Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 1897–1901, March 1984
Neurobiology

Time-resolved photolabeling by the noncompetitive blocker chlorpromazine of the acetylcholine receptor in its transiently open and closed ion channel conformations

(rapid kinetics/channel blocker/activation/desensitization)

THIERRY HEIDMANN AND JEAN-PIERRE CHANGEUX*

Neurobiologie Moléculaire and Laboratoire Associé au Centre National de la Recherche Scientifique, Interactions Moléculaires et Cellulaires, Institut Pasteur, Paris, France

Contributed by Jean-Pierre Changeux, November 29, 1983

ABSTRACT **A rapid-mixing photolabeling apparatus is developed to resolve the kinetics of association of the noncompetitive channel blocker [^{3}H]chlorpromazine (CPZ) with the membrane-bound acetylcholine (AcCho) receptor from *Torpedo marmorata* and to photolabel its subunits in the 100-milliseconds to seconds time range. Rapid mixing of AcCho and [^{3}H]CPZ with the receptor followed by brief (<20 msec) UV irradiation results in the selective labeling of the *four* chains of the AcCho receptor, according to a rapid bimolecular association process close to diffusion-controlled. Rapid association is not observed with the competitive antagonists *d*-tubocurarine or flaxedil or the snake venom α-toxins. Its initial rate increases with agonist concentration, with maxima of 0.6 for carbamoylcholine and 0.2 for phenyltrimethylammonium taking 1 for AcCho, with apparent dissociation constants of 30 μM, 400 μM, and 300 μM for AcCho, carbamoylcholine, and phenyltrimethylammonium, respectively, and with sigmoid shape (Hill coefficients of 1.1–1.3). Under conditions in which the receptor "desensitizes" and the ionic channel closes (preincubation with AcCho), rapid [^{3}H]CPZ association decreases in parallel. It is concluded that the agonist-dependent rapid association of [^{3}H]CPZ takes place at the level of a site common to all five subunits, which lies within the ion channel and becomes accessible when the channel opens.**

The elementary molecular unit that mediates the physiological response to acetylcholine (AcCho) at the neuromuscular junction and the electromotor synapse is a well-identified allosteric membrane protein: the AcCho receptor (AcChoR) (reviewed in ref. 1). Its light form, of M_r 270,000 (2), is a pentamer of four homologous polypeptide chains with an $\alpha_2\beta\gamma\delta$ stoichiometry (3–5). Labeling studies with snake venom α-toxins and covalent analogues of AcCho indicate that the AcCho binding sites are carried at least in part by the two α chains (reviewed in refs. 1 and 6). Functional reincorporation of the pure protein into artificial lipid membranes (reviewed in refs. 7 and 8) further demonstrates that this transmembrane oligomeric "bundle" also contains the ion channel.

The permeability response to AcCho is blocked by a group of compounds, referred to as "noncompetitive blockers" (NCBs), which differ from the typical competitive antagonists and provide potentially useful tools for the structural identification of the components of the ion channel (reviewed in refs. 9 and 10). Indeed, electrophysiological observations based on "noise" analysis, relaxation kinetics after voltage jump, or single channel recordings have led to the proposal that these effectors might, under defined conditions, enter the open ion channel, thereby sterically inhibiting ion transport (reviewed in ref. 10).

NCBs reversibly bind, under equilibrium conditions, to a few categories of sites on the membrane-bound AcChoR. The most prominent one (*i*) discloses a high affinity for the frog toxin histrionicotoxin and the hallucinogen phencyclidine, (*ii*) is allosterically regulated by cholinergic agonists and competitive antagonists, and (*iii*) is present as a single copy per AcChoR light form (reviewed in ref. 9). This high-affinity site was selectively labeled by a series of azido and mustard derivatives of the NCBs and by some unmodified NCBs upon UV irradiation (11–15). Depending on the species of *Torpedo*, the α, β, or δ chain incorporated the radioactive NCBs preferentially, but not exclusively. With [^{3}H]chlorpromazine (CPZ), UV irradiation resulted in the selective labeling of the *four* chains under conditions of occupancy of the high-affinity NCB site, suggesting that *all* of them contribute to this unique site (9, 11).

An important feature disclosed by *in vivo* electrophysiological recordings and *in vitro* rapid ion flux measurements is that the ion channel opens only transiently upon sustained application of AcCho. In the fraction-of-a-second to seconds time range the AcChoR undergoes a two-step transition toward a "desensitized" conformation where the ion channel is closed (reviewed in ref. 16). Accordingly, rapid kinetic techniques are required to monitor the interaction of NCBs with the transient and functionally relevant states of the AcChoR. Yet, binding of radioactive NCBs in the seconds–minute time range, using manual filtration techniques, disclosed a rapid but unresolved association of NCBs with a transient state of the AcChoR selectively populated by cholinergic agonists but not by competitive antagonists (17, 18). Also, stopped-flow experiments with a fluorescent NCB, quinacrine, revealed a fast fluorescence increase unique to cholinergic agonists (19).

To analyze directly and quantitatively the interaction of NCBs with the AcChoR, we have developed a rapid-mixing photolabeling apparatus that allows the resolution of [^{3}H]CPZ association with the membrane-bound AcChoR and the photolabeling of its subunits in the 100-msec time scale, under conditions, then, in which the agonists selectively open the ion channel.

MATERIALS AND METHODS

Materials. AcChoR-rich membranes were purified from freshly dissected *Torpedo marmorata* electric organ as described in Sobel *et al.* (20), with protease inhibitors and che-

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; NCB, noncompetitive blocker; CPZ, chlorpromazine; $NPhMe_3$, phenyltrimethylammonium; Tetram, *O,O'*-diethyl-*S*-(β-diethylamino)ethyl phosphorothiolate.

*To whom reprint requests should be addressed at: Neurobiologie Moléculaire, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France.

lating agents to limit proteolysis (21). Purified membranes were stored in liquid nitrogen at a concentration of 15–25 μM α-toxin sites and thawed at room temperature immediately before use. [^{3}H]CPZ (15–30 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear, and carbamoylcholine, AcCho, *d*-tubocurarine, and phenyltrimethylammonium ($NPhMe_3$) were from Sigma.

Rapid-Mixing Photolabeling Apparatus. The rapid-mixing photolabeling apparatus was constructed by combining modified elements of the Durrum stopped-flow and multimixing spectrophotometers (models D110 and D133), with a 500-W high-pressure mercury lamp (HBO 500W/2, OSRAM) to deliver intense UV light. The fluorescence observation chamber of the Durrum D110 spectrophotometer was inserted between the second mixing chamber and the collection valve block of the Durrum D133 multimixing equipment, and the image of the lamp arc was formed on the quartz cylinder (20 × 2 mm) of the chamber, along its axis to obtain maximal illumination. The sequential rapid mixing of up to three solutions was achieved in mixing chambers 1 and 2 (see Fig. 1) and was time-controlled by the Durrum multimixing control module, using both the *continuous* and *interrupt* modes and varying the length of the delay tube. The mixed solutions were UV irradiated while passing in the fluorescence observation chamber, and part of it (400 μl) was collected in the collect syringe. Flow rate was adjusted by varying the pressure of the air ram, and a value of 1 μl/msec corresponding to a duration of irradiation of <20 msec was selected. In some experiments, an additional rapid-mixing step, after irradiation, was achieved in the collect syringe itself, which was then prefilled with 400 μl of the appropriate solution and whose entrance was narrowed for efficient mixing (16).

Determination of the Extent of Photolabeling. All photolabeling experiments were performed at 20°C in *Torpedo* saline solution (250 mM NaCl/5 mM KCl/4 mM $CaCl_2$/2 mM $MgCl_2$/5 mM phosphate buffer, pH 7.4), supplemented with 50 μM *O,O'*-diethyl-*S*-(β-diethylamino)ethyl phosphorothiolate (Tetram) to inhibit acetylcholinesterase activity. Binding measurements were made with AcChoR-rich membranes at a concentration of 0.05–0.15 μM in α-toxin sites and [^{3}H]CPZ (isotopically diluted or not) at a concentration of 0.1–1 μM. After photolabeling, the membranes in the collect syringe were centrifuged for 15 min in an Eppendorf Minifuge, and the pellet was resuspended in sample loading buffer (2% $NaDodSO_4$/0.06 M Tris·HCl, pH 6.8/5% 2-mercaptoethanol/0.001% bromophenol blue) and submitted to $NaDodSO_4$/polyacrylamide (10% acrylamide/0.26% bisacrylamide) gel electrophoresis as in ref. 12. Radioactivity was detected by fluorography or by cutting out appropriate sections of the dried gel as in ref. 12.

RESULTS

Kinetics of [^{3}H]CPZ Labeling. Figs. 1 and 2 show the kinetics of [^{3}H]CPZ labeling of AcChoR subunits. [^{3}H]CPZ solution supplemented with either AcCho (0.1 mM) or *d*-tubocurarine (0.1 mM) was rapidly mixed with AcChoR-rich membranes and UV irradiated at various times after mixing. The radioactivity of [^{3}H]CPZ covalently bound to the AcChoR was detected after gel electrophoresis by fluorography (Fig. 1) or by determining cpm in the appropriate bands of the gel (Figs. 2–5). As seen in Figs. 1 and 2, the α, β, δ, and, to a lesser extent, the γ chains of the AcChoR were labeled by [^{3}H]CPZ, and the labeling was almost completely prevented by an excess of unlabeled phencyclidine, a highly specific ligand of the high-affinity site for NCBs (9). With AcCho, the radioactivity incorporated into all four chains first increased rapidly to a maximum within 200–500 msec and then decreased, in the seconds time range, to reach a plateau. With the competitive antagonist *d*-tubocurarine, the rapid association process was not observed, although the equilibrium values were close to those observed with AcCho.

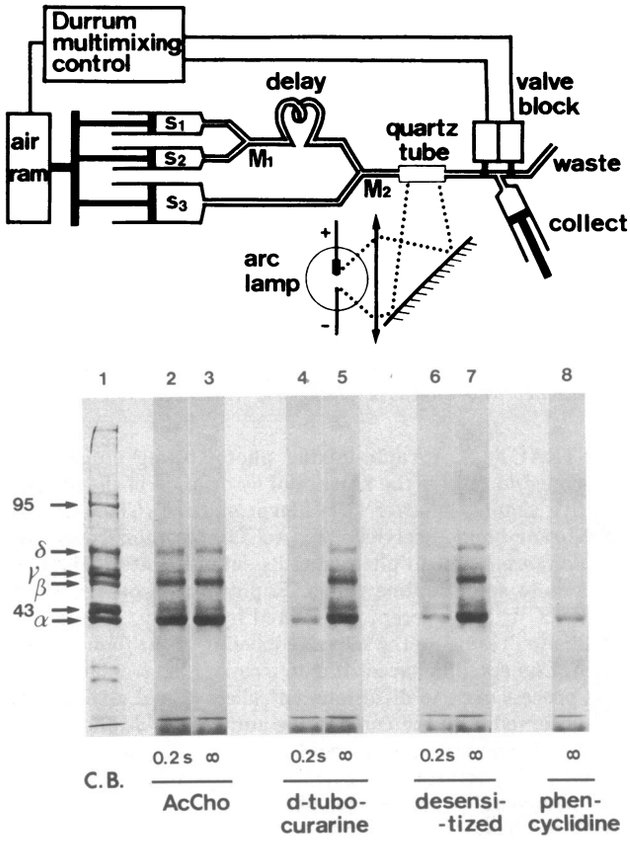

FIG. 1. (*Upper*) Schematic drawing of the rapid-mixing photolabeling apparatus. S, drive syringe; M, mixing chamber. (*Lower*) Time-dependent incorporation of [^{3}H]CPZ into the proteins of the AcChoR-rich membrane fragments in the presence of various pharmacological agents. Lane 1, Coomassie blue (C.B.) stain of the 10% acrylamide/$NaDodSO_4$ gel of the AcChoR-rich membranes. Lanes 2–8, fluorogram of the gel. AcChoR-rich membranes (0.20 μM α-toxin sites) in *Torpedo* saline solution were placed in syringe S_1 and [^{3}H]CPZ (1.0 μM, 1:10 isotopic dilution) was placed in syringe S_2. Effectors were added in the syringes as follows. Lanes 2 and 3, 0.2 mM AcCho in S_2; lanes 4 and 5, 0.2 mM *d*-tubocurarine in S_2; lanes 6 and 7, 0.1 mM AcCho in S_1 and S_2; lane 8, 0.1 mM AcCho and 50 μM phencyclidine in S_1 and S_2. Rapid mixing (1:1) in M_1 (M_2 not used) and UV irradiation at the times indicated; the ∞ time values correspond to 5 min of equilibration. Labeling of the 43-kilodalton proteins was systematically observed, as in ref. 11, but was found to be phencyclidine-insensitive when a better separation from the α chain was obtained (loading less proteins on the gel and determining directly cpm from the cut band).

The kinetic analysis of the photolabeling reaction is limited by the duration of the UV irradiation period (<20 msec) and the life time of the photoactivated compounds. This last parameter was estimated as follows. First, 50 msec after UV irradiation (the shortest possible delay), the [^{3}H]CPZ-labeled membranes were rapidly mixed in the collect syringe (see *Materials and Methods*) with a solution containing a high concentration (0.1 mM) of unlabeled CPZ, to quench eventual further covalent attachment of [^{3}H]CPZ molecules to the high-affinity NCB site. Comparison with the control (no CPZ in the collect syringe) did not reveal any significant difference (i.e., <5%, if any) in both the time course and the extent of [^{3}H]CPZ labeling. In a second series of experiments, a solution of [^{3}H]CPZ and AcCho was preirradiated while flowing in the irradiation chamber and, after the shortest possible delay (50 msec), was rapidly mixed in the collect syringe with AcChoR-rich membranes. In this case, the four chains were only slightly labeled, to an extent that represented 5–10% of that obtained when UV irradiation *followed* and

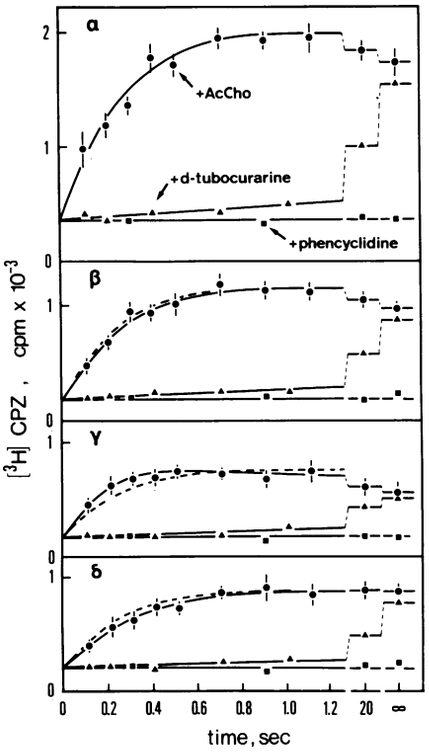

FIG. 2. Kinetics of [^{3}H]CPZ incorporation into each polypeptide chain of the AcChoR. S_1, AcChoR-rich membranes (0.20 μM α-toxin sites) supplemented (■) or not supplemented (●, ▲) with 50 μM phencyclidine; S_2, [^{3}H]CPZ (0.8 μM, 1:5 isotopic dilution) supplemented with 0.2 mM AcCho (●), 0.2 mM *d*-tubocurarine (▲), or 50 μM phencyclidine with 0.1 mM AcCho (■). Rapid mixing (1:1) in M_1. Each point is the mean of duplicates (▲, ■) or triplicates (●; standard errors indicated by the bars). The dashed lines in β, γ, and δ give the time course of [^{3}H]CPZ incorporation in the α chain adjusted to the corresponding maximal amplitudes.

not *preceded* the rapid mixing of the membranes with CPZ. In turn, no labeling could be detected when the membranes instead of CPZ were preirradiated.

These results indicate that (*i*) the formation of photoactivated CPZ molecules is the essential step in the photolabeling of the AcChoR, as expected from the large peak of absorption of CPZ in the short UV range, and (*ii*) the half-life of the excited states of CPZ free in solution is <50 msec and most likely much shorter in the presence of membranes and acceptor groups. The maximal error in the analysis of the time course of CPZ covalent association with the AcChoR should therefore not exceed 50 msec.

This conclusion legitimates a detailed analysis of the rapid association of CPZ with the AcChoR. As shown in Fig. 3, the rate of [^{3}H]CPZ association to *all* four chains of the AcChoR [as well as to the individual chains (not shown)] increased with CPZ concentration, at least in the explored range of concentrations. In this experiment, CPZ concentrations were ≈10 times higher than the concentration of the high-affinity NCB sites (≈0.025 μM), and in these pseudo-first order conditions, the binding curves were fitted by single exponentials in the 0- to 1-sec time range (solid lines in Fig. 3). A plot of the apparent rate constant as a function of CPZ concentration is linear (see Fig. 3 *Inset*), as expected for a simple bimolecular association process. The slope yields an *on* rate constant of 1.1 ± 0.2 10^7 $M^{-1}\cdot sec^{-1}$, close to that expected for a diffusion-controlled reaction. The intercept with the ordinate axis gives the *off* rate, which can be estimated as <1 sec^{-1}. Accordingly, an upper limit for the equilibrium dissociation constant of CPZ for its high-affinity site in the transient state of the AcChoR is 0.1 μM.

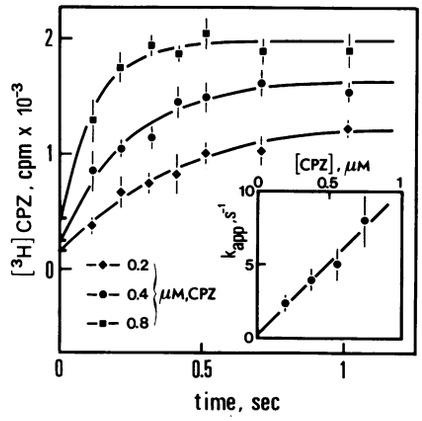

FIG. 3. Concentration dependence of the rate of [^{3}H]CPZ rapid association. S_1, AcChoR-rich membranes (0.1 μM α-toxin sites); S_2, 1:10 isotopically diluted [^{3}H]CPZ supplemented with 0.2 mM AcCho; the final CPZ concentrations after 1:1 rapid mixing in M_1 are indicated; ordinate, sum of the cpm incorporated in all four chains of the AcChoR; each point is the mean of triplicates (standard errors indicated by the bars); the zero-time values were determined by independent measurement of [^{3}H]CPZ incorporation in the presence of 50 μM phencyclidine added to the membranes 30 min before rapid mixing. The solid lines are the best fit by single exponentials, which were forced through the zero-time values derived as indicated above. (*Inset*) Plot of the apparent rate constant, k_{app}, of the exponential fit as a function of CPZ *free* concentration; this last value was derived independently, after centrifugation of aliquots of the CPZ–membrane complex in a Beckman airfuge and determination of cpm in both supernatant and pellet.

Finally, close analysis of the time course of CPZ labeling of each individual chain of the AcChoR (see dashed lines in Fig. 2) revealed slight but reproducible differences, with the maximal rate for the γ chain and the minimal rate for the δ chain. Analysis of the CPZ concentration dependence of these rates of association, as in Fig. 3, further indicates that the differences noticed between chains originate essentially from slight variations in individual k_{off} values.

Agonists and Competitive Antagonists: Concentration–Effect Curves. The initial rate of [^{3}H]CPZ covalent association with the AcChoR was followed as a function of the concentration of three agonists, AcCho, carbamoylcholine, and $NPhMe_3$, and of two competitive antagonists, *d*-tubocurarine and flaxedil (Fig. 4). With the competitive antagonists, as already mentioned, no significant effect was detected (except possibly a limited but reproducible increase with *d*-tubocurarine in the 100-μM range). With agonists, on the other hand, a very large increase was systematically observed, which disappeared completely after preincubation of the AcChoR-rich membranes, before rapid mixing, with *N. nigricollis* α-toxin.

The concentration–effect curves obtained with the three agonists tested differed by (*i*) the maximal incorporation of [^{3}H]CPZ: ≈0.6 for carbamoylcholine and 0.2 for $NPhMe_3$, taking 1.0 as a reference for AcCho, (*ii*) the apparent dissociation constant, K_{app}: ≈30 μM for AcCho, ≈400 μM for carbamoylcholine, and ≈300 μM for $NPheMe_3$, and (*iii*) the shape of the curves, characterized by Hill coefficients, n_H, of 1.3 ± 0.2 for AcCho and carbamoylcholine and 1.1 ± 0.2 for $NPhMe_3$.

Effect of Desensitization on the Initial Rate of CPZ Association. The kinetics of AcCho-triggered [^{3}H]CPZ association changed when the AcChoR-rich membranes were preincubated, before rapid mixing, with AcCho or carbamoylcholine (Figs. 1 and 5). Under these conditions, in which "desensitization" of the ion channel takes place (9), the rapid binding process was no longer observed and [^{3}H]CPZ association proceeded at a rate comparable to—although systematically

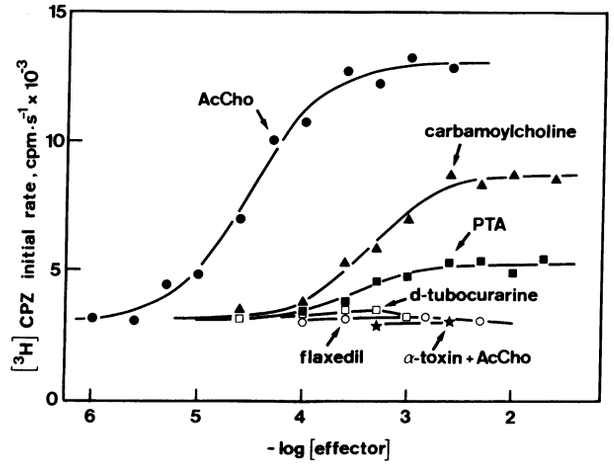

FIG. 4. Effect of various effectors on the initial rate of [^{3}H]CPZ rapid association. S_1, AcChoR-rich membranes (0.2 μM α-toxin sites) supplemented in one case ("α-toxin + AcCho") with 2 μM *Naja nigricollis* α-toxin 30 min before rapid mixing; S_2, [^{3}H]CPZ (0.2 μM, no isotopic dilution) supplemented with effectors as indicated (PTA = $NPhMe_3$); rapid mixing (1:1) in M_1. Ordinate, initial rate, defined as the sum of the cpm incorporated in the β, γ, and δ chains 100 msec after rapid mixing {α was omitted due to possible incorporation of [^{3}H]CPZ at the level of the AcCho binding sites (see ref. 9) in the low-agonist and competitive-antagonist concentration range}; under these conditions, the 100-msec determination is in the linear region of the [^{3}H]CPZ binding curves (see Fig. 3).

larger than—that measured upon simultaneous (as well as prior) addition of *d*-tubocurarine and [^{3}H]CPZ. The reduction of the initial rate of [^{3}H]CPZ association was further analyzed by first rapidly mixing the AcChoR-rich membranes with AcCho (1–10 μM) and, then, after the periods of time indicated in Fig. 5, rapid mixing again with [^{3}H]CPZ with AcCho (1 mM). Under these conditions, the initial rate of CPZ association decreased in the fraction-of-a-second to seconds time range, as a function of the concentrations of AcCho added prior to [^{3}H]CPZ addition. A quantitative analysis of the data yielded apparent rate constants in the 0.1–1 sec^{-1} range.

DISCUSSION

Rapid association of [^{3}H]CPZ to all four chains of the AcChoR (*i*) is agonist-specific, (*ii*) develops in the high-agonist concentration range, in a concentration-dependent manner, and (*iii*) is transient. All of these features are as well characteristic of the permeability response elicited *in vivo* and *in vitro* by AcCho. In both instances, the concentration–effect curves are sigmoid, with Hill coefficients in the 1.1–2.0 range and with apparent dissociation constants in the 50 μM range for AcCho and 500 μM range for carbamoylcholine and $NPhMe_3$ (10, 22–26). Also, saturating concentrations of different agonists stimulate CPZ rapid association to different *maximal* extents, with rank order of potency (AcCho > carbamoylcholine > $NPhMe_3$) identical to that found *in vitro* by initial ion flux measurements (10, 22, 25). With both methods, carbamoylcholine and $NPhMe_3$ behave as *partial* agonists. Moreover, the rapid CPZ association process disappears upon prolonged exposure of the AcChoR to AcCho, with decay rates close to that of channel closure measured *in vivo* and *in vitro* (0.1–1 sec^{-1} range for micromolar AcCho concentrations; reviewed in refs. 26 and 27). Clearly, the initial rate of CPZ association parallels the number of channels initially opened for varying concentrations of agonists, and, for a fixed concentration of agonist, parallels the number of channels that remain open in the course of AcChoR desensitization. Thus, the agonist-dependent rapid association of [^{3}H]CPZ takes place with the AcChoR in its "open channel" conformation. In addition, the CPZ concentration dependence and the absolute value of the rate of CPZ rapid association are close to those derived from *in vivo* electrophysiological measurements, for the blockade by NCBs of the open channel (10, 28). Therefore, the rapid association of CPZ most likely occurs at the level of the ion channel itself.

As a typical NCB, [^{3}H]CPZ binds to a high-affinity site, sensitive to histrionicotoxin and present as a single copy per molecule of AcChoR (9). The evidence presented here that the labeling of the four chains follows closely related time courses supports our previous conclusion that this site is located in the axis of "crypto-symmetry" of the AcChoR oligomer, possibly in the hydrophilic pit revealed by electron mi-

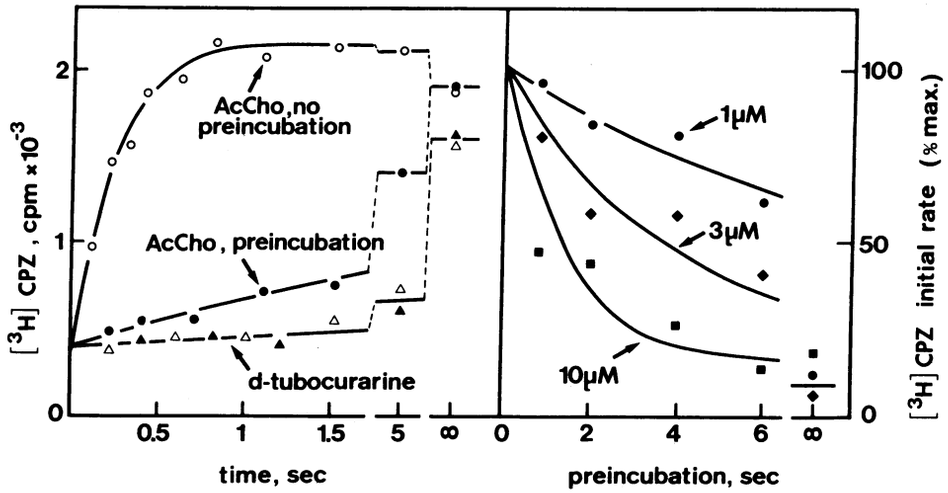

FIG. 5. Effect of desensitization on [^{3}H]CPZ association. (*Left*) Effect of prolonged application of cholinergic effectors. S_1, AcChoR-rich membranes (0.2 μM α-toxin sites) supplemented at least 30 min before rapid mixing with 0.1 mM AcCho (●), 0.1 mM *d*-tubocurarine (▲), or no effector (○, △); S_2, [^{3}H]CPZ (0.8 μM, 1:10 isotopic dilution) supplemented with 0.1 (●) or 0.2 (○) mM AcCho or 0.1 (▲) or 0.2 (△) mM *d*-tubocurarine; rapid mixing (1:1) in M_1. Ordinate, sum of the cpm incorporated in all four chains of the AcChoR. (*Right*) Time course of the decrease of [^{3}H]CPZ rapid association. S_1, AcChoR-rich membranes (0.2 μM α-toxin sites); S_2, AcCho at concentrations twice those indicated in the figure; S_3, [^{3}H]CPZ (0.5 μM, no isotopic dilution) supplemented with 0.2 mM AcCho; rapid mixing (1:1) in M_1, followed at the times indicated by rapid mixing (1:1) in M_2; [^{3}H]CPZ initial rate is the sum of the cpm incorporated in all four chains of the AcChoR ≈10 msec after the rapid mixing in M_2.

croscopy at the center of the rosette (29). Although a high degree of primary structure homology exists between the chains (2, 5), minor but reproducible differences were detected in the kinetics of labeling of each individual chain. They might be relevant to different locations of appropriate acceptor groups on the polypeptide chains, as a consequence of discrete dissimilarities in primary structure or tertiary organization or both.

Under conditions in which the ion channel is closed—i.e., upon addition of a competitive antagonist or upon *prolonged* application of agonists—association of CPZ was found to be very slow, suggesting that in the closed channel conformation structural barriers restrict the accessibility of [^{3}H]CPZ to its site (18). The slightly faster rate of CPZ association to the AcChoR observed after prolonged application of AcCho as compared to that with *d*-tubocurarine further suggests that either (*i*) the accessibility of the NCB site slightly differs in the resting and desensitized conformations of the AcChoR or (*ii*) NCBs reach their site exclusively if the channel opens (see ref. 30) and, then, the occurrence of this "active" conformation differs, at equilibrium, in the presence of AcCho or *d*-tubocurarine. In the framework of this second alternative, the slight effect reported with *d*-tubocurarine on the stimulation of CPZ association (Fig. 4) might be compared with the ability of this effector to promote, to some extent, channel opening of AcChoRs in rat myotubes and embryonic muscle (31, 32).

The slow decrease in the seconds time range observed after the rapid increase of [^{3}H]CPZ labeling elicited by AcCho might simply reflect the slow, diffusion-restricted release of CPZ molecules trapped within the ion channel. Accordingly, the affinity of the NCB site in the open conformation would have to be much larger than in the closed conformation to account for the release of already bound molecules upon AcChoR desensitization. This fits with the upper estimate of the affinity of the NCB site in the open channel conformation (0.1 μM), as derived from the kinetic analysis in Fig. 3, a value lower than that previously derived under *equilibrium* conditions with the ion channel closed (0.6 and 5 μM; see ref. 9). Accordingly, the differences in affinity already observed for several NCBs (including CPZ) between the *resting* and the two *desensitized* conformations of the AcChoR should be extended to the *active* state.

In conclusion, rapid labeling data of the AcChoR subunits by [^{3}H]CPZ support the proposal that the high-affinity site for NCBs is common to all five subunits, lies within the ion channel, and becomes accessible when the channel opens.

We thank Dr. Charlier (Centre National de la Recherche Scientifique, Orleans) for lending his 500-W HBO arc lamp, J. Daty and A. Assimon (Institut Pasteur) for their help in the construction of the photolabeling apparatus, Dr. Bazin and Prof. Le Pecq for helpful discussions, Prof. Lazdunski for phencyclidine, and Dr. Menez for *N. nigricollis* α-toxin. This work was supported by grants from the Muscular Dystrophy Association of America, the Fondation de France, the Collège de France, the Ministère de la Recherche et de l'Industrie, the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, and the Commissariat à l'Energie Atomique.

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