D. (1979) *Biochemistry 18*, 780-789.
Sarges, R., & Witkop, B. (1964) *J. Am. Chem. Soc. 86*, 1862-1863.
Scandella, C. J., Devaux, P., & McConnell, H. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2056-2060.
Tokutomi, S., Lew, R., & Ohnishi, S. (1981) *Biochim. Biophys. Acta 643*, 276-282.
Van Dijck, P. W. M., De Kruijff, B., Verkleij, A. J., Van Deenen, L. L. M., & De Gier, J. (1978) *Biochim. Biophys. Acta 512*, 84-96.

Multiple Sites of Action for Noncompetitive Blockers on Acetylcholine Receptor Rich Membrane Fragments from *Torpedo marmorata*†

Thierry Heidmann, Robert E. Oswald, and Jean-Pierre Changeux*

ABSTRACT: A vast series of compounds, different from the typical nicotinic antagonists, block in a noncompetitive manner the permeability response of several cholinergic synapses (electromotor synapse, neuromuscular junction) to acetylcholine. The interaction of several of these noncompetitive blockers was investigated in vitro with membrane fragments rich in acetylcholine receptor prepared from *Torpedo marmorata* electric organ. First, their effect on the conformational transitions of the membrane-bound receptor was monitored with a fluorescent cholinergic agonist and a rapid-mixing technique. All the compounds tested stabilized to various extents a "desensitized" state of the receptor exhibiting a high affinity for the agonist with minimal effect but with a high affinity in the case of the frog toxin perhydrohistrionicotoxin (H_{12}HTX). H_{12}HTX was then used to distinguish between two categories of effects of the noncompetitive blockers: (1) with some of them (phencyclidine, meproadifen, Triton X-100), the stabilization of the high-affinity state was blocked by H_{12}HTX; (2) with others (chlorpromazine, trimethisoquin), the shift of the conformational equilibrium was insensitive to H_{12}HTX. In the first instance, the stabilization of the high-affinity state took place without noticeable cooperative effects, while in the second, significant positive cooperativity was systematically observed. In a second series of experiments, binding studies were carried out under equilibrium conditions with tritiated derivatives of H_{12}HTX, phencyclidine, meproadifen, Triton X-100, chlorpromazine, and trimethisoquin. All these tritiated ligands bound to "allosteric" sites distinct from the acetylcholine receptor site, but they also bound to some extent to the receptor site. Two main categories of allosteric sites for noncompetitive blockers were distinguished: (1) a "high-affinity" site which was present in one copy per acetylcholine receptor light form (250000 daltons) and blocked by H_{12}HTX and (2) a population of "low-affinity" sites present in large numbers (10-30 sites per molecule of receptor) and insensitive to H_{12}HTX. Comparison of the conformational and binding data led to the conclusion that the high-affinity site was responsible for the H_{12}HTX-sensitive effect of noncompetitive blockers on the conformational transition while the low-affinity sites were engaged in the H_{12}HTX-insensitive ones. In the cases of chlorpromazine and trimethisoquin, low-affinity H_{12}HTX-insensitive binding was significant at concentrations at which the effects on conformational transitions occurred. Reconstitution experiments indicated that the number of these low-affinity sites depended on the lipid to protein ratio, suggesting that these sites are located at the interface of the receptor protein with membrane lipids. It was also shown that the unique high-affinity site for noncompetitive blockers is in contact with all four types of subunits of the receptor molecule. The possibility is considered that this site lies within the central depression of the molecule which potentially may serve as an ion channel.

At the neuromuscular junction and the electromotor synapse, the permeability response of the postsynaptic membrane to the neurotransmitter acetylcholine (AcCh)[1] is blocked by two series of pharmacological agents: the competitive antagonists like *d*-tubocurarine or flaxedil and a heterogeneous group of compounds referred to as noncompetitive blockers (NCBs) [review in Changeux (1981)]. This class of effectors includes aminated local anesthetics such as procaine, lidocaine, dibucaine, proadifen, or dimethisoquin [see Weber & Changeux (1974), Cohen et al. (1974), Weiland et al. (1977), Heidmann & Changeux (1979), Krodel et al. (1979), and Sine & Taylor (1982)], sedatives such as chlorpromazine (Koblin & Lester, 1979), antimalarial drugs such as quinacrine (Grünhagen & Changeux, 1976; Adams & Feltz, 1980), hallucinogens such as phencyclidine (Kloog et al., 1980; Albuquerque et al., 1980; Karpen et al., 1982; Oswald & Changeux, 1981a,b), a frog toxin, histrionicotoxin (Daly et al., 1971; Elliott & Raftery, 1977; Eldefrawi et al., 1980b; Spivak et al., 1982), the lipophilic cation triphenylmethylphosphonium (Lauffer & Hucho, 1982), and a number of

† From the Neurobiologie Moléculaire et Laboratoire Associé au Centre National de la Recherche Scientifique, Interactions Moléculaires et Cellulaires, Institut Pasteur, 75724 Paris Cedex 15, France (T.H., R.E.O., and J.-P.C.), and the Department of Pharmacology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853 (R.E.O.). *Received November 15, 1982; revised manuscript received March 3, 1983.* This work was supported by grants from the Muscular Dystrophy Association of America, the Fondation Fyssen, 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.

[1] Abbreviations: AcCh, acetylcholine; AcChR, acetylcholine receptor; H_{12}HTX, perhydrohistrionicotoxin; Dns-C_6-Cho, 6-[5-(dimethylamino)-1-naphthalenesulfonamido]hexanoic acid β-(methobromide) ethyl ester; NCB, noncompetitive blocker; 5A[^{3}H]T, 5-azido[^{3}H]trimethisoquin; $C_{12}E_8$, octaoxyethylene glycol dodecyl monoether; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; α-[^{125}I]BGT, ^{125}I-labeled α-bungarotoxin; $NaDodSO_4$, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole; Temed, *N,N,N',N'*-tetramethylethylenediamine.

0006-2960/83/0422-3112$01.50/0

structurally unrelated compounds usually considered as primarily interacting with lipids such as detergents and fatty acids (Brisson et al., 1975; Andreasen & McNamee, 1980), phospholipases (Bon et al., 1979; Andreasen et al., 1979), and general anesthetics and alcohols (Gage, 1976; Bradley et al., 1980; Young & Sigman, 1982). All these compounds block the response to AcCh in a noncompetitive manner, that is, by decreasing the amplitude of the response without significantly changing the apparent affinity for AcCh.

A number of physiological observations based on the analysis of miniature end-plate currents in the presence of these effectors under voltage-clamp conditions (Steinbach, 1968), on noise (Katz & Miledi, 1975), voltage-jump (Adams, 1977; Koblin & Lester, 1979), and single-channel (Neher & Steinbach, 1978) recordings, led to the suggestion that NCBs (both charged and neutral ones) could transiently enter and plug the open ionic channel, thus blocking ion translocation by steric hindrance [reviewed in Adams (1978)]. Other sites and/or mechanisms of action for NCBs were also considered [see Katz & Miledi (1980)]. Koblin & Lester (1979) have shown by the voltage-jump technique that, at low negative membrane potentials, the potency of several NCBs simply parallels their lipid solubility, whereas at high negative potentials this correlation is no longer observed. They suggested, therefore, that, in addition to a direct interaction with the receptor–channel complex, NCBs could also block the physiological response by an indirect interaction via the lipids surrounding the receptor. Finally, as pointed out by Neher & Steinbach (1978), a scheme where the NCBs do not *sterically* inhibit ion translocation but *allosterically* favor a conformation of the receptor where the channels are closed may also account for the electrophysiological data.

In this respect, in vitro studies of the effects of NCBs on highly purified AcChR-rich membranes from electric fish led to the conclusion that these effectors may regulate, in an *allosteric* manner, the conformational transitions of the AcChR: (1) Under equilibrium conditions, NCBs increase the binding of [^{3}H]AcCh [Cohen et al., 1974; Krodel et al., 1979; reviewed in Changeux 1981)]. (2) Kinetic experiments show that the rate of slow conversion of the AcChR from a state of low affinity for AcCh to a state of high affinity, which corresponds to the physiological processes of "desensitization" [Weber et al., 1975; reviewed in Changeux (1981)], is, as observed in vivo, accelerated by NCBs (Grünhagen & Changeux, 1976; Weiland, et al., 1977; Heidmann & Changeux, 1979; Sine & Taylor, 1982) with the exception of tetracaine (Blanchard et al., 1979). (3) Finally [Heidmann & Changeux, 1979; Cohen et al., 1980; Young & Sigman, 1982; Sine & Taylor, 1982; but see Dunn et al. (1981)], NCBs may shift the equilibrium between preexisting conformations of the AcChR, in the absence of AcCh, and stabilize high-affinity desensitized conformations of the AcChR. It was shown, however (Heidmann et al., 1980; Cohen et al., 1980; Heidmann & Changeux, 1981), that the effects of several NCBs were additive, suggesting that, in agreement with the hypothesis of Koblin & Lester (1979), these compounds do not act at a single class of sites.

Binding sites for noncompetitive blockers have been identified on AcChR-rich membrane fragments by using the fluorescent NCB quinacrine (Grünhagen & Changeux, 1976) and a variety of radiolabeled derivatives such as [^{14}C]- or [^{3}H]trimethisoquin (Cohen, 1978; Sobel et al., 1980), [^{14}C]-meproadifen (Krodel et al., 1979), [^{3}H]perhydrohistrionicotoxin (H_{12}HTX) (Eldefrawi et al., 1977, 1978, 1980b; Elliott & Raftery, 1977, 1979; Aronstam et al., 1981; Oswald & Changeux, 1981a), or [^{3}H]phencyclidine (Kloog et al., 1980; Eldefrawi et al., 1980a; Oswald & Changeux, 1981a,b). Although, in several cases, heterogeneous binding was observed, the analysis primarily concerned a high-affinity binding which takes place in the micromolar range and is inhibited, for all noncompetitive blockers tested, by the potent noncompetitive blocker histrionicotoxin. Cholinergic ligands increased the affinity of the NCBs for these sites. The values reported for the stoichiometry of these high-affinity sites ranged from approximately 0.25 site per α-toxin binding sites (Elliott & Raftery, 1977; Krodel et al., 1979; Elliott et al., 1979) to 0.5–0.7 (Eldefrawi et al., 1978; Sobel et al., 1980; Medynski & Cohen, 1981) and 2 (Eldefrawi et al., 1977). Finally, reconstituted vesicles containing only the purified AcChR and lipids (Sobel et al., 1980) exhibit binding properties for NCBs identical with those for the native membranes. Thus, at least part of the sites for NCBs are carried by the AcChR and not by nonreceptor polypeptides (Neubig et al., 1979) present in the AcChR-rich membranes such as the 43 000-dalton polypeptides (Sobel et al., 1977, 1978). An unambiguous demonstration that the site(s) for the pharmacological action of NCBs is (are) localized on the AcChR molecule has been presented by Oswald & Changeux (1981a,b) by using a radioactive photoaffinity derivative of the potent local anesthetic trimethisoquin, 5A[^{3}H]T, synthesized by Waksman et al. (1980a). This compound selectively labels the δ subunit of the AcChR, and this labeling is inhibited by an excess of trimethisoquin and histrionicotoxin and enhanced by cholinergic agonists and some antagonists but not by the snake α-toxins. Simultaneous measurements of the covalent labeling of the δ chain and of the reversible binding of [^{3}H]phencyclidine to its high-affinity sites on the AcChR-rich membrane fragments led to the conclusion that the δ chain of the receptor carries at least part of the high-affinity NCB sites.

Covalent labeling of the δ chain of the AcChRs was also achieved by simple UV irradiation of the AcChR-rich membrane fragments in the presence of unmodified noncompetitive blockers such as [^{3}H]H_{12}HTX, [^{3}H]phencyclidine, [^{3}H]trimethisoquin, and [^{3}H]chlorpromazine (Oswald & Changeux, 1981a). In all cases, the labeling was enhanced by the agonist carbamoylcholine and decreased by HTX. Interestingly, [^{3}H]chlorpromazine labeled the three other chains of the AcChR (α, β, γ) in addition to the δ chain.

In this series of two papers, the mode of action and the binding properties of NCBs to AcChR-rich membranes from *Torpedo marmorata* are investigated in a detailed manner. The first paper deals with the different sites for NCBs and the second with the different conformations of the membrane-bound AcChR with which they selectively interact. Structural and kinetic models of the AcChR as an "allosteric" membrane protein are discussed.

Materials and Methods

Materials. Tritium-substituted *Naja nigricollis* α-toxin was a gift from Dr. A. Menez (C.E.A., France). [^{3}H]Trimethisoquin (6.5 Ci/mmol) and [^{3}H]meproadifen (20.5 Ci/mmol) were synthesized by methylation of dimethisoquin and proadifen, respectively, with [^{3}H]methyl iodide from C.E.A. (France). [^{3}H]H_{12}HTX (46 Ci/mmol) was synthesized by reduction of dihydrohistrionicotoxin with $^3H_2$ from C.E.A. (France). [^{3}H]Phencyclidine (40–48 Ci/mmol), ^{3}H-labeled Triton X-100 (1.0 mCi/mmol), and [^{3}H]chlorpromazine (20–25 Ci/mmol) were purchased from New England Nuclear; [^{14}C]$C_{12}E_8$ (46 mCi/mmol) was from C.E.A. (France) and [^{14}C]decanol (7.5 mCi/mmol) from ICN. The purity

(>97%) of all these radiolabeled analogues as well as the comigration with the corresponding unlabeled analogues was verified by thin-layer chromatography as indicated by the manufacturers.

Naja nigricollis α-toxin was a gift from Dr. A. Menez, erabutoxin b was from Professor Tamiya, Dns-C_6-Cho was from Dr. G. Waksman, who synthesized it (Waksman et al., 1980b), phencyclidine was from Professors M. Lazdunski and M. Sololovsky, histrionicotoxin and perhydrohistrionicotoxin were from Professor J. Daly, prilocaine, lidocaine, and dimethisoquin were from Laboratory Roger Bellon (France), proadifen was from Smith Kline and French (Philadelphia, PA), $C_{12}E_8$ was from C.E.A. (France), and flaxedil was from Rhone-Poulenc (France). Trimethisoquin and meproadifen were prepared by methylation of the free base tertiary amine precursors and were a gift from Dr. G. Waksman. Nonidet P-40 was from BDH Chemicals Ltd., dibucaine and carbamoylcholine chloride were from K and K Labs, Triton X-100, Lubrol PX, chlorpromazine, methanol, decanol, aprotinine, pepstatin, *p*-aminobenzoate, and PMSF were from Sigma, 2-methyl-2-nitrosopropane was from Fluka, and acrylamide, bis(acrylamide), and Temed were obtained from Kodak. Live *Torpedo marmorata* were provided by the Biological Station of Arcachon (France).

Preparation of AcChR-Rich Membranes and Reconstituted Vesicles. Membranes enriched in AcChR were purified from freshly dissected *Torpedo marmorata* electric organ as described by Sobel et al. (1977) in the presence of protease inhibitors and chelating agents to limit proteolysis (buffer A: 50 mM Tris-HCl, pH 7.5, 3 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 5 units/mL aprotinine and 5 μg/mL pepstatin; Saitoh et al., 1980). The membranes were stored until use in liquid nitrogen at a concentration of 15–25 μM α-toxin sites and were thawed at room temperature immediately before use.

Reconstituted vesicles containing the purified AcChR were prepared as described in Sobel et al. (1980), after depletion of the AcChR-rich membranes of nonreceptor peptides by alkaline treatment (Neubig et al., 1979). Variable amounts of exogenous lipids [asolectin; see Epstein & Racker (1978)] solubilized in sodium cholate (10% w/v asolectin/10% w/v sodium cholate) were added to the solubilized AcChR extract in 1% (w/v) sodium cholate (0–0.2% w/v final asolectin concentration), and after a 30-min incubation, the solutions were layered on Sephadex G-50 columns equilibrated with 100 mM NaCl and 10 mM Tris-HCl, pH 7.5 (buffer B). The columns were developed in buffer B and reconstituted liposomes containing AcChR eluted in the void volume free of sodium cholate. Phospholipid content was determined by using the Fiske Subba-Row technique (Böttcher et al., 1961).

The concentration of α-toxin binding sites was routinely determined by using commercially available α-[^{125}I]BGT and column filtration as described by Sobel et al. (1977). For stoichiometry determinations, two different methods were used. They are described under Results and in the legend of Figure 5.

Initial Rate of ^{125}I-Labeled α-Toxin Binding to Membrane Fragments. This rate was measured at 20 °C by filtration as described by Oswald & Freeman (1979) by using EGWP Millipore filters. The concentration of α-[^{125}I]BGT was 0.8 nM, and that of α-toxin binding sites was 4 nM. Under these conditions, the amount of α-toxin bound was linear with time for at least 5 min, and the initial rate of binding was determined by filtering aliquots at 15-s intervals after mixing.

Rapid Kinetics of Dns-C_6-Cho Binding. Rapid kinetics of Dns-C_6-Cho and analysis of the fluorescence signals were carried out as previously described (Heidmann & Changeux, 1979) with a Gibson-Durrum stopped-flow rapid-mixing apparatus equipped for fluorescence detection. AcChR-rich membrane fragments diluted in *Torpedo* physiological solution (buffer C: 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate buffer, pH 7) were incubated with (or without) NCBs for at least 10 min before rapid mixing in the stopped-flow apparatus with solutions of Dns-C_6-Cho (1–5 μM) in the same medium. Experiments were carried out at 20 °C.

Equilibrium Binding of Radioactive Ligands. The reversible binding of radiolabeled noncompetitive blockers was measured as described by Krodel et al. (1979) with minor modifications. Membrane fragments were resuspended to a final concentration of approximately 2–3 μM α-toxin binding sites in a *Torpedo* physiological solution with or without the indicated cholinergic effectors (carbamoylcholine, erabutoxin b, excess unlabeled noncompetitive blocker, etc.), and 50-μL aliquots were pipetted into Beckman airfuge polyallomer tubes. Aliquots (50 μL) of a *Torpedo* physiological solution containing the radioactively labeled noncompetitive blocker to be tested were then added, and the suspension was equilibrated for at least 1 h at 20 °C. Unless otherwise specified (see α-Toxin and NCB Site Stoichiometry under Results), the ratio of the concentration of radiolabeled vs. unlabeled noncompetitive blocker in the aliquot was less than 0.05 so that the actual concentration of noncompetitive blocker (i.e., radiolabeled plus unlabeled) was not significantly different from the concentration of the unlabeled analogue. In all cases, this ratio was held constant for a given binding experiment. All binding experiments were performed at 20 °C.

Bound ligand was separated from free ligand by centrifugation in a Beckman airfuge at 30 psi ($\sim$130000*g*) for 10 min. After centrifugation, duplicate 20-μL aliquots of the supernatant were removed and assayed for radioactivity in 4 mL of BioFluor. The remainder of the supernatant was then aspirated, and the tube was wiped with a cotton swab and left inverted on the swab for 30 min. Fifty microliters of 10% (w/v) Triton X-100 was then added to the tubes for at least 30 min, and the solution was transferred to a counting vial. The tube was then washed with two 50-μL aliquots of 0.01% (w/v) sodium dodecyl sulfate and the wash combined with the Triton X-100 extract. Samples were assayed for radioactivity in BioFluor (NEN) by using an Intertechnique SL 30 or a Kontron β counter.

Covalent Labeling of the AcChR by [^{3}H]Chlorpromazine. Covalent labeling of the AcChR by [^{3}H]chlorpromazine was performed as described in Oswald & Changeux (1981a,b). Membranes were resuspended to a final concentration of 7–8 μM α-toxin binding sites in a *Torpedo* physiological solution supplemented with carbamoylcholine at a final concentration of 0.3 mM. To 20 μL of the suspension was added 20 μL of *Torpedo* physiological solution with or without nonradioactive NCBs and/or scavenging reagents followed by 20 μL of a solution of [^{3}H]chlorpromazine at a concentration of 1.5 μM. Thirty-microliter aliquots of the solutions were placed in a chamber, flushed with nitrogen for 15 min, and irradiated for 5 min with a Mineralight shortwave UV lamp (254 nm). After illumination, 30 μL of $NaDodSO_4$ buffer was added, and 25-μL samples were loaded on $NaDodSO_4$/10% acrylamide/0.26% bis(acrylamide) gels [see Saitoh et al. (1980)]. Radioactivity was detected by cutting out appropriate sections of the dried gel and counting in a medium containing 900 mL of toluene/PPO, 100 mL of tissue solubilizer, and 20 mL of 4 M NH_4OH. In parallel, 30-μL aliquots of nonirradiated

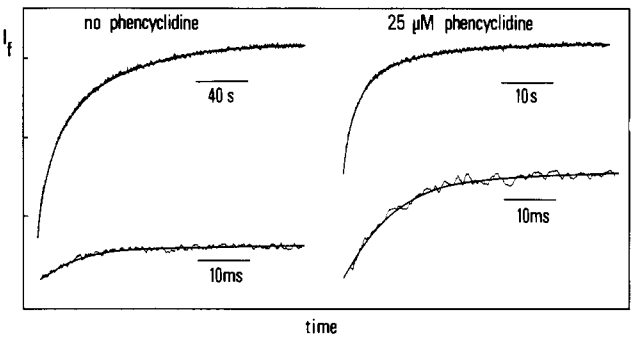

FIGURE 1: Fast kinetic titration of the low- and high-affinity conformations of the AcChR in the absence or presence of a NCB. Increases in fluorescence intensity after 1:1 rapid mixing in the stopped-flow apparatus of a suspension of AcChR-rich membrane fragments (0.3 μM α-toxin binding sites) in *Torpedo* physiological solution supplemented (right) or unsupplemented (left) with 25 μM phencyclidine with a solution of Dns-C_6-Cho (3 μM) in the same medium [λ_{ex} = 290 nm, λ_{em} > 540 nm; see Heidmann & Changeux (1979)]. The rapid relaxation processes are represented on expanded time scales, and the smooth lines are the least-squares fit of the experimental traces by a sum of exponentials. Fractional amplitudes of the rapid relaxation processes are 0.20 and 0.53, respectively, in the absence and presence of 25 μM phencyclidine.

solutions were centrifuged for 5 min in a Beckman airfuge at maximum speed, and the amount of bound [^{3}H]chlorpromazine was measured by pipetting duplicate 10-μL aliquots of the supernatants after centrifugation.

Results

Multiple Effects of NCBs on the Conformational Transitions of the Membrane-Bound AcChR. (A) Quantitative Analysis of the Effect of NCBs. As illustrated in Figure 1 and previously described (Heidmann & Changeux, 1979), rapid mixing of membrane-bound AcChR with micromolar solutions of the fluorescent cholinergic agonist Dns-C_6-Cho results in the diffusion-controlled binding of the agonist, in the millisecond time scale, to a fraction of receptors existing prior to mixing with the agonist in a high-affinity desensitized state, followed by the slow interconversion in the second time range of the rest of the sites, of lower affinity, toward the high-affinity state. The fractional concentration, $\bar{D}$, of receptor sites existing prior to agonist addition in the high-affinity state, which is then equal to the fractional amplitude of the rapid relaxation process, ranges from approximately 0.2 in the absence of any ligand up to a maximum of 0.9–1.0 after preincubation of the AcChR-rich membranes or reconstituted vesicles with NCBs (Heidmann & Changeux, 1979).

The variation of $\bar{D}$ as a function of NCB concentrations is presented in Figure 2 for a series of NCBs which includes H_{12}HTX (Figure 2A), meproadifen (Figure 2B), phencyclidine (Figure 2C), Triton X-100 (Figure 2D), trimethisoquin (Figure 2E), and chlorpromazine (Figure 2F). With all NCBs tested, $\bar{D}$ increases with increasing NCB concentration, but both the concentration of NCB giving half-maximum variation (apparent dissociation constant) and the shape of the concentration effect curve are highly dependent on the nature of the NCB. Apparent dissociation constants (see Table I) vary from the micromolar range for H_{12}HTX (0.7 μM), meproadifen (3.4 μM), phencyclidine (4.3 μM), and chlorpromazine (4.3 μM) to the millimolar range for prilocaine (1.5 mM; Heidmann & Changeux, 1979) and the molar range for methanol (1.85 M). With phencyclidine and H_{12}HTX, $\bar{D}$ has only a limited variation whereas with trimethisoquin, Triton X-100, chlorpromazine, meproadifen, methanol, or decanol $\bar{D}$ reaches values close to unity. With meproadifen or Triton X-100, the concentration effect curves do not deviate from

Table I: Binding Properties and Allosteric Effects of Various NCBs on Membrane-Bound AcChR

	AcChR site, K (μM)[a]	R ⟷ D transition[b]			binding of radiolabeled NCB[c]			
					high affinity		low-affinity	nonsaturable[d]
		K_{app} (μM)	K_D(calcd) (μM)	K_R(calcd) (μM)	$K_{carbamoylcholine}$ (μM)	$K_{\alpha\text{-toxin or no effector}}$ (μM)	K (μM)	$P_{NCB}/P_{phencyclidine}$
H_{12}HTX	120 ± 20	0.7 ± 0.2	0.4 ± 0.1	0.9 ± 0.2	0.16 ± 0.05	0.25 ± 0.05 (0.23 ± 0.05)	>100	1.8
phencyclidine	250 ± 30	4.3 ± 1.0	1.5 ± 0.5	7.6 ± 1.5	0.8 ± 0.2	5.1 ± 0.8 (3.6 ± 0.8)	170 ± 50	1.0
trimethisoquin	12 ± 3	6.2 ± 1.5			1.2 ± 0.3	3.2 ± 0.9	39 ± 15	2.2
meproadifen	54 ± 11	3.4 ± 1.0	0.8 ± 0.2	33 ± 7	0.5 ± 0.2	6.2 ± 3	150 ± 30	1.4
chlorpromazine	24 ± 5	4.3 ± 1.5			0.6 ± 0.2	4.9 ± 1.3	19 ± 5	12.1
Triton X-100	>150	21 ± 3	6.8 ± 2	105 ± 25	4.9 ± 1.0	31 ± 8		6.2
$C_{12}E_8$	>150	14 ± 3	2.8 ± 1	110 ± 45	2.5 ± 1.5			62.2
decanol		32 ± 7						53.4

[a] K derived from the decrease of the initial rate of ^{125}I-labeled α-toxin binding and from the reduction of the apparent rate constant of the "rapid relaxation" process monitored with Dns-C_6-Cho (see Conformational Transitions Associated with the Interaction of NCBs with the AcCh Binding Site under Results). [b] Parameters for the effect of NCBs on the allosteric transitions of the AcChR monitored by the rapid kinetics of Dns-C_6-Cho binding (see Figure 2); K_{app} is the NCB concentration for the half-maximum effect; K_D and K_R are calculated values obtained by a least-squares fit of the data as in Figure 2 by eq 2 (see Regulation via the High-Affinity Site for NCBs under Discussion). [c] K for the high- and low-affinity equilibrium binding in the presence of no effector (numbers in parentheses), α-toxin, or carbamoylcholine derived from binding curves as in Figure 4 (see Saturable Binding of NCBs under Results). [d] Nonsaturable partitioning (bound/free NCB in the presence of excess unlabeled NCB) relative to that of phencyclidine (see Nonsaturable Binding of NCBs under Results).

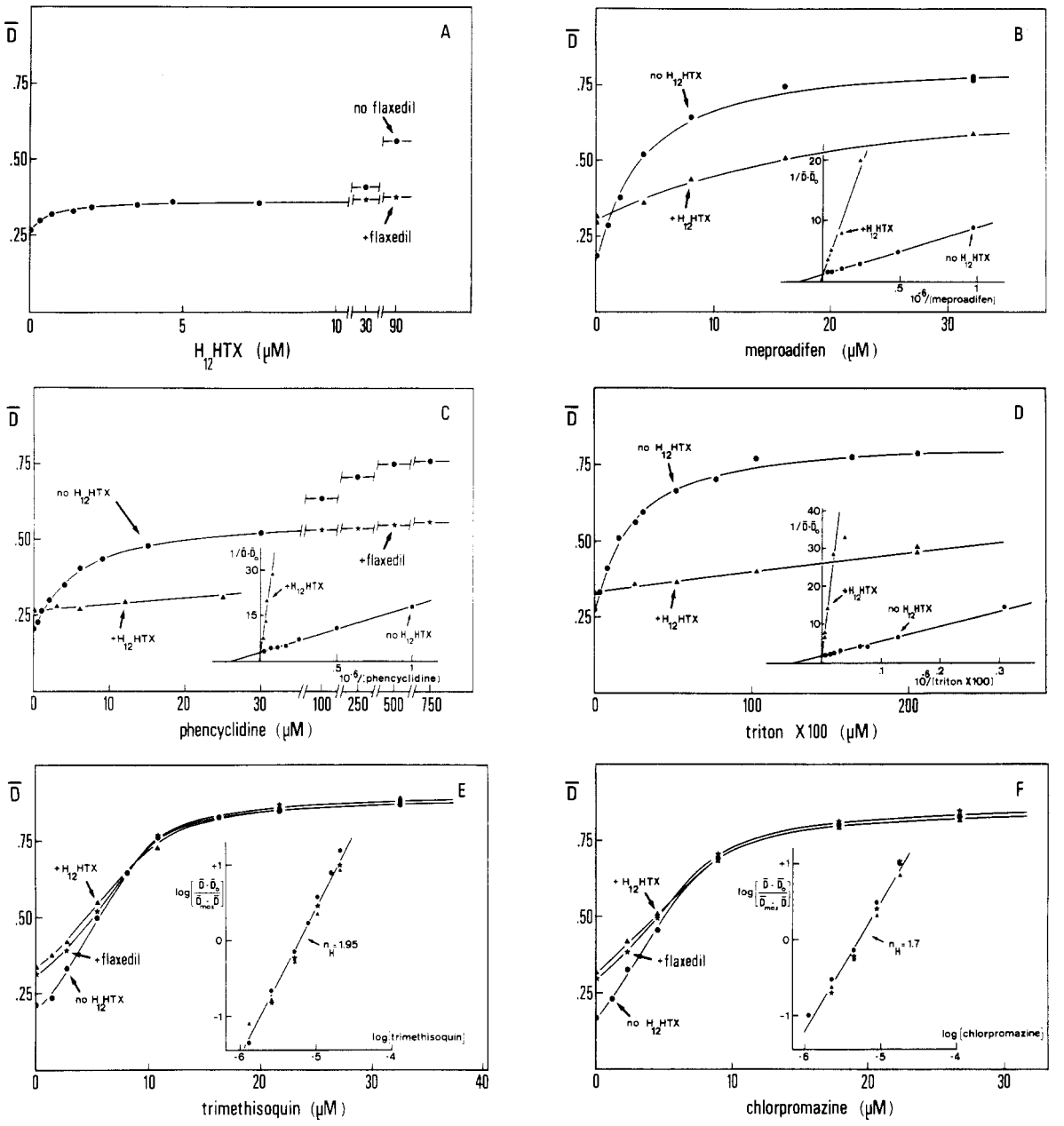

FIGURE 2: Allosteric regulations by a series of NCBs of the fractional concentration of AcChRs in the high-affinity conformation. The experimental conditions are the same as those in Figure 1. Prior to the addition of 3 μM Dns-C_6-Cho, AcChR-rich membrane fragments were incubated with increasing concentrations of the indicated NCBs alone (●), with 10 μM H_{12}HTX (▲), or with 2 mM flaxedil (★). The NCBs used were H_{12}HTX (A), meproadifen (B), phencyclidine (C), Triton X-100 (D), trimethisoquin (E), and chlorpromazine (F). The solid lines in Figure 2A–D (up to the break on the abscissa axis) are least-squares fits of the data by a rectangular hyperbola (see Regulation via the High-Affinity Site for NCBs under Discussion). Inserts show double-reciprocal (B–D) or Hill plots (E, F) of the data (see Appendix 1).

rectangular hyperbolas (n_H = 1; see solid line in Figure 2B,D). With trimethisoquin, chlorpromazine, and methanol, the concentration effect curves are sigmoidal with Hill coefficients respectively equal to 1.95, 1.7, and 2.5 (see Figure 2E,F and data not shown). On the other hand, with phencyclidine (Figure 2C) and H_{12}HTX (Figure 2A), the curves can be correctly fitted in the low concentration range by a rectangular hyperbola (n_H = 1; see solid line), but deviations are clearly observed in the high NCB concentration range. These results suggest that NCBs interact with the membrane-bound AcChR at the level of several classes of sites which might include the AcCh binding site itself and several categories of allosteric sites distinct from the AcCh binding site.

(B) Conformational Transitions Associated with the Interaction of NCBs with the AcCh Binding Site. In agreement with the early findings of Weber & Changeux (1974), NCBs reduce the initial rate of α-[^{125}I]BGT binding to the AcChR and the rate constant of the rapid signal which corresponds to Dns-C_6-Cho binding to the AcChR in its high-affinity state (Heidmann & Changeux, 1979). Apparent dissociation constants for the AcCh binding sites are given in Table I and are in all cases larger than those at which the NCBs exert their primary allosteric effects. To test for possible contributions of the interaction of NCBs with the AcCh binding sites on the conformational transitions of the AcChR, conditions were searched for which eliminate NCB binding to the AcCh binding site but still allow measurements of the effects of NCBs on the state transitions of the AcChR. It was found that following prior incubation of membrane fragments with saturating concentrations of flaxedil (2 mM), the fractional amplitude of the rapid signal, monitored upon rapid mixing with Dns-C_6-Cho, was still small (approximately 0.3 as compared to 0.2 with control membranes without flaxedil) and that the rate constant for the rapid signal decreased approximately

10-fold, assuring that the flaxedil concentration was at least 10-fold above the equilibrium dissociation constant for the AcCh binding site in the conditions of the experiment [see Heidmann & Changeux (1979)]. With these conditions, for two of the NCBs tested, i.e., phencyclidine and $H_{12}HTX$ (panels A and C, respectively, of Figure 2), flaxedil caused a simplification of the concentration effect curves with an almost complete elimination of the deviation from a single hyperbola observed with high concentrations of these NCBs and was without much effect in the low concentration range (identical maximal extents and less than a 2-fold difference in apparent dissociation constants). This strongly suggests that part of the effects observed with $H_{12}HTX$ and phencyclidine in their high concentration range is caused by their interaction with the AcCh binding sites. With other NCBs such as trimethisoquin, meproadifen, or chlorpromazine, addition of flaxedil had no significant effect, at least in the domain of NCBs concentrations explored (see Figure 2E,F). Thus, the interaction of some NCBs with the AcCh binding site may affect the conformational transitions of the AcChR protein, but only to a limited extent.

(C) Distinction of Two Classes of Effects Associated with the Interaction of NCBs with "Allosteric" Sites of the AcChR Protein. Major effects of NCBs are exerted at the level of sites distinct from the AcCh binding site. Recent observations, however, suggest that these effects might involve multiple sites (Heidmann & Changeux, 1981). To test for possible heterogeneity, advantage was taken of the limited extent of the maximal effect for $H_{12}HTX$ and of the low value of its apparent dissociation constant. The additivity of the effect of NCBs with that of $H_{12}HTX$ was thus explored in the following manner. Membrane fragments were incubated with $H_{12}HTX$ at concentrations at least 10-fold higher than its apparent dissociation constant prior to the addition of increasing concentrations of other NCBs (Figure 2B–F). Two classes of effects were distinguished. With NCBs such as trimethisoquin (Figure 2E), lidocaine (Heidmann & Changeux, 1981), chlorpromazine (Figure 2F), or the detergent Lubrol PX (Heidmann & Changeux, 1981), the stabilization of the high-affinity state of the receptor was unaffected by the presence of $H_{12}HTX$; the apparent dissociation constants were close to those measured in the absence of $H_{12}HTX$. On the other hand, $H_{12}HTX$ almost completely abolished the effect of meproadifen (Figure 2B), dibucaine (Heidmann & Changeux, 1981), phencyclidine (Figure 2B), and the detergents Triton X-100 (Figure 2D) and Nonidet P-40 (Heidmann & Changeux, 1981) in the domain of NCB concentrations explored. These results indicate that two different classes of effects of NCBs on the transition of the AcChR can be distinguished on the basis of their sensitivity to $H_{12}HTX$. In the case of the "$H_{12}HTX$-resistant" action of NCBs, the possibility that the observed effect results from an interaction with the AcCh binding site was ruled out by experiments where both flaxedil and $H_{12}HTX$ were present, indicating that the effect is mediated by a category of sites distinct from the AcCh and $H_{12}HTX$ binding sites.

The competitive character of the interaction between $H_{12}HTX$ and phencyclidine, meproadifen, or Triton X-100 was tested by replotting the data from Figure 2 by using the "double-reciprocal" representation (see Appendix 1 and Figure 2B–D). The intercepts with the ordinate axis of the concentration effect curves determined in the presence and in the absence of $H_{12}HTX$ were identical. Therefore, the antagonism between $H_{12}HTX$ and the NCBs tested is a typical competitive interaction, this second class of NCBs (including $H_{12}HTX$)

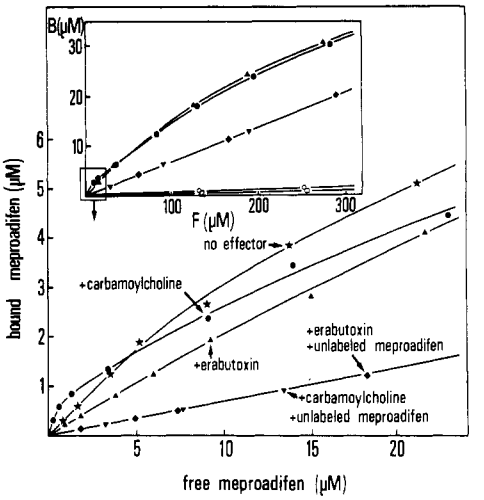

FIGURE 3: Binding of the NCB [^{3}H]meproadifen to the AcChR-rich membrane fragments. Binding of [^{3}H]meproadifen to AcChR-rich membranes was followed as described under Materials and Methods in the absence of effector (★) or after incubation with 10 μM erabutoxin b (▲), 1 mM carbamoylcholine (●), 10 μM erabutoxin b and 2 mM unlabeled meproadifen (♦), or 1 mM carbamoylcholine and 2 mM unlabeled meproadifen (▼). Insert: Experimental conditions are the same as in the remainder of the figure (and same symbols) except that the range of meproadifen concentrations is greater. Also shown is the binding of [^{3}H]meproadifen to the centrifugation tubes in the absence of AcChR-rich membranes, in the presence (□) or absence (○) of 2 mM unlabeled meproadifen.

exerting their effect on the AcChR at a common class of $H_{12}HTX$-sensitive sites which are distinct from the "$H_{12}HTX$-resistant" sites. The equilibrium inhibition constant (K_i) for $H_{12}HTX$ antagonism was derived from double-reciprocal plots as in Figure 2C, in the presence of varying concentrations of $H_{12}HTX$ (0.1–0.6 μM), and was found equal to 0.2 ± 0.05 μM.

In conclusion, the analysis of the effect of NCBs on the interaction of Dns-C_6-Cho with the AcChR indicates that the NCBs may stabilize the high-affinity state of the AcChR at two distinct categories of allosteric sites (in addition to the AcCh binding site). One is unaffected by $H_{12}HTX$, while the other binds this toxin with a high affinity.

Binding Sites for NCBs on AcChR-Rich Membrane Fragments. (A) Binding Assay for NCBs. Equilibrium binding of several radiolabeled NCBs was analyzed over a wide range of concentrations (0.05–300 μM) by using the centrifugation technique described under Materials and Methods. The NCBs tested include [^{3}H]trimethisoquin, [^{3}H]meproadifen, [^{3}H]-chlorpromazine, [^{3}H]phencyclidine, [^{3}H]$H_{12}HTX$, and ^{3}H-labeled Triton X-100. This series comprises NCBs which possess different chemical structures and also exert (as shown in the preceding section) different effects on the conformational transitions of the membrane-bound AcChR. In a typical binding experiment [see Figure 3 for [^{3}H]meproadifen], the amount of radioactive ligand bound to the membrane fragments (associated with the pellet) was plotted as a function of the amount of free ligand (present in the supernatant) under the following experimental conditions: (1) in the absence of effector; (2) in the presence of an excess of erabutoxin b (10 μM) and carbamoylcholine (1 mM) which both bind to the AcCh binding site but behave, respectively, as antagonist and as agonist of the physiological response; (3) in the presence of a large excess (2 mM) of the unlabeled NCB to distinguish between "saturable" and "nonsaturable" binding.

As NCBs are hydrophobic molecules and, as such, interact with the walls of the centrifuge tubes, control binding assays were thus performed under conditions similar to that of Figure

3, but in the absence of membranes. The experiments were carried out exactly as in the standard assay, and the "pseudopellet" was resuspended and counted. Under these conditions, as pointed out by several authors [see Krodel et al. (1979) and Sobel et al. (1980)] and as illustrated in Figure 3, both saturable and nonsaturable binding took place on the tube walls. With [^{3}H]meproadifen, this binding represented less than 5% of the total binding measured in the presence of AcChR-rich membranes. Similar values were obtained for [^{3}H]H_{12}HTX, [^{3}H]phencyclidine, and [^{3}H]trimethisoquin. For ^{3}H-labeled Triton X-100 and [^{3}H]chlorpromazine, slightly higher values were found (5% for Triton X-100 and approximately 15% for chlorpromazine). Although negligible in most cases, this nonspecific binding was systematically quantitated and subtracted from the results.

(*B*) *Nonsaturable Binding of NCBs.* In the presence of an excess of unlabeled NCB, a linear incorporation of radiolabeled NCBs was observed with all NCBs tested. No saturation occurred in the domain of concentrations tested, i.e., up to approximately 300 μM. After subtraction of the binding to the centrifugation tubes, the amount of ligand incorporated into the membrane fragments reached values as high as 100-fold the concentration in α-toxin binding sites. Binding was found to be independent of the nature of the cholinergic effector bound to the AcCh binding site (i.e., erabutoxin b or carbamoylcholine; see Figure 3) but depended on the nature of the NCB tested. The relative incorporation of the various NCBs was quantitated (within a given membrane preparation) by an apparent partition coefficient, P, equal to the ratio of bound vs. free ligand in the presence of an excess of unlabeled NCB. The values obtained for eight radiolabeled NCBs are listed in Table I and vary by almost 100-fold, with the smallest value for [^{3}H]phencyclidine and the largest for the detergent [^{14}C]$C_{12}E_8$. No correlation was found between the value of P and the sensitivity to H_{12}HTX (see previous section).

(*C*) *Saturable Binding of NCBs.* Saturable binding, defined as the amount of ligand bound which is displaced in the presence of an excess of nonradioactive ligand, was demonstrated with all the NCBs tested. As shown in Figure 3, the saturable binding of [^{3}H]meproadifen involves at least two classes of sites (concentration range of 0.1–100 μM), more clearly evidenced in the Scatchard plot of the data in the presence of carbamoylcholine after subtraction of the nonsaturable binding (Figure 4). These sites include (1) binding sites which will be referred to as "high affinity" to which the NCBs tested bind with a dissociation constant in the range of a fraction of, to several, micromolar and (2) binding sites which will be referred to as "low affinity" particularly evident in the case of [^{3}H]trimethisoquin and [^{3}H]chlorpromazine and for which the dissociation constants are in the tens of micromolar to hundreds of micromolar range. Dissociation constants and the number of sites, for each class of sites, were calculated by using a nonlinear least-squares program (see solid lines in Figure 4) according to the following equation:

$$B = B_1/(1 + K_{d1}/F) + B_2/(1 + K_{d2}/F) \qquad (1)$$

where B_1 and B_2 are the concentrations of the high- and low-affinity sites, respectively, K_{d1} and K_{d2} are their equilibrium dissociation constants, and F is the concentration of free NCB. The properties of these high- and low-affinity "saturable" sites are examined below.

(*D*) *High-Affinity Binding.* High-affinity binding was observed in the presence of carbamoylcholine with *all* NCBs tested (Figure 4 and Table I) including the detergent Triton X-100 (Figure 4) at concentrations at least 10 times below its critical micellar concentration. The dissociation constants in the presence of carbamoylcholine (Table I) range from approximately 0.15 μM for H_{12}HTX to 5 μM for Triton X-100. When tested on the same membrane preparation and under conditions of at least 20-fold isotopic dilution of radiolabeled NCBs (see Materials and Methods), the concentrations of high-affinity sites were similar for all NCBs tested (Figure 4). These values correspond to approximately half of the number of α-toxin sites (see α-Toxin and NCB Site Stoichiometry).

Since the NCBs studied possess strikingly different structures, their high-affinity binding sites could be either common or distinct. The effect of H_{12}HTX on this high-affinity binding was tested to distinguish between these alternatives. H_{12}HTX was selected because of the low value of its equilibrium dissociation constant for the high-affinity sites and its negligible binding to the low-affinity ones (see Figure 4A). In the presence of 10 μM H_{12}HTX (approximately 50 times its dissociation constant for the high-affinity sites), the high-affinity binding observed with all the NCBs tested was no longer observed (see Figure 4, inserts). Similar results were observed with 30 μM phencyclidine, supporting the conclusion that the high-affinity binding takes place, for all the NCBs tested, at a common class of binding sites.

Another series of observations supports this conclusion. As shown in Figure 4 and Table I, the binding of the NCBs to their high-affinity sites is regulated in a similar manner by cholinergic ligands bound to the AcCh binding sites. In all cases, the equilibrium dissociation constant was higher in the presence of erabutoxin b than in the presence of carbamoylcholine with an intermediate value in the absence of an effector (see Table I). The amplitude of the variation differed with the NCB tested: the ratios of the dissociation constants measured in the presence of erabutoxin b vs. carbamoylcholine ranged from 1.5 for H_{12}HTX to 10 for Triton X-100, chlorpromazine, and meproadifen, with an intermediate value of 5 for phencyclidine.

(*E*) *Low-Affinity Binding.* Low-affinity binding was observed with all of the NCBs tested (except Triton X-100 for technical reasons) but was investigated in detail with [^{3}H]-trimethisoquin and [^{3}H]chlorpromazine. The dissociation constants, listed in Table I, range from 15–45 μM for chlorpromazine and trimethisoquin to more than 100 μM for H_{12}HTX, phencyclidine, or meproadifen. A critical feature of the low-affinity binding is the large number of binding sites involved. In native AcChR-rich membrane fragments, they represent approximately 10–20 times the number of α-toxin sites, in the case of trimethisoquin and chlorpromazine, and similar or slightly lower values were found for the other NCBs tested but with poor resolution. Only limited effects of cholinergic ligands were observed on the low-affinity binding of the NCBs studied. These effects consisted of a slight decrease (10–20%) in the affinity for NCBs in the presence of erabutoxin b as opposed to the presence of carbamoylcholine and a slightly larger number of sites in the presence of erabutoxin b than in the presence of carbamoylcholine.

(*F*) *α-Toxin and NCB Site Stoichiometry.* Considerable confusion exists in the literature concerning the stoichiometry of α-toxin and reversible NCB binding sites. The possible sources of errors include *technical* errors, such as the radiolysis of labeled compounds (especially in the case of α-toxins) and uncertainties in the specific activities of commercially available ligands, and *theoretical* reasons, such as a limited uncoupling between components in a multiple sites interaction or even a lack of intrinsic correlation between the concentrations of α-toxin sites and the concentration of some of the sites for

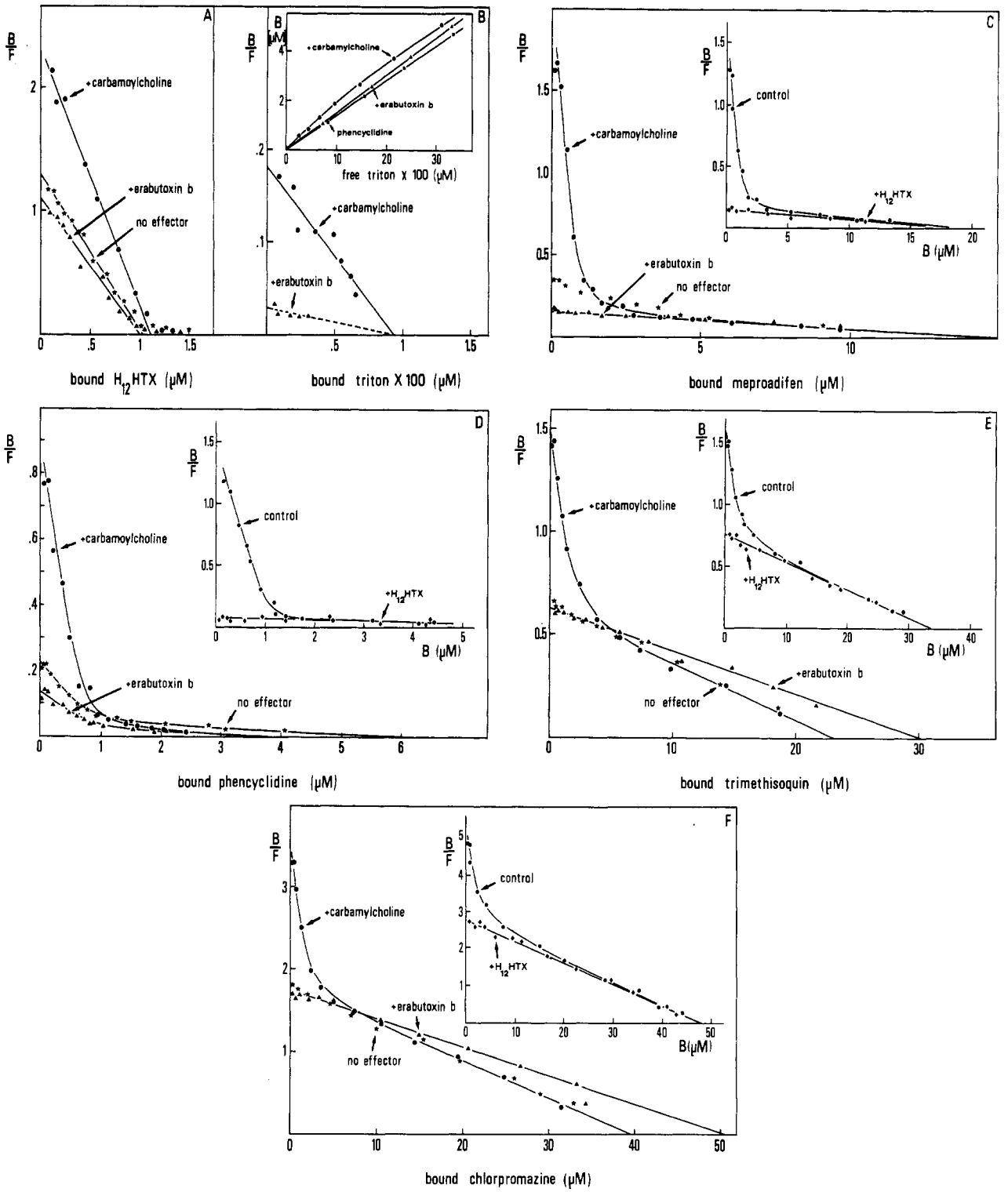

FIGURE 4: Scatchard plot of the saturable binding of a series of radiolabeled NCBs to the AcChR-rich membranes. The experimental conditions were the same as those in Figure 3. AcChR-rich membranes were incubated in the absence of effectors (★) or in the presence of 10 μM erabutoxin b (▲) or 1 mM carbamoylcholine (●) and (inserts) 1 mM carbamoylcholine (●, control) or 1 mM carbamoylcholine and 10 μM $H_{12}HTX$ (♦). The amount of bound NCB (B) is that displaced by an excess of the corresponding unlabeled NCB for all NCBs tested, except for ^{3}H-labeled Triton X-100 where it refers to the amount of bound ^{3}H-labeled Triton X-100 displaced by 30 μM phencyclidine (see insert of panel B). Solid lines are least-squares fits of the data according to eq 1 (see Saturable Binding of NCBs under Results).

NCBs. Precise stoichiometries were measured only for the high-affinity sites. [^{3}H]Phencyclidine was selected, among NCBs, because of its minimal interaction with both the non-saturable and the low-affinity sites. The concentrations of binding sites for both α-toxin and phencyclidine were measured on the same day under closely related experimental conditions by using the same membrane preparation at the same dilution and by using several independent procedures.

The concentration of α-toxin sites was measured by two independent methods (see Figure 5A,B): (1) a fluorescence titration assay which does not depend on any radiolabeled ligand and (2) a binding assay performed under conditions similar to those used with [^{3}H]phencyclidine binding, i.e., at least 20-fold isotopic dilution and controlled radiochemical purity. The unlabeled α-toxin used was highly purified *Naja nigricollis* α-toxin, whose purity was higher than 95% as de-

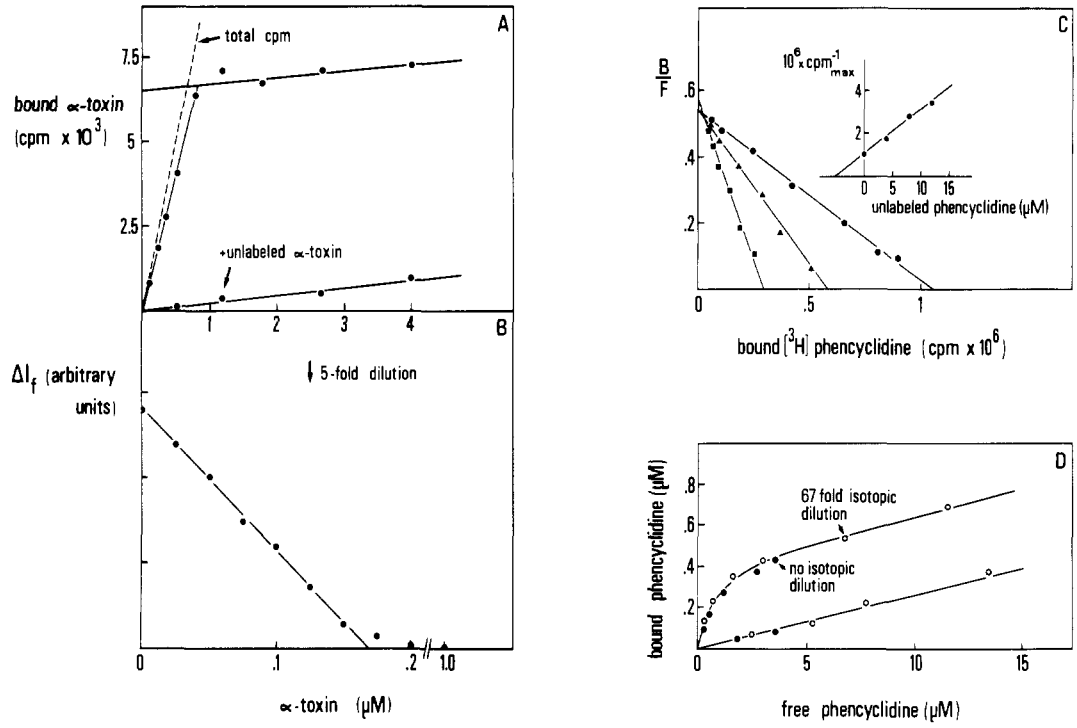

FIGURE 5: Stoichiometry of the AcChR and the high-affinity phencyclidine binding sites. The same AcChR-rich membrane suspension was used for the determination of the α-toxin site concentration (A and B), the determination of the specific activity of the stock [^{3}H]phencyclidine solution (C), and the determination of the binding of [^{3}H]phencyclidine under conditions of 0- and 67-fold isotopic dilution (D). (A) Binding of ^{3}H-labeled *Naja nigricollis* α-toxin under conditions of at least 50-fold isotopic dilution to the AcChR-rich membrane suspension in the absence or presence of an excess (50 μM) of unlabeled *Naja nigricollis* α-toxin. The dashed line corresponds to the total cpm in the centrifugation tube and yields the percent of active toxin (approximately 81%) and the relation between cpm and active α-toxin concentration. The α-toxin site concentration was 0.82 μM in this case. (B) Spectroscopic titration of the AcChR sites. The same membrane suspension as that in the legend to Figure 5A was diluted 5-fold, and aliquots were incubated for at least 1 h with unlabeled *Naja nigricollis* α-toxin (concentrations indicated on the abscissa). The mixture was then mixed rapidly in the stopped-flow apparatus with a solution of 0.5 μM Dns-C_6-Cho, and the total amplitude of the fluorescence signal is plotted (arbitrary units on the ordinate) as a function of the α-toxin concentration. The intercept with the abscissa axis yields the concentration of α-toxin binding sites. (C) Determination of the concentration of a stock [^{3}H]phencyclidine solution. Binding of [^{3}H]phencyclidine to the AcChR-rich membrane suspension as in the legend to Figure 5A was performed as described in Figure 4D in the presence of 1 mM carbamoylcholine starting from a [^{3}H]phencyclidine stock solution supplemented with 0 (●), 4 (▲), or 12 μM (■) unlabeled phencyclidine. The Scatchard representation (as in Figure 4D) yields the amount of [^{3}H]phencyclidine bound to the high-affinity NCB site at saturation (cpm_{max}). Insert: Plot of cpm_{max}^{-1} as a function of the concentration of unlabeled phencyclidine in the isotopic dilution of the [^{3}H]phencyclidine stock solution. The intercept with the abscissa axis yields (see Appendix 2) the concentration of [^{3}H]phencyclidine in the stock solution (approximately 5.1 μM). (D) High-affinity binding to AcChR-rich membranes of [^{3}H]phencyclidine with (○) or without (●) isotopic dilution. Same experimental conditions as those given in the legend to Figure 5C. The results shown in Figure 5C allowed the conversion of measured cpm to phencyclidine concentrations. The concentration of high-affinity phencyclidine binding sites on the same AcChR-rich membrane suspension as in (A) was equal to 0.38 μM.

termined by isoelectric focusing and whose concentration was determined by optical absorption (ϵ_{280nm} = 8900 M^{-1} cm^{-1}) to circumvent the problems of possible hydration. Labeled α-toxin was tritium substituted *Naja nigricollis* α-toxin which has been demonstrated to have binding properties identical with those of the unlabeled analogue (Weber & Changeux, 1974). The values obtained by both methods with three different membrane preparations did not differ by more than 10%.

[^{3}H]Phencyclidine binding was measured as described under Materials and Methods under conditions of at least 20-fold isotopic dilution. The purity of both [^{3}H]phencyclidine and unlabeled phencyclidine was checked by thin-layer chromatography less than 2 days before stoichiometry measurements and yielded identical R_f values for both compounds. Identity between the radiolabeled and the unlabeled phencyclidine was further controlled by performing binding experiments with isotopic dilutions ranging from 0- to 75-fold (larger isotopic dilutions yielded insufficient counts). The concentrations of unlabeled phencyclidine were verified by light absorption (ϵ_{262nm} = 3 × 10^2 M^{-1} cm^{-1}). The concentration of [^{3}H]-phencyclidine (same concentration in the stock solutions for each isotopic dilution) was determined as follows: (1) Scatchard plots of the binding curves were drawn (Figure 5C) which yielded the cpm bound to the NCB high-affinity sites at saturation (cpm_{max}) for each isotopic dilution; cpm_{max}^{-1} was then plotted as a function of the unlabeled phencyclidine concentration in the stock solutions. Under these conditions, a straight line was obtained (see Figure 5C), and its intercept with the abscissa gave the concentration of radiolabeled [^{3}H]phencyclidine in the stock solutions (see Appendix 2 for equations). The binding curves were then corrected for isotopic dilution by the multiplicative factor corresponding to the actual [^{3}H]phencyclidine to unlabeled phencyclidine ratio. As observed in Figure 5D, the corrected curves for 0- and 67-fold isotopic dilutions superimposed, suggesting that radiolabeled and unlabeled phencyclidine have identical structural and functional properties. Under these conditions, the stoichiometry was found equal to 0.46 ± 0.09 high-affinity phencyclidine site per α-toxin site. Similar results were obtained with [^{3}H]chlorpromazine (Figure 6), yielding a stoichiometry of 0.44 ± 0.12 high-affinity site per α-toxin site.

Biochemical Characterization of the Different Categories of NCB Binding Sites. (A) Interaction of NCBs with Boiled AcChR-Rich Membranes. To distinguish between the various classes of sites, we treated AcChR-rich membrane fragments

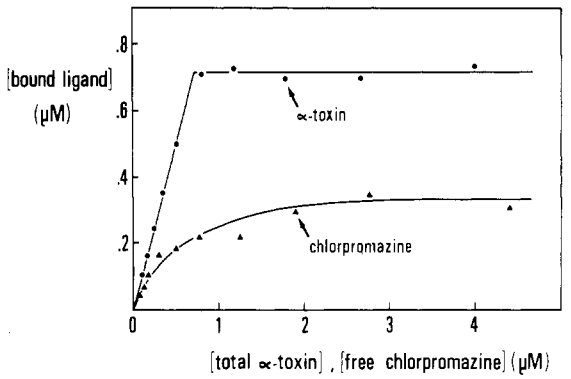

FIGURE 6: Stoichiometry of the AcChR and the high-affinity $H_{12}HTX$-sensitive chlorpromazine binding site. AcChR sites were assayed as in Figure 5A, and the concentration of bound ^3H-labeled α-toxin displaced by an excess of unlabeled α-toxin is plotted as a function of the total α-toxin concentrations. The high-affinity chlorpromazine binding site was assayed under conditions of approximately 110-fold isotopic dilution, and the amount of bound [^3H]chlorpromazine displaced by $H_{12}HTX$ (10 μM) is plotted as a function of the free chlorpromazine concentration.

Table II: Reversible Equilibrium Binding of [^3H]Trimethisoquin to AcChR Reconstituted with Varying Amounts of Exogenous Lipids[a]

samples	phospholipids/ α-toxin sites (mol/mol)	nonsaturable partitioning (bound/free)	high-affinity site concn (μM)	low-affinity site concn (μM)
1	86 ± 7	0.21 ± 0.02	0.9 ± 0.3	27 ± 5
2	118 ± 10	0.29 ± 0.02	1.0 ± 0.3	40 ± 5
3	155 ± 10	0.39 ± 0.02	0.8 ± 0.3	59 ± 5

[a] See Interaction of NCBs with AcChR Reconstituted with Different Phospholipid/Receptor Ratios under Results.

at 100 °C for 5 min in the absence of ligand, and routine NCB binding measurements were carried out at 20 °C in the presence of carbamoylcholine, with [^3H]chlorpromazine and [^3H]trimethisoquin. The following results were obtained: (1) nonsaturable binding is not affected by the heat treatment; (2) binding to the saturable low-affinity sites is preserved with almost unmodified characteristics; and (3) high-affinity binding completely disappears.

(B) Interaction of NCBs with AcChR Reconstituted with Different Phospholipid/Receptor Ratios. Reconstitution of the AcChR in particulate or vesicular forms was performed as described by Sobel et al. (1980) after removal of nonreceptor peptides, and binding of [^3H]trimethisoquin to these preparations was measured as previously described except that the centrifugation was prolonged for 15 min. The binding curves obtained under these conditions at the same concentration of α-toxin sites show (Table II) the following: (1) the nonsaturable binding of [^3H]trimethisoquin increases with increasing phospholipid concentration in the reconstituted material, with a linear dependence of the "apparent partition coefficient" *P* as a function of phospholipid concentration; (2) high-affinity binding is observed with all reconstituted preparations with both the affinity and the number of sites independent of the phospholipid concentration; (3) low-affinity binding is still present with similar affinities, but the number of low-affinity sites increases with increasing phospholipid concentration.

(C) Interaction of NCBs with the α, β, γ, and δ Chains of the AcChR. As previously shown by Oswald & Changeux (1981a), all four chains of the AcChR can be labeled covalently by simple UV irradiation of the AcChR-rich membranes in the presence of [^3H]chlorpromazine. Since [^3H]chlorpromazine interacts with several classes of sites (see preceding

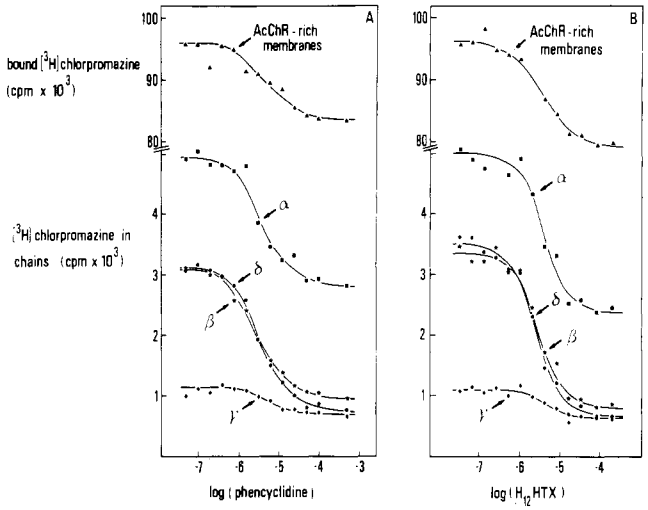

FIGURE 7: Covalent labeling of the polypeptide chains of the AcChR by [^3H]chlorpromazine. AcChR-rich membrane fragments (7–8 μM α-toxin sites) supplemented with 0.5 μM [^3H]chlorpromazine and the indicated concentrations of phencyclidine (A) or $H_{12}HTX$ (B) were UV irradiated as described under Materials and Methods. After gel electrophoresis, the amount of [^3H]chlorpromazine covalently attached to each polypeptide chain of the receptor was determined (see Materials and Methods) and plotted as a function of the total phencyclidine or $H_{12}HTX$ concentration. Negligible amounts of [^3H]chlorpromazine (<250 cpm) were found in each chain upon addition of high concentrations (0.5 mM) of unlabeled chlorpromazine before UV irradiation. Upper curve: amount of [^3H]chlorpromazine bound to the AcChR-rich membranes as a function of the total phencyclidine or $H_{12}HTX$ concentration in the membrane suspensions before UV irradiation.

sections), it was further tested whether the labeling of the four chains of the AcChR could be assigned to the interaction of [^3H]chlorpromazine with one or several of these sites.

AcCh-rich membranes supplemented with 0.1 mM carbamoylcholine were therefore incubated with a fixed concentration of [^3H]chlorpromazine and increasing concentrations of two NCBs highly selective for the high-affinity NCB binding site, i.e., phencyclidine and $H_{12}HTX$. The amount of [^3H]chlorpromazine incorporated into each chain following UV irradiation was determined as described under Materials and Methods and plotted as a function of phencylidine or $H_{12}HTX$ concentration (see Figure 7). As shown previously by Oswald & Changeux (1981a), incorporation of [^3H]-chlorpromazine is not identical in all chains, but an essential feature disclosed in Figure 7 is that strictly identical concentration dependences are observed for the inhibition of the labeling of *all four chains*. In each case, [^3H]chlorpromazine incorporation decreases in a dose-dependent manner, with identical apparent inhibition constants (IC_{50}). The absolute values of these inhibition constants, in the 4–6 μM range, are rather high but consistent with the equilibrium dissociation constants for $H_{12}HTX$ and phencyclidine binding to the high-affinity NCB site, taking into consideration that the values on the abscissa are *total* rather than free NCB concentrations and that the high-affinity NCB site concentration was 3–4 μM. To further ascertain that the observed inhibition of [^3H]chlorpromazine labeling was mediated by $H_{12}HTX$ and phencyclidine interaction with their high-affinity site, the inhibition of the reversible binding of [^3H]chlorpromazine was followed in parallel by ultracentrifugation. Binding measurements performed on the same material and under conditions of identical binding site and total ligand concentrations (see Materials and Methods) disclosed that the displacement of reversibly bound [^3H]chlorpromazine follows exactly the same concentration dependence as the inhibition of the covalent

labeling of the four chains, with *apparent* inhibition constants for $H_{12}HTX$ and phencyclidine again in the 4–6 μM range (see Figure 7). In addition, the amount of $H_{12}HTX$- or phencyclidine-sensitive labeling of the chains was found close to the amount of $H_{12}HTX$- or phencyclidine-sensitive reversible binding to the nonirradiated membranes (50–60% recovery of radioactivity in the polypeptide chains after irradiation).

In conclusion, [^{3}H]chlorpromazine shares the same high-affinity sites as $H_{12}HTX$ or phencyclidine, and, when bound to this site, chlorpromazine can be attached covalently to any of the four chains of the AcChR. The exact mechanism of covalent attachment still is unknown, but (a) the excited states of chlorpromazine are shortlived (smaller than microseconds; Navaratnam et al., 1978), (b) the wavelength of UV irradiation corresponds to a major peak of free chlorpromazine absorption ($\epsilon_{254nm} \simeq 2 \times 10^4$ M^{-1} cm^{-1}), and (c) the rate of NCB dissociation from this site is exceptionally slow [$t_{1/2} \simeq$ 1 min for phencyclidine; see Oswald et al. (1983)]. This strongly suggests that covalent attachment takes place locally and therefore that the *four* polypeptide chains (and not only the δ chain covalently labeled by the azido derivative of the NCB trimethisoquin; Oswald et al., 1980) contribute to the unique high-affinity NCB site on the AcChR protein. It renders unlikely the alternative hypothesis, which would require that only chlorpromazine molecules bound to the high-affinity NCB site are photoactivated and dissociate sufficiently rapidly from this site to nonspecifically attach to remote regions of the α, β, and γ chains. This possibility was nevertheless tested by using two "scavenging" reagents, *p*-aminobenzoate and 2-methyl-2-nitrosopropane (Ruoho et al., 1973; Jonhstone et al., 1971). UV irradiation was performed in the absence and presence of 10^{-2} M solutions of these two reagents and the covalent incorporation of [^{3}H]chlorpromazine measured as described previously in the presence and absence of saturating concentrations of phencyclidine (0.2 mM). Although with both scavengers a reduction of the labeling was observed due to, possibly, absorption of the UV irradiation by the scavengers ($\epsilon_{254} \simeq 9.6 \times 10^3$ and 10^2 M^{-1} cm^{-1} for *p*-aminobenzoate and 2-methyl-2-nitrosopropane, respectively) or in situ inactivation of the photoactivated chlorpromazine molecules, the *relative* labeling of the four chains was not significantly different from that of the control: percent reduction of phencyclidine-sensitive labeling with 2-methyl-2-nitrosopropane, α, 27%, β, 34%, γ, 27%, δ, 36%; percent reduction with *p*-aminobenzoate, α, 48%, β, 57%, γ, 53%, δ, 60%.

Finally, the residual labeling observed for each chain at saturating concentration of either phencyclidine or $H_{12}HTX$ (see Figure 7) and which can be decreased to negligible values upon addition of unlabeled chlorpromazine (0.5 mM) before UV irradiation possibly corresponds to the "nonsaturable" or to the "low-affinity" binding of [^{3}H]chlorpromazine observed by direct binding measurements. The relatively intense $H_{12}HTX$-unsensitive labeling observed with the α chain as compared to the other chains might simply reflect the $\alpha_2\beta\gamma\delta$ stoichiometry of the AcChR molecule (Reynolds & Karlin, 1978).

Discussion

Several Classes of Binding Sites Are Involved in the Regulation of AcChR Transitions by NCBs. The data presented in this paper show that NCBs bind to several classes of sites on AcChR-rich membrane fragments. These compounds also exert different classes of effects on the transitions of the membrane-bound receptor, which all result in the stabilization of a high-affinity state for agonists. An important issue is to relate the binding of NCBs to a given class of sites with a given effect on the conformational transitions of the receptor protein.

In agreement with the early findings of Weber & Changeux (1974), NCBs bind, in the high concentration range, to the AcCh binding site itself. With some of the NCBs tested, the stabilization of the high-affinity state is partially blocked by flaxedil, a competitive antagonist of the AcCh binding site. This blocking effect occurs with phencyclidine and $H_{12}HTX$ in the high concentration range which coincides with the range of the equilibrium dissociation constants of the NCBs binding to the AcCh binding site. Like typical cholinergic agonists, NCBs may thus stabilize the high-affinity state of the AcChR when they bind to the AcCh binding site.

In addition, NCBs interact with both saturable and nonsaturable sites on AcChR-rich membranes which are distinct from the AcCh binding site. For a series of NCBs (including phencyclidine, $H_{12}HTX$, meproadifen, and Triton X-100), the effect on the transitions of the AcChR (1) can be fitted by a single hyperbola (i.e., $n_H = 1$), (2) is blocked by $H_{12}HTX$ in a competitive manner with a dissociation constant for the inhibition close to 0.2 μM, and (3) takes place with a rank order of potency (*apparent* dissociation constants in parentheses) $H_{12}HTX$ ($\sim$0.8 μM) > meproadifen ($\sim$3 μM) > phencyclidine ($\sim$4 μM) > Triton X-100 ($\sim$21 μM). Binding of these NCBs to the saturable *high-affinity* NCB site takes place with closely related characteristics: (1) the stoichiometry of this binding site is *one* common high-affinity site per two α-toxin sites, i.e., one per receptor light form; (2) the binding is inhibited by $H_{12}HTX$ or phencyclidine with a K_d for $H_{12}HTX$ close to 0.2 μM; and (3) the rank order of potency is (equilibrium dissociation constants in the presence of carbamoylcholine in parentheses) $H_{12}HTX$ ($\sim$0.2 μM) > meproadifen ($\sim$0.5 μM) > phencyclidine ($\sim$0.8 μM) > Triton X-100 ($\sim$5 μM); the relative potencies of each of these NCBs are qualitatively and quantitatively (when compared, for instance, with $H_{12}HTX$) similar whether binding or effects on the transitions are considered. The binding of these NCBs to the high-affinity site thus accounts for their $H_{12}HTX$-sensitive effects on the conformational transitions of the membrane-bound receptor.

For the other NCBs, such as trimethisoquin or chlorpromazine, the situation is more complex: (a) the concentration effect curves for the stabilization of the high-affinity state are *sigmoidal* with Hill coefficients of 1.5–3.0; and (b) these effects are not significantly altered by $H_{12}HTX$ or phencyclidine. Although trimethisoquin and chlorpromazine bind to the high-affinity site for NCBs, it must then be assumed that, at low concentrations, they exert their effect at the level of site(s) distinct from the high-affinity site. Direct binding measurements with these NCBs give only two possible targets: the nonsaturable sites and the low-affinity saturable sites. Close examination of the data shows the following: (a) trimethisoquin and chlorpromazine are among all NCBs tested those which have the highest affinity (almost 10-fold difference) for the low-affinity NCB sites; (b) other NCBs such as the detergent $C_{12}E_8$ possess a partition coefficient approximately 20-fold higher than that of trimethisoquin but do not exert any major effect on the transitions of the AcChR (in the presence of 10 μM $H_{12}HTX$) at concentrations up to 100 μM (the same is observed for Triton X-100); (c) the ratio of the potencies of chlorpromazine and trimethisoquin for the regulation of AcChR transitions is 1.5 $\pm$ 0.8; this is closer to the ratio of their K_D's for the low-affinity sites (2.0 $\pm$ 1) than to the ratio of their apparent partition coefficients (6 $\pm$ 1) which can be taken as a measure of their binding to the nonsaturable sites. These observations suggest that the in-

teraction of these two NCBs with the nonsaturable sites is not responsible for the stabilization of the high-affinity state but rather that this stabilization is mediated by their binding to the low-affinity ones. In conclusion, NCBs regulate the conformational state of the membrane-bound AcChR via three categories of sites: the AcCh binding sites and two distinct populations of allosteric sites which include the high-affinity H_{12}HTX-sensitive site and the low-affinity H_{12}HTX-insensitive ones.

Quantitative Correlation between NCBs Binding to Their Respective Sites and the Stabilization of the High-Affinity State of the AcChR. (A) Regulation via the High-Affinity Site for NCB. The four-state allosteric model developed by Heidmann & Changeux (1979, 1980) and Neubig & Cohen (1980) provides a straightforward theoretical basis for a qualitative and quantitative analysis of both binding and state transitions of the AcChR. Since the data presented in this paper concern experiments carried out under equilibrium conditions, a simplified version of the model will be used [see Changeux (1981)]. Only two states will be taken into consideration: the resting (R) and the desensitized (D) state of the AcChR, in reversible equilibrium R $\rightleftharpoons$ D. If the equilibrium dissociation constants of the NCB for the *unique* high-affinity site are K_R and K_D in the resting and desensitized states, respectively, then the fraction $\bar{D}$ of AcChR in the D state in the presence of a concentration F of free NCB should be equal to

$$\bar{D} = \frac{1 + F/K_D}{1 + F/K_D + L_0(1 + F/K_R)} \quad (2)$$

and the fractional concentration $\bar{B}$ of NCB binding sites which are occupied should follow a classical Langmuir isotherm [see, e.g., Monod et al. (1965)]:

$$\bar{B} = \frac{F}{F + K_{app}} \quad (3)$$

with

$$K_{app} = K_D(1 + L_0)/(1 + L_0K_D/K_R) \quad (4)$$

L_0, which is the isomerization constant between the R and D states in the absence of NCB, depends on the state of occupancy of the AcCh binding site and varies from less than unity in the presence of agonist ($L_0 \simeq 0.015$) (Heidmann & Changeux, 1979; Boyd & Cohen, 1980) to about $L_0 \simeq 4$ in the absence of effector (same references) and even to higher values ($L_0 > 4$) in the presence of α-toxin [see Grünhagen & Changeux (1976) and Oswald & Changeux (1981b)].

Figure 2A–D shows (see solid lines) that the model adequately fits the results obtained for the experimental variation of the fraction of AcChR in the high-affinity state ($\bar{D}$) as a function of NCB concentration: (a) the variation of $\bar{D}$ is hyperbolic, as expected from eq 2; (b) the *increase* of $\bar{D}$ with NCB concentration only requires that NCBs have a higher affinity for the AcChR in the D conformation than in the R conformation (i.e., $K_D < K_R$; see eq 2); and (c) the *partial* stabilization of the D state observed with H_{12}HTX and phencyclidine can be accounted for by assuming that for these two NCBs K_D and K_R are close to each other [H_{12}HTX and phencyclidine then behave as "nonexclusive" effectors which bind to both conformations of the AcChR; see Rubin & Changeux (1966) and Heidmann & Changeux (1979)]. Quantitatively, the intrinsic equilibrium dissociation constants K_R and K_D can be unambiguously derived by direct computer fit of the curves in Figure 2A–D according to eq 2 (see solid lines), and their values are listed in Table I.

Conversely, Figure 4 shows that the model also adequately fits the results obtained for the binding of the corresponding radiolabeled NCBs to the high-affinity site: (a) according to eq 3, binding of NCBs to the high-affinity site is linear in the Scatchard representation; (b) binding takes place with higher affinity in the presence of carbamoylcholine ($L_0 = 0.015$) than in the presence of erabutoxin b or in the absence of cholinergic effector ($L_0 \geq 4$), consistent with the notion that $K_D < K_R$ (see eq 4), and for H_{12}HTX, and to a lesser extent for phencyclidine, only limited variation in affinity is observed whether binding is measured in the presence of carbamoylcholine or erabutoxin b, consitent with the notion of a nonexclusive interaction of these NCBs with the AcChR (i.e., K_D close to K_R; see eq 4). Quantitatively, since $K_D < K_R$ and since L_0 in the presence of carbamoylcholine is very low ($L_0 = 0.015$), the apparent dissociation constant K_{app} for binding in the presence of carbamoylcholine should be equal to K_D (within less than 2% with $L_0 = 0.015$; see eq 4), whereas K_{app} in the presence of erabutoxin b should be between K_D and K_R and close to K_R if $L_0 \gg K_R/K_D$ (see eq 4). These values can then be compared with the K_D and K_R values derived from the fitting of the conformational transitions of the AcChR (see above and Table I): the dissociation constants for binding in the presence of carbamoylcholine are indeed in good agreement with the K_D values derived from the concentration effect curves, and the dissociation constants for the binding in the presence of erabutoxin b are in the expected concentration range when compared with the corresponding K_R values. Thus, the model qualitatively and quantitatively accounts both for the binding of NCBs to the high-affinity site and for the relevant H_{12}HTX-sensitive effects of the NCBs on the transition of the AcChR.[2]

(B) Regulation via the Low-Affinity Sites for NCBs. The same two-state model can be used to fit both the binding of the NCBs to the low-affinity sites and their H_{12}HTX-insensitive effects on the conformational transitions of the receptor. The additional postulate that there exists several (n) low-affinity sites for NCBs per AcChR molecule is included. For the sake of simplicity, it will be assumed that all low-affinity sites have identical binding properties, with dissociation constants K_R and K_D for the R and D conformations. Accordingly, the fraction $\bar{D}$ of AcChR molecules in the D conformation for a concentration F of free NCB is given by

$$\bar{D} = \frac{(1 + F/K_D)^n}{[1/(F/K_D)]^n + L_0(1 + F/K_R)^n} \quad (5)$$

and the fractional concentration, $\bar{B}$, of NCB binding sites occupied by the NCB is

$$\bar{B} = \frac{(1 + F/K_D)^{n-1}(F/K_D) + L_0(1 + F/K_R)^{n-1}(F/K_R)}{(1 + F/K_D)^n + L_0(1 + F/K_R)^n} \quad (6)$$

The model should account for (a) the sigmoidal shape of the concentration effect curves for the stabilization of the high-affinity state of the AcChR while the curves for the

[2] In turn, the value of L_0 in the presence of erabutoxin b can be estimated from the phencyclidine binding data for which precise evaluations of binding constants are available not only in the presence of carbamoylcholine and erabutoxin b but also in the absence of effector. Combination of eq 4 for the three experimental cases (no effector, + carbamoylcholine, + erabutoxin b) mentioned above yields an L_0 value equal to 8 ± 1 in the presence of erabutoxin b, i.e., a value 2 times higher than the corresponding value without effector ($L_0 = 4$). Erabutoxin b is therefore able to reduce the concentration of AcChR preexisting in the D conformation before agonist addition from approximately 20% to approximately 10%.

binding of the radiolabeled NCBs to the low-affinity sites are hyperbolic (straight lines in Scatchard plots), (b) the large extent of the shift toward the high-affinity state of the receptor as compared to the limited extent of the changes in binding affinity in the presence of carbamoylcholine or erabutoxin b, and (c) the lower (approximately 5-fold) apparent dissociation constants for the stabilization of the high-affinity state than the dissociation constants for binding to the low-affinity sites.

The variations of $\bar{D}$ and $\bar{B}$ have been calculated according to eq 5 and 6 for $n = 30$, $K_D/K_R = 0.6$, and three values of L_0, i.e., $L_0 = 8$, $L_0 = 4$, and $L_0 = 0.015$. For $L_0 = 4$, which corresponds to the experimental situation of Figure 2E (trimethisoquin) and Figure 2F (chlorpromazine), the variation of $\bar{D}$ is sigmoidal ($n \simeq 2.0$) with an apparent dissociation constant for the effect approximately 5 times lower than K_D, and a maximal extent close to unity. For $L_0 = 8$ and $L_0 = 0.015$, which corresponds to the experimental situations where binding of the radiolabeled ligands was assayed in the presence of erabutoxin b or carbamoylcholine, respectively, binding is hyperbolic ($n \simeq 1.05$) with apparent dissociation constants for binding equal to approximately K_D: in other words, the essential features of the H_{12}HTX-insensitive effect of NCBs on the conformational transitions of the AcChR are fit qualitatively and to a large extent quantitatively by the model (although a direct computer fit of the data in Figure 2E,F was not assayed, due to the large uncertainty on n) via the interaction of NCBs with a large number of low-affinity binding sites.

The model also accounts for the rather paradoxical observation that some NCBs exert an H_{12}HTX-insensitive effect on the conformation of the AcChR even though they bind to the high-affinity sites in an H_{12}HTX-sensitive manner. Equations 2, 5 and 3, 6 indicate that trimethisoquin and chlorpromazine, respectively, should bind to the high-affinity site in the absence of cholinergic effector in a range of concentrations (namely, $K_{app} = 4.0$ and 2.6 μM for trimethisoquin and chlorpromazine, respectively) where they already interact with the low-affinity sites and stabilize the high-affinity, D, conformation of the AcChR in an H_{12}HTX-insensitive manner. Conversely, NCBs which cause an H_{12}HTX-sensitive shift of the AcChR in favor of the D state also interact with the low-affinity H_{12}HTX-insensitive sites but in a higher range of concentrations. In other words, NCBs are expected to exert mixed effects on the AcChR via the two populations of allosteric sites described above. The first effect observed on the conformational transition would coincide with the interaction with the particular population of sites for which they exhibit the highest affinity: (1) the high-affinity sites in the case of H_{12}HTX, meproadifen, phencyclidine, and Triton X-100; (2) the low-affinity ones in the case of trimethisoquin and chlorpromazine. Finally, the model also predicts that the allosteric effects triggered via the AcCh binding sites should take place in a range of concentrations much higher than those where these NCBs interact with either the high- or the low-affinity NCB sites. With phencyclidine or H_{12}HTX for which there is only a limited shift of the R $\rightleftharpoons$ D equilibrium mediated via the NCB sites, these effects can indeed be observed at high concentrations, and, in this case, a very good correlation exists between the measured apparent dissociation constant for the stabilization of the high-affinity state of the AcChR (i.e., approximately 150 μM for phencyclidine, see Figure 2B) and the theoretical value predicted by the model (namely, 170 μM) according to eq 5 with $n = 2$ (two AcCh binding sites per AcChR molecule) and the K_D value for the binding of phencyclidine to the AcCh binding sites in Table I (i.e., 250 μM).

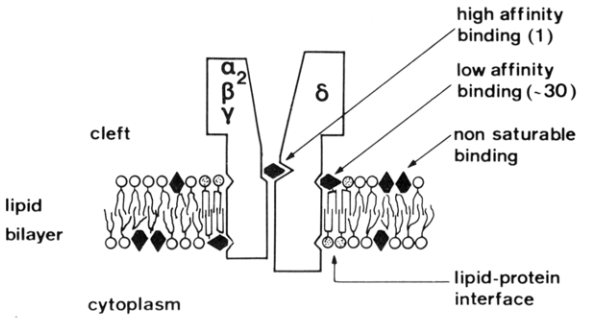

FIGURE 8: Model for the AcChR and NCB interactions. The three modes of interactions of NCBs with the AcChR-rich membrane fragments are illustrated: the high-affinity binding to a unique site in close vicinity to all four chains of the AcChR and most likely located in the ion channel; the low-affinity binding to a large number of sites most likely located at the lipid–protein interface (schematized by the presence of lipids of different physical and/or chemical properties); and the nonsaturable partitioning in the lipid bilayer.

Identification and Localization of the High-Affinity Binding Site for NCBs on the AcChR Protein. Binding studies carried out with a large series of NCBs under identical conditions and experiments designed for a precise determination of the stoichiometry of NCB vs. α-toxin binding sites disclose that *all* NCBs tested, including the detergents Triton X-100 and $C_{12}E_8$ or the sedative chlorpromazine, bind to *one* common high-affinity site per two α-toxin sites. Based on the accepted stoichiometry of the four chains of the AcChR, 2α, 1β, 1γ, and 1δ (Reynolds & Karlin, 1978; Lindstrøm et al., 1979; Raftery et al., 1980) and on covalent labeling experiments using reagents specific for the AcCh binding sites [review Karlin (1980) and Changeux (1981)] which primarily label the α chains, the proposed stoichiometry of one NCB to two α-toxin sites is thus consistent with the occurrence of one high-affinity NCB site per 250 000-dalton light form of the AcChR.

The covalent labeling of the AcChR chains by [^{3}H]chlorpromazine raises an important question. In agreement with the findings of Oswald & Changeux (1981a), this NCB is incorporated into the *four* chains of the receptor upon UV irradiation of the membrane fragments. Chlorpromazine, however, binds in a significant manner to the low-affinity sites, and the possibility exists that the observed labeling results primarily from the interaction of [^{3}H]chlorpromazine with this particular class of sites. This, however, appears unlikely for the following reasons: (a) covalent attachment of [^{3}H]-chlorpromazine to *all four* chains decreases in the presence of two NCBs, H_{12}HTX and phencyclidine, which have been demonstrated to interact in a highly selective manner with the high-affinity site; and (b) the concentration dependence of this decrease correlates with the dissociation of [^{3}H]chlorpromazine-reversible binding to the high-affinity site. Thus, the labeling of the AcChR by [^{3}H]chlorpromazine primarily results from the interaction of this NCB with the high-affinity site. The reason why [^{3}H]chlorpromazine labels all four chains of the AcChR while other NCBs such as the azido derivative of trimethisoquin (Oswald et al., 1980; Oswald & Changeux, 1981b) synthesized by Waksman et al. (1980a), as well as [^{3}H]phencyclidine, [^{3}H]trimethisoquin, and [^{3}H]H_{12}HTX (Oswald & Changeux, 1981a), label only the δ chain upon UV irradiation yet is not understood. The establishment of covalent bonds between chlorpromazine and the protein may result from both the high reactivity of the ligand and the presence of closely located acceptor groups in the protein.

Model for the AcChR and NCB Interactions. The model of the AcChR and NCB interactions shown in Figure 8 is

based on the known structural and functional properties [reviewed in Changeux (1981), Anholt et al. (1983), and Kistler et al. (1982)] and on the data presented in this paper and in the following one [see Oswald et al. (1983)].

The five subunits of the M_r 250000 AcChR oligomer traverse the membrane bilayer [see Wennogle & Changeux (1980), Strader & Raftery (1980), Froehner (1981), and Saint-John et al. (1982)] and are packed in a cylinder with the long axis perpendicular to the plane of the membrane. The molecule viewed "en face" appears as a rosette with a central hydrophilic crevice and "de côté" as a thick funnel with a central "channel" at least 55 nm deep (Cartaud et al., 1978; Klymkowsky & Stroud, 1979; Kistler et al., 1982). The high-affinity NCB site would be located within this central depression. This depression constitutes a unique region of the molecule where the distances to all five chains of the AcChR are minimum and where, being hydrophilic [see Devillers-Thiery et al. (1983)], charged compounds may easily enter and traverse the membrane. A rather similar location for an allosteric site has been described with hemoglobin: the unique site for diphosphoglycerate present per $\alpha_2\beta_2$ tetramer is located on the symmetry axis of the molecule and limited by the four subunits [see Arnone (1972)]. The high degree of homology between the four chains of the AcChR (Raftery et al., 1980; Noda et al., 1983) and the high reactivity of chlorpromazine upon UV irradiation would then simply account for the covalent labeling of this site.

Binding of NCBs at the level of this high-affinity site stabilizes the high-affinity desensitized state of the AcChR and consequently inhibits channel activation. This effect could account for the slow time-dependent inhibition of the permeability response by NCBs and the so-called "blockade of closed channels" [reviewed in Spivak & Albuquerque (1982)]. Taking into further consideration the in vivo data on the direct, steric channel blockade by NCBs [the "blockade of open channels"; reviewed in Adams (1981)] and the in vitro pharmacological and kinetic data presented in the following paper (Oswald et al. 1983), it is proposed that the central cavity of the receptor molecule is part of the ion channel itself.

The presence of a lipid domain with distinct physical and/or chemical properties at the level of the interface between the receptor protein and the bulk lipid phase is also postulated. The general existence of such a domain in integral membrane proteins was initially proposed on the basis of preferential interactions between these proteins and given lipids, or of steric modulation of lipid chain order by these proteins (Owicki et al., 1978), and subsequently demonstrated by ESR spectroscopy for several membrane-bound proteins [reviewed in Jost & Griffith (1980) and Davoust & Devaux (1982)]. In the case of the membrane-bound AcChR, the low-affinity saturable binding of NCBs may take place at the level of this lipid/protein domain. On the other hand, the nonsaturable binding of these NCBs would correspond to their partitioning into the bulk lipid phase. The interaction of NCBs at the level of the lipid–AcChR interface would account for the "H_{12}HTX-resistant" stabilization of the high-affinity desensitized state of the AcChR by some NCBs and result in channel inactivation as above. It would also account for the lack of effect of heat treatment on this low-affinity binding. The increased number of low-affinity NCB binding sites observed with increasing phospholipid to AcChR molar ratios in reconstituted vesicles would then result from either a disruption of protein–protein interactions upon dilution with increasing lipid concentrations or an increased accumulation of lipids of adequate chemical properties supplied by the complex mixture of exogenous lipids added for reconstition in increasing amounts [see Owicki et al. (1978) for a theoretical treatment].

The number of low-affinity sites for NCBs falls in the same range as the number of phospholipid molecules bound per molecule of cytochrome oxidase or ATPase at the level of discrete binding or contact sites of their hydrophobic surface (Brotherus et al., 1981). A similar number of sites for Triton X-100 was also reported for the delipidated Ca^{2+}-ATPase at submicellar detergent concentrations (Dean & Suarez, 1981). Chang & Bock (1979) have also found that about 20 molecules of phospholipid per α-toxin site remain associated with the detergent-purified receptor. Finally, Popot et al. (1978) have shown that the purified AcChR selectively discriminates between cholesterol and closely related sterols and between phospholipids of varying chain lengths sprayed as monolayers at an air–water interface. Interestingly, denaturation of the AcChR does not destroy this discriminative power which might thus be determined by short hydrophobic sequences rather insensitive to conformation and directly in contact with the hydrophobic phase of the lipid bilayer in the native membrane.

The occurrence of two possible and nonexclusive modes of allosteric interactions of NCBs with the AcChR—via a strictly proteic high-affinity hydrophilic site and via the lipid–protein interface—also accounts for the known effect of membrane potential on the relative potency of NCBs and on the occurrence of a correlation between potency and lipid solubility (Koblin & Lester, 1979). It also accounts for some of the observed deviations [see Richards et al. (1978)] from simple theories which postulate that nonspecific partitioning of amphiphile molecules in the bulk lipid phase of biological membranes [reviewed in Seeman (1972)] is the general mechanism for the pharmacological action of local and general anesthetics.

Acknowledgments

We thank Dr. C. Bon, Dr. C. Hirth, and Professors B. Roques and J. B. Le Pecq for helpful discussions, Dr. F. Labeyrie for her kind hospitality in the Laboratoire d'Enzymologie Physico-chimique of the Centre de Génétique Moléculaire at Gif-sur-Yvette and for her help in the maintenance of the stopped-flow equipment, Dr. G. Waksman for the gift of Dns-C_6-Cho, Professor J. Daly for perhydrohistrionicotoxin, Professors M. Lazdunski and M. Sokolovsky for phencyclidine, Dr. Menez for *Naja nigricollis* α-toxin, and Professor Tamiya for erabutoxin b.

Appendix

1. Equation 2 under Discussion can be rewritten as

$$\bar{D} - \bar{D}_0 = \frac{F}{F + K_{app}} \qquad \text{(A2)}$$

where $\bar{D}_0$ is equal to the fraction ($\bar{D}$) of AcChR in the D state in the absence of NCB [$\bar{D}_0 = (1 + L_0)^{-1}$] and where K_{app} is given by eq 4 under Discussion. Equation A2 is formally equivalent to a binding isotherm relating the amount of bound ligand (B) to that of free ligand (F). Accordingly, all the classical representations of the binding data such as "Scatchard" or "double-inverse" can be used by taking as corresponding variables $\bar{D} - \bar{D}_0$ and F.

2. The actual concentration (x^*) of a stock solution of radiolabeled NCB can be derived as described under Results and Figure 5, by using the binding capacity of the high-affinity NCB site. In a typical isotopic dilution experiment, the specific binding of a radiolabeled NCB to the high-affinity site was determined in the presence of varying concentrations of the corresponding nonradioactive NCB, and the amount of ra-

diolabeled NCB bound at saturation (cpm_{max}) was determined. Upon addition of the nonradioactive NCB, cpm_{max} should remain proportional to $x^*/(x^* + x)$ where x is the concentration of the nonradioactive NCB added to the solution of the radiolabeled NCB at a fixed concentration x^*. A plot of the reciprocal of cpm_{max} as a function of x should then be linear, and its intercept with the abscissa axis should be equal to x^*.

Registry No. Phencyclidine, 77-10-1; meproadifen, 56538-56-8; Triton X-100, 9002-93-1; chlorpromazine, 50-53-3; trimethisoquin, 69311-91-7.

References

Adams, P. R. (1977) *J. Physiol. (London) 268*, 291–318.

Adams, P. R. (1981) *J. Membr. Biol. 58*, 161–174.

Adams, P. R., & Feltz, A. (1980) *J. Physiol. (London) 306*, 261–281, 283–306.

Albuquerque, E. X., Tsai, M.-C., Aronstam, R., Witkop, B., Eldefrawi, A. T., & Eldefrawi, M. E. (1980) *Mol. Pharmacol. 18*, 159–178.

Andreasen, T. J., & McNamee, M. G. (1980) *Biochemistry 19*, 4719–4726.

Andreasen, T. J., Doerge, D. R., & McNamee, M. G. (1979) *Arch. Biochem. Biophys. 194*, 468–480.

Anholt, R., Lindstrøm, J., & Montal, M. (1983) in *Enzymes of Biological Membranes* (Martonosi, A., Ed.) 2nd ed., Plenum Press, New York.

Arnone, A. (1972) *Nature (London) 231*, 146–149.

Aronstam, R., Eldefrawi, A. T., Pessah, I. N., Daly, J. D., Albuquerque, E. X., & Eldefrawi, M. E. (1981) *J. Biol. Chem. 256*, 2843–2850.

Blanchard, S. G., Elliott, J., & Raftery, M. A. (1979) *Biochemistry 18*, 5880–5885.

Bon, C., Changeux, J.-P., Jeng, T. W., & Fraenkel-Conrat, H. (1979) *Eur. J. Biochem. 99*, 471–481.

Böttcher, C. J. F., Van Gent, C. M., & Pries, C. (1961) *Anal. Chim. Acta 24*, 203–204.

Boyd, N. D., & Cohen, J. B. (1980) *Biochemistry 19*, 5344–5353.

Bradley, R., Peper, K., & Sterz, R. (1980) *Nature (London) 284*, 60–62.

Brisson, A. D., Devaux, P. F., & Changeux, J.-P. (1975) *C. R. Hebd. Seances Acad. Sci., Ser. D 280*, 2153–2156.

Brotherus, J. R., Griffith, O. H., Brotherus, M. O., Jost, P. C., Silvius, J. R., & Hokin, L. E. (1981) *Biochemistry 20*, 2561–2567.

Cartaud, J., Benedetti, L., Sobel, A., & Changeux, J.-P. (1978) *J. Cell Sci. 29*, 313–337.

Chang, H. W., & Bock, E. (1979) *Biochemistry 18*, 172–179.

Changeux, J.-P. (1981) *Harvey Lect. 75*, 85–254.

Cohen, J. B. (1978) in *Molecular Specialization and Symmetry in Membrane Function* (Solomon, A. K., & Karnovsky, M., Eds.) pp 99–127, Harvard University Press, Cambridge, MA.

Cohen, J. B., Weber, M., & Changeux, J.-P. (1974) *Mol. Pharmacol. 10*, 904–932.

Cohen, J. B., Boyd, N. D., & Shera, N. S. (1980) in *Progress in Anesthesiology* (Fink, B. R., Ed.) Vol. 2, pp 165–175, Raven Press, New York.

Daly, J. W., Karle, J., Myers, C. W., Tokuyama, T., Waters, J. A., & Witkop, B. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1870–1875.

Davoust, J., & Devaux, P. F. (1982) *J. Magn. Reson. 48*, 475–494.

Dean, W. L., & Suarez, C. P. (1981) *Biochemistry 20*, 1743–1747.

Devillers-Thiery, A., Giraudat, J., Bentaboulet, M., & Changeux, J.-P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2067–2071.

Dunn, S. M. J., Blanchard, S. G., & Raftery, M. A. (1981) *Biochemistry 20*, 5617–5624.

Eldefrawi, A. T., Eldefrawi, M. E., Albuquerque, E. X., Oliveira, A. C., Mansour, N., Adler, M., Daly, J. W., Brown, G. B., Burgermeister, W. B., & Witkop, B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2172–2176.

Eldefrawi, M. E., Eldefrawi, A. T., Mansour, N. A., Daly, J. W., Witkop, B., & Albuquerque, E. X. (1978) *Biochemistry 17*, 5474–5483.

Eldefrawi, M. E., Eldefrawi, A. T., Aronstam, R. S., Maleque, M. A., Warnick, J. E., & Albuquerque, E. X. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7458–7462.

Eldefrawi, M. E., Aronstam, R. S., Bakry, N., Eldefrawi, A. T., & Albuquerque, E. X. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2309–2313.

Elliott, J., & Raftery, M. A. (1977) *Biochem. Biophys. Res. Commun. 77*, 1347–1353.

Elliott, J., & Raftery, M. A. (1979) *Biochemistry 18*, 1868–1874.

Elliott, J., Dunn, S. M. J., Blanchard, S. G., & Raftery, M. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2576–2579.

Epstein, M., & Racker, E. (1978) *J. Biol. Chem. 253*, 6660–6662.

Froehner, S. C. (1981) *Biochemistry 20*, 4905–4915.

Gage, P. W. (1976) *Physiol. Rev. 56*, 177–247.

Grünhagen, H. H., & Changeux, J.-P. (1976) *J. Mol. Biol. 106*, 497–516.

Heidmann, T., & Changeux, J.-P. (1979) *Eur. J. Biochem. 94*, 255–279, 281–296.

Heidmann, T., & Changeux, J.-P. (1980) *Biochem. Biophys. Res. Commun. 97*, 889–896.

Heidmann, T., & Changeux, J.-P. (1981) *FEBS Lett. 131*, 239–244.

Heidmann, T., Sobel, A., Popot, J. L., & Changeux, J.-P. (1980) *Eur. J. Biochem. 110*, 35–55.

Johnstone, R. A. W., Neville, A. F., & Russell, P. J. (1971) *J. Chem. Soc. B*, 1183.

Jost, P. C., & Griffith, O. H. (1980) *Ann. N.Y. Acad. Sci. 348*, 391.

Karlin, A. (1980) in *The Cell Surface and Neuronal Function* (Poste, G., Nicolson, G., & Cotman, C., Eds.) pp 191–260, Elsevier/North-Holland Biomedical Press, New York.

Karpen, J. W., Aoshima, H., Abood, L. G., & Hess, G. P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2509–2513.

Katz, B., & Miledi, R. (1975) *J. Physiol. (London) 249*, 269–284.

Katz, B., & Miledi, R. (1980) *INSERM Symp. No. 13*, 171–178.

Kistler, J., Stroud, R. M., Klymkowsky, M. W., Lalancette, R., & Fairclough, R. H. (1982) *Biophys. J. 37*, 371–383.

Kloog, Y., Kalir, A., Buchman, O., & Sokolovsky, M. (1980) *FEBS Lett. 109*, 125–128.

Klymkowsky, M. W., & Stroud, R. M. (1979) *J. Mol. Biol. 128*, 319–334.

Koblin, D. K., & Lester, H. A. (1979) *Mol. Pharmacol. 15*, 559–580.

Krodel, E. K., Beckman, R. A., & Cohen, J. B. (1979) *Mol. Pharmacol. 15*, 294–312.

Lauffer, L., & Hucho, F. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2408–2409.

Lindstrøm, J., Merlie, J., & Yogeeswaran, G. (1979) *Biochemistry 18*, 4465–4470.

Medynski, D. C., & Cohen, J. B. (1981) *Soc. Neurosci. Abstr. 7*, 345.

Monod. J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118.

Navaratnam, S., Parsons, B. J., & Phillips, G. O. (1978) *J. Chem. Soc. 74*, 1811–1819.

Neher, E., & Steinbach, J. H. (1978) *J. Physiol. (London) 277*, 153–176.

Neubig, R. R., & Cohen, J. B. (1980) *Biochemistry 19*, 2770–2779.

Neubig, R. R., Krodel, E. K., Boyd, N. D., & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690–694.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Hirose, T., Furutani, Y., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983) *Nature (London) 302*, 528–532.

Oswald, R. E., & Freeman, J. A. (1979) *J. Biol. Chem. 254*, 3419–3426.

Oswald, R. E., & Changeux, J.-P. (1981a) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3925–3929.

Oswald, R. E., & Changeux, J.-P. (1981b) *Biochemistry 20*, 7166–7174.

Oswald, R. E., Sobel, A., Waksman, G., Roques, B., & Changeux, J.-P. (1980) *FEBS Lett. 111*, 29–34.

Oswald, R. E., Heidmann, T., & Changeux, J.-P. (1983) *Biochemistry* (following paper in this issue).

Owicki, J. L., Springgate, M. W., & McConnell, H. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1616–1619.

Popot, J. L., Demel, R. A., Sobel, A., Van Deenen, L., & Changeux, J.-P. (1978) *Eur. J. Biochem. 85*, 24–42.

Raftery, M. A., Hunkapiller, M. W., Strader, C. B., & Hood, L. E. (1980) *Science (Washington, D.C.) 108*, 1454–1457.

Reynolds, J. A., & Karlin, A. (1978) *Biochemistry 17*, 2035–2038.

Richards, C. D., Martin, K., Gregory, S., Keightley, C. A., Hesketh, T. R., Smith, G. A., Warren, G. B., & Metcalfe, J. C. (1978) *Nature (London) 276*, 755–759.

Rubin, M. M., & Changeux, J.-P. (1966) *J. Mol. Biol. 21*, 265–274.

Ruoho, A. E., Kieffer, H., Roeder, P. E., & Singer, S. J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2567–2571.

Saint-John, P. A., Froehner, S. C., Goodenough, A., & Cohen, J. B. (1982) *J. Cell Biol. 92*, 333–342.

Saitoh, T., Oswald, R. E., Wennogle, L., & Changeux, J.-P. (1980) *FEBS Lett. 116*, 30–36.

Seeman, P. (1972) *Pharmacol. Rev. 24*, 583–655.

Sine, S. M., & Taylor, P. (1982) *J. Biol. Chem. 257*, 8106–8114.

Sobel, A., Weber, M., & Changeux, J.-P. (1977) *Eur. J. Biochem. 80*, 215–224.

Sobel, A., Heidmann, T., Hofler, J., & Changeux, J.-P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 510–514.

Sobel, A., Heidmann, T., Cartaud, J., & Changeux, J.-P. (1980) *Eur. J. Biochem. 110*, 13–33.

Spivak, C. E., & Albuquerque, E. X. (1982) in *Progress in Cholinergic Biology* (Hanin, J., & Goldberg, A. M., Eds.) pp 323–357, Raven Press, New York.

Spivak, C. E., Maleque, M. A., Oliveria, A. C., Masukawa, L. M., Tokuyama, T., Daly, J. W., & Albuquerque, E. X. (1982) *Mol. Pharmacol. 21*, 351–361.

Steinbach, A. B. (1968) *J. Gen. Physiol. 52*, 144–180.

Strader, C. D., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5807–5811.

Waksman, G., Oswald, R. E., Changeux, J.-P., & Roques, B. (1980a) *FEBS Lett. 111*, 23–28.

Waksman, G., Changeux, J.-P., & Roques, B. (1980b) *Mol. Pharmacol. 18*, 20–27.

Weber, M., & Changeux, J.-P. (1974) *Mol. Pharmacol. 10*, 35–40.

Weber, M., David-Pfeuty, T., & Changeux, J.-P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3443–3447.

Weiland, G., Georgia, B., Lappi, S., Chignell, C. E., & Taylor, P. (1977) *J. Biol. Chem. 252*, 7648–7656.

Wennogle, L., & Changeux, J.-P. (1980) *Eur. J. Biochem. 106*, 381–393.

Young, A. P., & Sigman, D. S. (1982) *Mol. Pharmacol. 20*, 498–505.