labels all of them in *T. marmorata* AcChR, and (2) the possible occurrence of local rearrangements and side-chain movements at the level of the NCB high-affinity site as indicated by the changes in affinity for NCBs between the various conformations of the receptor [reviewed in Heidmann et al. (1983b) and Boyd and Cohen (1984)].

Quantitatively, according to this model and as observed, the kinetics of labeling by [^{3}H]QA should be rate-limited by the A $\rightarrow$ I transition, provided that the reversible binding step itself is rapid as compared to this transition. The CPZ data indeed show that the labeling of the AcChR by [^{3}H]CPZ, and therefore a fortiori the reversible binding of CPZ to the A state, is close to diffusion-controlled and thus that the binding step for CPZ, and this is also most likely true for QA, is fast compared to the A $\rightarrow$ I transition.

In conclusion, the most likely interpretation of CPZ and QA data is that CPZ binds to the AcChR in a diffusion-controlled manner *and* reacts with its subunits when in the open-channel A conformation, whereas QA most likely binds to the AcChR in a diffusion-controlled manner when in the A conformation but reacts with it only in the I conformation. Thus, [^{3}H]QA cannot be considered as a probe of the *open*-channel confor-

mation. In contrast, the rapid labeling of the AcChR subunits by [^{3}H]CPZ unambiguously parallels the occurrence of the open-channel A conformation, and this should allow the use of this NCB to analyze the "gating" events at the structural level [see Giraudat et al. (1986)].

Registry No. Chlorpromazine, 50-53-3; acetylcholine, 51-84-3; carbamoylcholine, 462-58-8.

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