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# Selective Labeling of the δ Subunit of the Acetylcholine Receptor by a Covalent Local Anesthetic†

Robert E. Oswald‡ and Jean-Pierre Changeux*

ABSTRACT: A radioactive photoaffinity derivative of the potent local anesthetic trimethisoquin, 5-azido[$^3$H]trimethisoquin, was used to label the acetylcholine receptor from *Torpedo marmorata* electric organ. The product labeled the 66 000-dalton (δ) subunit of the receptor with the selectivity expected for an affinity label of the site for noncompetitive blockers. That is, the labeling was enhanced by cholinergic agonists and inhibited by other noncompetitive blockers. The 40 000-dalton (α) subunit of the receptor was labeled in a manner consistent with the attachment of 5-azido[$^3$H]trimethisoquin to an acetylcholine binding site as the incorporation of radioactivity into the α chain was inhibited by cholinergic agonists and antagonists, such as carbamylcholine, *d*-tubocurarine, and α-bungarotoxin. The reversible binding of [$^3$H]phencyclidine, a potent noncompetitive blocker, to acetylcholine receptor rich membranes resembled qualitatively and quantitatively the 5-azido[$^3$H]trimethisoquin labeling of the δ subunit and was inhibited by the prior covalent labeling of the membranes with nonradioactive 5-azidotrimethisoquin. Thus, 5-azido[$^3$H]-trimethisoquin labels at least a portion of the binding site for noncompetitive blockers at the level of the δ subunit. The functional significance of this site and the use of 5-azidotrimethisoquin in the study of acetylcholine receptor structure and function are discussed.

The physiological response to AcCh[1] of the subsynaptic membrane from the neuromuscular junction or the electromotor synapse is blocked by two distinct classes of pharmacological agents: (1) the competitive, nicotinic antagonists (e.g., *d*-tubocurarine and flaxedil) which decrease the apparent affinity for AcCh without changing the maximal response and which interact directly with the AcCh binding site [for reviews, see Nachmansohn (1955), Changeux (1975, 1980), and Heidmann & Changeux (1978)] and (2) the noncompetitive blockers which include the aminated local anesthetics (Weber & Changeux, 1974; Heidmann & Changeux, 1979; Krodel et al., 1979; Cohen et al., 1980a,b), perhydrohistrionicotoxin (Daly et al., 1971; Eldefrawi et al., 1980a), phencyclidine (Kloog et al., 1980; Albuquerque et al., 1980a,b; Eldefrawi et al., 1980b), amantadine (Tsai et al., 1978), and various detergents [for a review, see Changeux (1980)] cause a major decrease of the maximal response with little effect on the apparent dissociation constant. A number of physiological observations based on noise (Katz & Miledi, 1975), voltage jump (Adams, 1977), and single channel (Neher & Steinbach, 1978) analysis and the nonlinear current–voltage relationships noticed for the end plate current in the presence of these agents (Albuquerque et al., 1980a,b; Eldefrawi et al., 1980a,b) led

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[1] Abbreviations used: AcCh, acetylcholine; AcChR, acetylcholine receptor; 5AT, 5-azidotrimethisoquin; 5A[$^3$H]T, 5-azido[$^3$H]trimethisoquin; BrAcCh, bromoacetylcholine; α-[$^{125}$I]BGT, α-[$^{125}$I]bungarotoxin; DAPA, bis(3-azidopyridinium)-1,10-decane perchlorate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MBTA, [4-(*N*-maleimido)benzyl]-trimethylammonium; MPTA, [4-(*N*-maleimido)phenyl]trimethylammonium; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; TEA, tetraethylammonium ion; Temed, *N,N,N',N'*-tetramethylethylenediamine; TDF, trimethylammonium diazonium fluoroborate; Tris, tris(hydroxymethyl)aminomethane.

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to the suggestion that the noncompetitive blockers interact directly with a component of the ion channel. However, allosteric effects of these agents at the level of saturable sites [for a review, see Changeux (1980)] or via the lipid phase (Koblin & Lester, 1979; Heidmann et al., 1980; Cohen et al., 1980b) are as plausible.

Competitive antagonists and noncompetitive blockers exert their pharmacological action on the permeability response to cholinergic agonists exhibited in vitro by highly purified AcChR-rich membranes from *Torpedo* electric organ (observed by measuring $^{22}Na$ flux; Kasai & Changeux, 1971; Popot et al., 1976; Neubig & Cohen, 1980). These membrane fragments contain the four AcChR subunits ($\alpha$, $\beta$, $\gamma$, and $\delta$; approximate $M_r$ 40 000, 50 000, 60 000, and 66 000, respectively; Karlin, 1980, Raftery et al., 1980; Changeux, 1980), a structural protein (43 000; Sobel et al., 1977, 1978), and a contaminating 95 000 band. Because of its high degree of purity and its availability in decigram quantities, this preparation serves as a convenient material to identify the polypeptide chain(s) which carries (carry) the sites for the diverse blocking agents.

The *$\alpha$ subunit* of the AcChR is labeled by affinity reagents which are analogues of AcCh [TDF (Changeux et al., 1967; Weiland et al., 1979), MBTA (Karlin & Winnik, 1968; Reiter et al., 1972; Damle & Karlin, 1978), MPTA (Sobel et al., 1977), BrAcCh (Damle et al., 1978; Moore & Raftery, 1979), and DAPA (Witzemann & Raftery, 1977)] and thus carries part, or all, of an AcCh binding site.

Binding of noncompetitive blockers to the 43 000-dalton protein has been reported (Sobel et al., 1978; Blanchard & Raftery, 1979); however, following alkaline extraction of the 43 000 protein (Neubig et al., 1979), the sensitivity of the permeability response to noncompetitive blockers persisted, indicating that the 43 000-dalton protein is not directly involved in the pharmacological action of these compounds.

In an attempt to affinity label the site(s) for noncompetitive blockers, a radioactive photoaffinity derivative of the potent local anesthetic trimethisoquin, 5A[$^3$H]T, was synthesized (Waksman et al., 1980). This product was shown to label selectively the *$\delta$ subunit* of the AcChR (Oswald et al., 1980; Saitoh et al., 1980) in a manner similar to the reversible equilibrium binding of local anesthetics to *Torpedo* membrane fragments (Krodel et al., 1979; Sobel et al., 1980); that is, the labeling was inhibited by trimethisoquin and histrionicotoxin and enhanced by carbamoylcholine. Also, $\alpha$-Bgt blocked the enhancement by carbamoylcholine.

We report here a detailed characterization of the labeling of the $\delta$ subunit of the AcChR receptor by 5A[$^3$H]T. The concentration dependence of 5A[$^3$H]T labeling, its inhibition by various noncompetitive blockers, and its enhancement by a variety of nicotinic agonists and competitive antagonists are described and compared to the binding data obtained with the reversible noncompetitive blocker [$^3$H]phencyclidine.

## Materials and Methods

AcChR-rich membranes were purified from freshly dissected *Torpedo marmorata* electric organ in the presence of protease inhibitors and chelating agents (buffer A: 50 mM Tris-HCl, pH 7.5; 3 mM EDTA; 1 mM EGTA; 0.1 mM PMSF; 5 units/mL aprotinine; 5 $\mu$g/mL pepstatin) as described previously (Saitoh et al., 1980). The membranes were either used directly or stored until use in liquid nitrogen at a concentration of 20–25 $\mu$M in $\alpha$-[$^{125}$I]Bgt sites.

Affinity labeling was performed essentially as described previously (Saitoh et al., 1980). Membranes were washed once in distilled water by centrifugation at full speed in a Beckman airfuge and resuspension in *Torpedo* physiological solution, followed by centrifugation and finally resuspension in *Torpedo* physiological solution to a final concentration of 2–5 $\mu$M in $\alpha$-[$^{125}$I]Bgt sites. Ten microliters of membranes was added to a 0.32-cm$^2$ well of a Costar tissue culture cluster and then mixed with 10 $\mu$L of a pharmacological agent (or *Torpedo* physiological solution), followed by the addition, in the dark, of 10 $\mu$L of 5A[$^3$H]T. The solutions were then placed in a chamber, flushed with nitrogen for 15 min in the dark, and irradiated for 5 min (except where otherwise indicated) with a Mineralight short-wave ultraviolet lamp (Ultra-Violet Products, Inc.) placed outside the chamber at a distance of 15 cm (254 nm; 1200 $\mu$W/cm$^2$). A 5-min irradiation was chosen because the highest level of specific labeling was obtained after this time (Figure 1). Longer irradiations resulted in protein cross-linking and/or degradation with a decrease in the apparent incorporation in the presence of carbamoylcholine. The solutions were separated from the light source by a thin sheet of cellophane which absorbed less than 5% of the light. No significant incorporation of radioactivity was observed with membranes not exposed to light before denaturation in $NaDodSO_4$ and gel electrophoresis. The addition of 1 mg/mL bovine serum albumin had no effect on the labeling pattern.

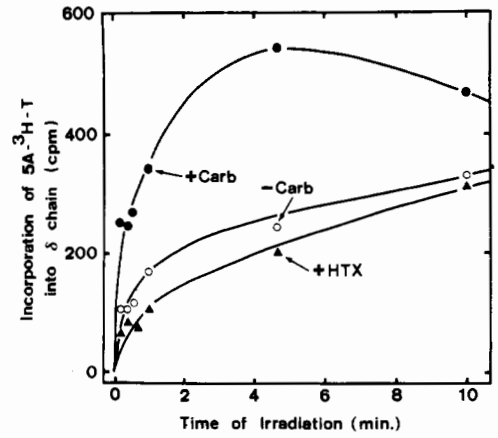


FIGURE 1: Incorporation of 5A[$^3$H]T into the $\delta$ subunit studied as a function of the time of UV irradiation at 254 nm. Incorporation was measured in the presence of $10^{-4}$ M carbamoylcholine (●), in the presence of $10^{-4}$ M histrionicotoxin (▲), and with the addition of neither carbamoylcholine nor histrionicotoxin (○). The 5A[$^3$H]T concentrations was 5 $\mu$M.

In the case of preparative labeling with nonradioactive 5AT, the volume of the reaction mixture was increased to 100 $\mu$L, and the solution was stirred during irradiation. Unbound 5AT was removed by five cycles of centrifugation (13 000 rpm for 15 min in a Sorvall SS34 rotor) and resuspension in 3 mL of *Torpedo* physiological solution or buffer A.

$NaDodSO_4$–polyacrylamide gel electrophoresis (10% acrylamide) was performed in 1.1–1.3 mm thick slabs by a modification of the Tris–glycine Laemmli (1970) system described previously (Sobel et al., 1977). Samples were dissolved in 0.06 M Tris-HCl, pH 6.8, 2% $NaDodSO_4$, 5% $\beta$-mercaptoethanol, and 0.001% bromphenol blue and were not heated prior to loading on the gel. After electrophoresis, the gels were stained and fixed simultaneously in 0.05% Coomassie blue R-250 dissolved in 20% (v/v) ethanol and 10% (v/v) glacial acetic acid and destained in the same solution minus Coomassie blue. Radioactivity was detected either by fluorography as described by Bonner & Laskey (1974) using Kodak X-Omat R film preflashed to an absorbance of 0.2 or by cutting out appropriate sections of the dried gel and counting in a medium containing 900 mL or toluene–PPO, 100 mL of tissue solu-

bilizer, and 10 mL of 4 M $NH_4OH$. Samples were counted after a 2-day incubation at room temperature by using an Intertechnique $\beta$ counter with standard tritium windows.

[$^3$H]Phencyclidine binding was measured by using a modification (Oswald & Changeux, 1981) of the assay described by Krodel et al. (1979) for the binding of [$^{14}$C]meproadifen. The extent of binding was estimated by the loss of radioactivity from the supernatant and by the amount of radioactivity in the pellet. [$^{14}$C]AcCh binding was measured as described by Weber & Changeux (1974) except that a 1-mL total volume was used and that centrifugations were performed at top speed for 15 min in an Eppendorf minifuge. $\alpha$-[$^{125}$I]Bgt binding was measured by the DEAE filter disk assay described by Maelicke et al. (1977) and by Sephadex G-75 chromatography (Sobel et al., 1980).

Phencyclidine was a gift of Professor M. Sokolovsky; histrionicotoxin was kindly supplied by Dr. G. Kato; 5-azidodimethisoquin, 5-azidotrimethisoquin, and trimethisoquin were gifts of Drs. G. Waksman and B. Roques (Waksman et al., 1980); Erabutoxin B was provided by Dr. Tamiya. 5-A[$^3$H]T (15 Ci/mmol) was synthesized from 5-azidodimethisoquin with [$^3$H]methyliodide at the CEA, Saclay, Service des Molécules Marquées. Purity was confirmed by thin-layer chromatography on silica gel plates using ethyl acetate–2-propanol–$H_2O$–formic acid (2:1:1:1%). [$^3$H]Phencyclidine was a gift of Professor E. F. Domino and was also purchased from New England Nuclear; $\alpha$-[$^{125}$I]Bgt was obtained from New England Nuclear. Aprotinine, PMSF, pepstatin, chlorpromazine, and TEA were products of Sigma Chemical Co.; acrylamide, bis(acrylamide), and Temed were obtained from Kodak; dibucaine and tetracaine were products of K & K Laboratories, Inc. Live *T. marmorata* were provided by the biological station of Arcachon, France.

## Results

*Differential Labeling of AcChR Subunits by 5A[$^3$H]T.* In a first series of experiments (Oswald et al., 1980), 5A[$^3$H]T was shown to label, in the presence of carbamoylcholine, two polypeptide chains of approximate $M_r$ 50 000 and 66 000, present in AcChR-rich membrane fragments. The pharmacological specificity of this labeling coincided with the binding properties of the high-affinity site for noncompetitive blockers. Further studies (Saitoh et al., 1980) indicated that, under conditions which prevent proteolytic nicking of AcChR subunits [see also Karlin (1980)], only the 66 000-dalton ($\delta$) subunit of the AcChR was labeled by 5A[$^3$H]T in the presence of carbamoylcholine; the labeled 50 000-dalton band was shown to result from the degradation of the labeled $\delta$ subunit. In Figure 2 are presented the labeling patterns of the polypeptide chains obtained, under the same conditions, by one-dimensional polyacrylamide gel electrophoresis in $NaDodSO_4$ of AcChR-rich membrane fragments in the presence (or absence) of characteristic pharmacological agents at a 5A[$^3$H]T concentration of 8.5 $\mu$M.

The *$\alpha$ subunit* was the most prominently labeled chain in the absence of carbamoylcholine. The incorporation of 5A-[$^3$H]T into this chain decreased significantly in the presence of 0.1 mM carbamoylcholine (Figure 2B, 5–8) and 5 $\mu$M erabutoxin B (Figure 2B, 4 and 8), but histrionicotoxin (Figure 2B, 2 and 3) had little effect, indicating that 5A[$^3$H]T interacted with the AcCh binding site. In addition, a faint labeling of the $\gamma$ chain was observed under the same experimental conditions as the labeling of the $\alpha$ chain (i.e., blocked by carbamoylcholine and erabutoxin B).

In the presence of carbamoylcholine, the most intensely labeled peptide was the *$\delta$ subunit* (Figure 2B, 5). A slight

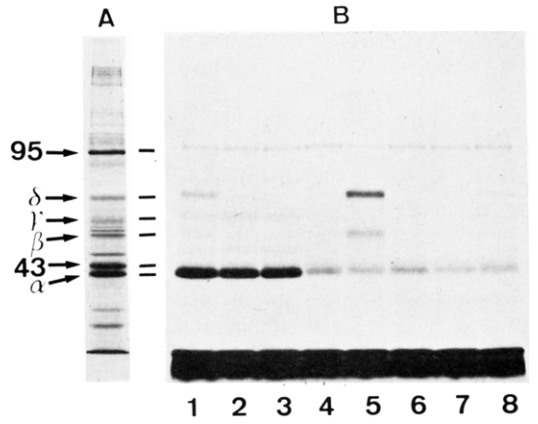


FIGURE 2: Incorporation of 8.5 $\mu$M 5A[$^3$H]T into the proteins of AcChR-rich membrane fragments in the presence of various pharmacological agents. (A) Coomassie blue protein stain of the 10% $NaDodSO_4$–polyacrylamide gel of receptor-rich membranes. (B) Fluorogram revealing the 5A[$^3$H]T radioactivity incorporated under the following conditions: 1, no addition; 2, 10 $\mu$M histrionicotoxin; 3, 50 $\mu$M histrionicotoxin; 4, 5 $\mu$M erabutoxin B; 5, 100 $\mu$M carbamoylcholine; 6, 100 $\mu$M carbamoylcholine plus 10 $\mu$M histrionicotoxin; 7, 100 $\mu$M carbamoylcholine plus 50 $\mu$M histrionicotoxin; 8, 100 $\mu$M carbamoylcholine plus 5 $\mu$M erabutoxin B. For the erabutoxin B conditions (4 and 8), the toxin was added 20 min before either carbamoylcholine (8 only) or 5A[$^3$H]T.

labeling in the same region of the gel as the $\beta$ subunit was observed but, as mentioned above (Saitoh et al., 1980), was associated with a proteolytic fragment of the $\delta$ chain. In preparations maintained continuously in the presence of EDTA and EGTA, this 50 000 fragment was present in quantities too low to be detected by Coomassie blue protein staining but was revealed by 5A[$^3$H]T labeling. In the absence of carbamoylcholine, the $\delta$ subunit was also labeled (Figure 2B, 1) but to a lesser extent than in the presence of carbamoylcholine. Histrionicotoxin (10 $\mu$M, Figure 2B, 2 and 6; 50 $\mu$M, Figures 2B, 3 and 7) reduced the labeling of the $\delta$ subunit to background level both in the presence and in the absence of carbamoylcholine. In the absence of carbamoylcholine, erabutoxin B (Figure 2B, 4 and 8) also decreased the labeling of the $\delta$ subunit, but not to as great an extent as histrionicotoxin.

A slight labeling of the 95 000-dalton chain and of the 43 000 chain was observed. In addition, radioactivity was seen at the level of the tracking dye which represents both the degradation products of the free reagent and the labeling of small peptides. Significant labeling at the position of the tracking dye remained even when the 5A[$^3$H]T-labeled AcChR was solubilized in nondenaturing detergent and purified on a sucrose gradient. The labeling of the 95 000 chain, the 43 000 chain, and the peptides at the tracking dye was insensitive to any of the pharmacological agents tested. Higher concentrations of 5A[$^3$H]T results in the increase in nonspecific labeling; however, with 5A[$^3$H]T concentrations of up to 500 $\mu$M, only labeling of the $\delta$ chain could be inhibited by up to 500 $\mu$M histrionicotoxin.

*Quantitative Labeling of the $\delta$ Subunit by 5A[$^3$H]T.* The labeling of the *$\delta$ subunit* by 5A[$^3$H]T was quantitatively studied by cutting out the appropriate section of the gel, solubilizing the gel slice, and counting for tritium (see Materials and Methods). Incorporation of tritium into the $\delta$ subunit as a function of total 5A[$^3$H]T concentration is shown in Figure 3. In the presence of 0.5 mM carbamoylcholine, the labeling reached a plateau between 50 and 100 $\mu$M 5A-[$^3$H]T. At the plateau, the number of sites labeled by 5A-[$^3$H]T and displaced by histrionicotoxin was approximately 0.25–0.30 the number of $\alpha$-[$^{125}$I]Bgt binding sites.[2] Half-

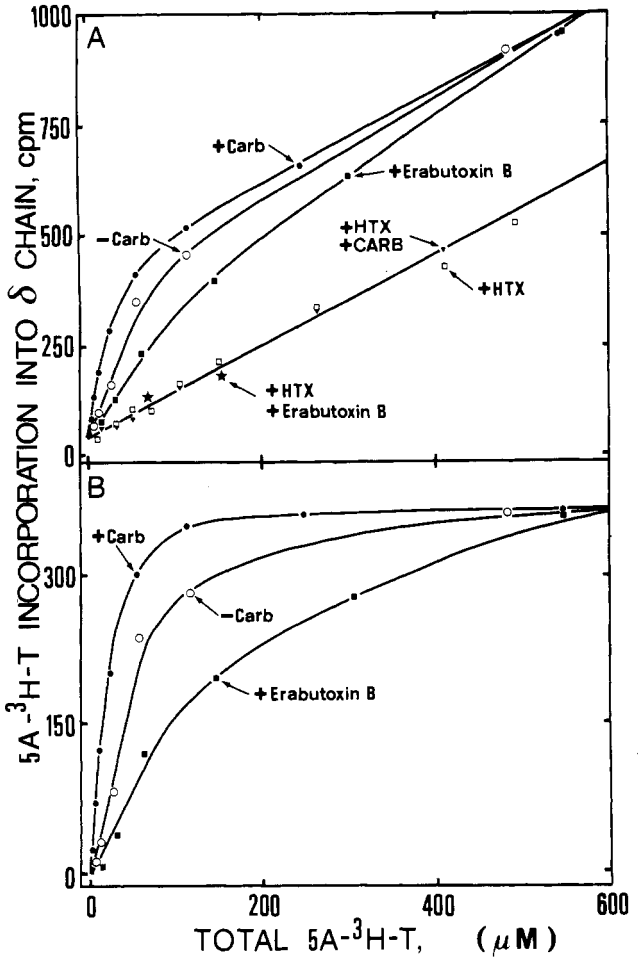


FIGURE 3: Incorporation of 5A[$^3$H]T into the $\delta$ subunit as a function of total 5A[$^3$H]T concentration. (A) Incorporation was measured under the following conditions: in the presence of 500 $\mu$M carbamoylcholine (●), in the absence of unlabeled effectors (○), in the presence of 5 $\mu$M erabutoxin B (■), in the presence of 500 $\mu$M histrionicotoxin (□), in the presence of 500 $\mu$M histrionicotoxin and 500 $\mu$M carbamoylcholine (▼), and in the presence of 5 $\mu$M erabutoxin B and 500 $\mu$M histrionicotoxin (★). (B) "Specific" incorporation calculated after subtracting incorporation in the presence of 500 $\mu$M histrionicotoxin from incorporation in its absence under the following conditions: 500 $\mu$M carbamoylcholine (●), no unlabeled effectors (○), and 5 $\mu$M erabutoxin B (■). The highest apparent affinity was found in the presence of carbamoylcholine (20 $\mu$M), with lower affinities found in the absence of effectors (60 $\mu$M) and in the presence of erabutoxin B (100 $\mu$M).

maximal labeling occurred at a total 5A[$^3$H]T concentration of 20–25 $\mu$M. Labeling in the absence of carbamoylcholine and in the presence of 10 $\mu$M erabutoxin B reached the same final level as that observed in the presence of carbamoylcholine. Half-maximum incorporation of 5A[$^3$H]T into the $\delta$ subunit took place at a higher concentration (60 $\mu$M) in the absence of carbamoylcholine than in its presence (20–25 $\mu$M) and a still higher concentration (100 $\mu$M) in the presence of erabutoxin B. This suggests that carbamoylcholine and erabutoxin B primarily modify, in opposite directions, the affinity of 5A[$^3$H]T for its binding site. In the presence of histrionicotoxin, a background level of radioactivity was found which increased linearly with 5A[$^3$H]T concentration. The same background was obtained when carbamoylcholine or erabutoxin B was included in addition to histrionicotoxin.

The incorporation of 5A[$^3$H]T into the *α subunit* in the *absence* of agonist (data not shown) took place with an apparent dissociation constant (35 $\mu$M) close to that of the $\delta$ chain labeling by 5A[$^3$H]T in the presence of agonist (20–25 $\mu$M). Under these conditions approximately twice as much label was recovered in the $\alpha$ subunit as that in the $\delta$ subunit, consistent with the 2:1 chain stoichiometry (Reynolds & Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980).

*Comparison of δ Subunit Labeling by 5A[$^3$H]T with the Reversible Binding of [$^3$H]Phenylcyclidine to AcChR-Rich Membrane Fragments.* So that the relationship between the site labeled by 5A[$^3$H]T on the $\delta$ subunit and the binding site for noncompetitive blockers could be established in more detail, the characteristics of 5A[$^3$H]T covalent labeling of the $\delta$ chain were compared to the reversible equilibrium binding of [$^3$H]phencyclidine to AcChR-rich membranes. Phencyclidine was selected because it binds to AcChR-rich membranes (Kloog et al., 1980; Albuquerque et al., 1980a,b; Eldefrawi et al., 1980b) in a manner similar to that of histrionicotoxin and interacts with this toxin in a competitive manner (Albuquerque et al., 1980a,b; Eldefrawi et al., 1980b) most likely at the same site. Also, like 5A[$^3$H]T, it covalently labels the $\delta$ chain of the AcChR after UV irradiation (Oswald & Changeux, 1981). Moreover, phencyclidine has been shown to act as a potent noncompetitive blocker of the electrophysiological response to acetylcholine both at the neuromuscular junction (Albuquerque et al., 1980b) and with isolated electroplaque preparation of *Electrophorus electricus* (Oswald & Changeux, 1981).

The equilibrium binding curves of [$^3$H]phencyclidine to AcChR-rich membranes are shown in Figure 4. In the absence of effector, half-maximal binding occurs at a [$^3$H]-phencyclidine concentration of 3 $\mu$M. In the presence of carbamoylcholine, the number of sites are equal to 0.5 of those for $\alpha$-[$^{125}$I]Bgt.[3] On the other hand, carbamoylcholine at saturation decreases the $K_D$ for phencyclidine to 0.9 $\mu$M while 5 $\mu$M erabutoxin B increases it to 5 $\mu$M. The same background level of binding was observed in the presence of a 50-fold excess of both unlabeled phencyclidine and histrionicotoxin and did not change further when erabutoxin B or carbamoylcholine was included in addition to histrionicotoxin. The resemblance of the equilibrium binding curves of phencyclidine to the AcChR-rich membranes with the labeling curves of the $\delta$ subunit of 5A[$^3$H]T is striking.

*Pharmacological Specificity of 5A[$^3$H]T Labeling and [$^3$H]Phencyclidine Binding and of Their Regulation by Cholinergic Ligands.* The cholinergic agonist, carbamoylcholine, and the antagonist, *d*-tubocurarine, have been reported to increase the high affinity binding of the aminated local anesthetic [$^{14}$C]meproadifen (Krodel et al., 1979) and of [$^3$H]perhydrohistrionicotoxin (Eldefrawi et al., 1980a; Elliott & Raftery, 1977, 1979). Figure 5 demonstrates that not only carbamoylcholine and *d*-tubocurarine but also decamethonium, hexamethonium, and flaxedil potentiate the labeling of the $\delta$ chain by 5A[$^3$H]T. In this case the labeling was studied at a fixed concentration of radioactive ligand (6 $\mu$M), and the concentrations of agonists and antagonists were varied. The $I_{50}$ values for the affinity increases were in the range of 0.5–1 $\mu$M for carbamoylcholine, decamethonium, and *d*-tubocurarine and 100–150 $\mu$M for hexamethonium and flaxedil. The agonists decamethonium and carbamoylcholine gave a slightly higher plateau level than the three antagonists. Similar results

[2] This estimate is based on control experiments with [$^3$H]MPTA-labeled AcChR which indicate that approximately 50% of the specifically and covalently bound [$^3$H]MPTA radioactivity added to the gel can be recovered after solubilization in the medium described under Materials and Methods. This technique assumes that recoveries for the $\delta$ chain, 5A[$^3$H]T, and the $\alpha$ chain, [$^3$H]MPTA, are similar.

[3] An additional binding site for [$^3$H]phencyclidine can be observed at concentrations greater than 40 $\mu$M (data not shown).

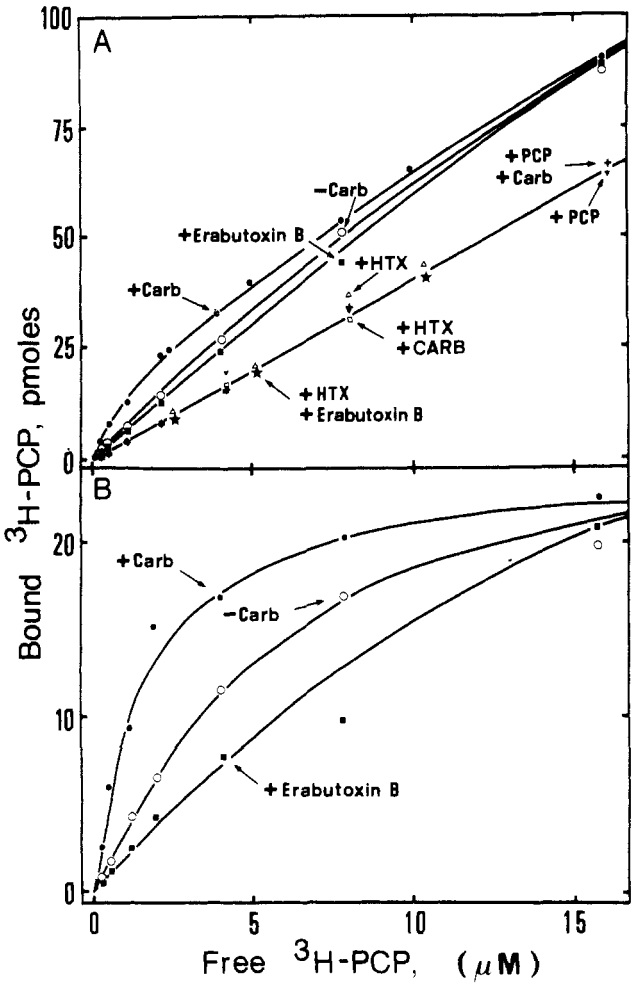


FIGURE 4: Reversible binding of [$^3$H]phencyclidine to AcChR-rich membranes as a function of free [$^3$H]phencyclidine concentration. (A) Binding was measured under the following conditions: in the presence of 100 μM carbamoylcholine (●), in the absence of unlabeled effectors (○), in the presence of 5 μM erabutoxin B (■), in the presence of 500 μM histrionicotoxin (△), in the presence of 500 μM histrionicotoxin and 100 μM carbamoylcholine (□), in the presence of 500 μM unlabeled phencyclidine (▼), in the presence of 500 μM unlabeled phencyclidine and 100 μM carbamoylcholine (+), and in the presence of 5 μM erabutoxin B and 500 μM histrionicotoxin (★). (B) "Specific" binding calculated after subtacting the binding observed in the presence of 500 μM histrionicotoxin from the total binding in its absence under the following conditions: 100 μM carbamoylcholine (●), no unlabeled effectors (○), and 5 μM erabutoxin B (■). The same number of sites were found in each case, but the affinity was higher in the presence of carbamoylcholine (0.9 μM) than in its absence (3 μM) and even lower in the presence of erabutoxin B (5 μM).

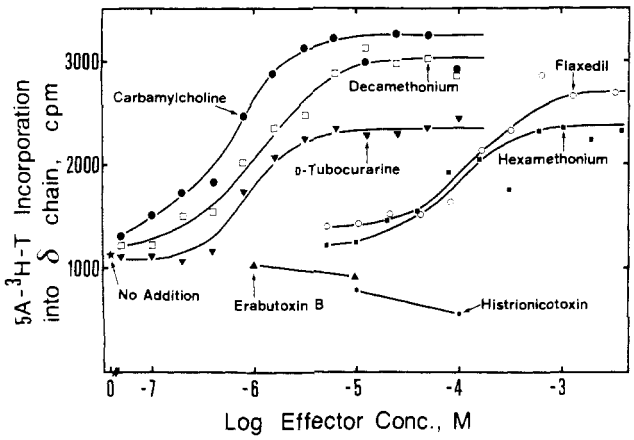


FIGURE 5: Effect of various effectors on the incorporation of 5A[$^3$H]T into the δ subunit. Incorporation was measured in the presence of carbamoylcholine (●), decamethonium (□), *d*-tubocurarine (▼), flaxedil (○), hexamethonium (■), erabutoxin B (▲), and histrionicotoxin (•), as well as in the absence of unlabeled effectors (★). The concentration of 5A[$^3$H]T was 5 μM, and all determinations were made in parallel on the same membrane preparation.

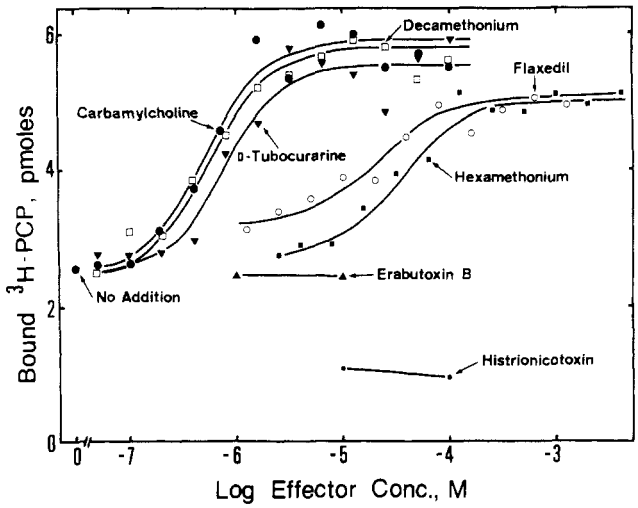


FIGURE 6: Effect of various effectors on the reversible binding of [$^3$H]phencyclidine to AcChR-rich membranes. Binding was measured in the presence of carbamoylcholine (●), decamethonium (□), *d*-tubocurarine (▼), flaxedil (○), hexamethonium (■), erabutoxin B (▲), and histrionicotoxin (•) as well as in the absence of unlabeled effectors (★). The concentration of [$^3$H]phencyclidine was 1 μM, and all determinations were made in parallel on the same membrane preparation.

were obtained for the pharmacological specificity of the potentiation of [$^3$H]phencyclidine binding (1 μM). As shown in Figure 6, the agonists, decamethonium, and carbamoylcholine, as well as the antagonists, hexamethonium, flaxedil, and *d*-tubocurarine, increased the affinity of the AcChR for [$^3$H]phencyclidine. The $I_{50}$ values were approximately the same as those found with 5A[$^3$H]T labeling of the δ chain for carbamoylcholine, decamethonium, and *d*-tubocurarine but were lower for flaxedil and hexamethonium (10–30 μM). Again the agonists gave a slightly higher plateau value than the antagonists. The affinity labeling reagent, MPTA, both covalently and noncovalently bound, showed similar potentiating effects.

The pharmacological specificity of the site labeled by 5A-[$^3$H]T on the δ subunit and of the binding of [$^3$H]phencyclidine to AcChR-rich membranes was investigated at a fixed concentration of radioactive ligand and in the presence of 100 μM carbamoylcholine. All the noncompetitive blockers tested (Figure 7) inhibited the labeling by 6 μM 5A[$^3$H]T, with $I_{50}$ values ranging from 1 mM for procaine to 800 μM for histrionicotoxin. Figure 8 shows a parallel set of experiments performed which measured the binding of [$^3$H]phencyclidine. The rank order (procaine < TEA < tetracaine < chlorpromazine ≈ trimethisoquin < dibucaine < histrionicotoxin) was the same as that found for 5A[$^3$H]T (except for TEA and procaine). Thus the specificity of 5A[$^3$H]T labeling and [$^3$H]phencyclidine binding appears similar and is that expected for the high-affinity binding site for noncompetitive blockers.

*Consequences of Prelabeling by 5AT on Subsequent 5A-[$^3$H]T Labeling and [$^3$H]Phencyclidine Binding.* If 5AT actually labels the high-affinity site for noncompetitive blockers, the covalent attachment of 5AT should interfere both with the subsequent labeling by 5A[$^3$H]T and with the reversible binding by a radioactive noncompetitive blocker like [$^3$H]phencyclidine.

So that this possibility could be tested, membrane fragments were first incubated with unlabeled 5AT, UV irradiated, and

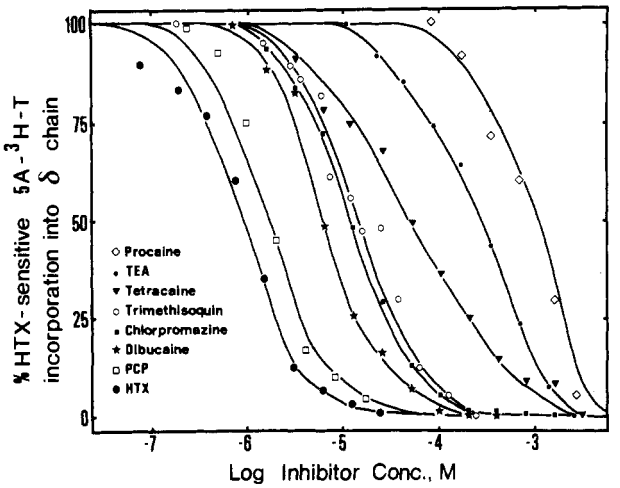


FIGURE 7: Inhibition of 5A[$^3$H]T incorporation into the δ chain by other noncompetitive blockers. Incorporation was measured in the presence of 100 μM carbamoylcholine and varying concentrations of the following agents: procaine (◇), TEA (•), tetracaine (▼), trimethisoquin (○), chlorpromazine (■), dibucaine (★), phencyclidine (□), and histrionicotoxin (●). A 5A[$^3$H]T concentration of 5 μM was used, and the data were normalized as described in the legend to Figure 8.

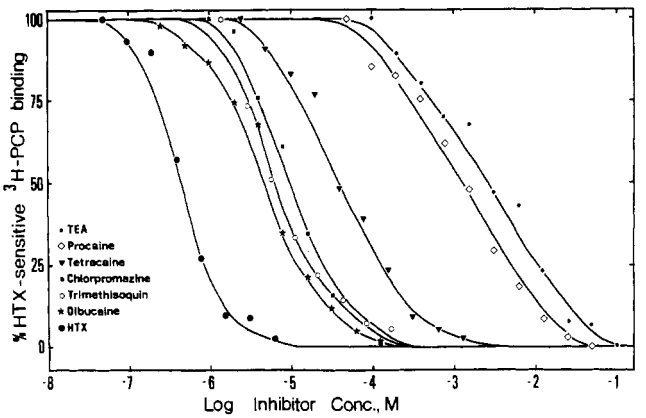


FIGURE 8: Inhibition of [$^3$H]phencyclidine binding by other noncompetitive blockers. Binding was measured in the presence of 100 μM carbamoylcholine and varying concentrations of the following agents: TEA (•), procaine (◇), tetracaine (▼), chlorpromazine (■), trimethisoquin (○), dibucaine (★), and histrionicotoxin (●). A concentration of 2 μM [$^3$H]phencyclidine was used. The data were normalized by taking the binding in the presence of 100 μM carbamoylcholine only as 100% and that in the presence of 100 μM carbamoylcholine and 100 μM histrionicotoxin as 0%.

then exposed to 5A[$^3$H]T and irradiated again. Such experiments are meaningful only if, after the first UV irradiation, the free reagent has been removed. A series of pilot experiments were carried out with radioactive trimethisoquin (no azido moiety) to test this point. They indicated that five cycles of centrifugation and resuspension in 3 mL of buffer over a period of 12 h removed essentially all the radioactivity from the pellet. The labeling of the δ chain by 5A[$^3$H]T was thus measured under these conditions after preincubation with increasing concentrations of unlabeled 5AT. Figure 9 shows that prior treatment by 5AT quantitatively inhibited subsequent labeling by 5A[$^3$H]T.

In the course of these experiments, as a control, the membrane fragments were UV irradiated in the presence of unlabeled trimethisoquin (no azido moiety) and then labeled with 5A[$^3$H]T. Unexpectedly, this treatment interfered with the subsequent labeling with 5A[$^3$H]T though to a smaller extent than after UV irradiation with the azido compound. As discussed in a separate paper (Oswald & Changeux, 1981),

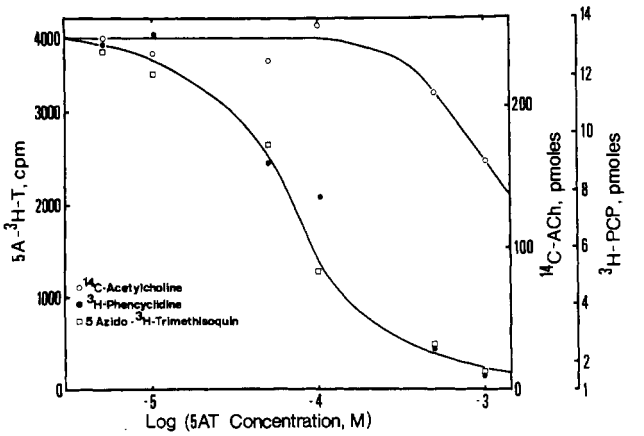


FIGURE 9: Binding of [$^{14}$C]AcCh (○) and [$^3$H]phencyclidine (●) and the incorporation of 5A[$^3$H]T (□) into the δ chain following prior treatment with unlabeled 5AT. Membranes were treated with varying concentrations of unlabeled 5AT, irradiated, washed to remove unreacted 5AT, and subjected to binding assays. The following concentrations were used: [$^{14}$C]AcCh, 0.4 μM; [$^3$H]phencyclidine, 1 μM; 5A[$^3$H]T, 5 μM.

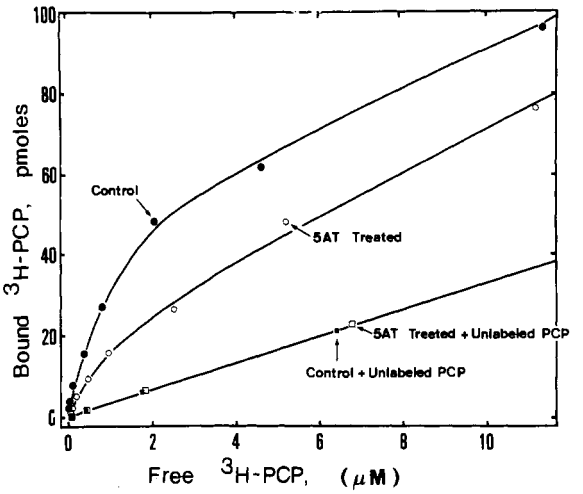


FIGURE 10: Binding of [$^3$H]phencyclidine to control (●) and 5AT-treated membranes (○) as a function of free [$^3$H]phencyclidine concentration. 5AT-treated membranes were incubated with 100 μM 5AT, irradiated, and washed. Control membranes were irradiated and washed. In some cases (□; ■), 100 μM unlabeled phencyclidine was added to the [$^3$H]phencyclidine incubation medium to control for nonspecific binding.

under these conditions, trimethisoquin established a covalent link with the δ chain though with a lower yield than in the case of 5AT.

The effect of pretreatment of the AcChR-rich membranes with 5AT on the reversible binding of [$^3$H]phencyclidine is shown in Figure 9 and 10. 5AT treatment reduced the number of [$^3$H]phencyclidine binding sites without significantly changing its affinity (Figure 10). In Figure 9 are compared the effects of 5AT pretreatment on subsequent [$^3$H]phencyclidine and [$^{14}$C]AcCh binding and 5A[$^3$H]T labeling. The decrease of [$^3$H]phencyclidine binding sites closely followed that of 5A[$^3$H]T and took place at a much lower range of concentrations than the observed decline of [$^{14}$C]AcCh binding. These data support the interpretations that (1) 5AT binds to the same site as phencyclidine and (2) this site is structurally distinct from the high-affinity AcCh binding sites inhibited by snake α-neurotoxins.

## Discussion

The significance of the four different, though homologous (Raftery et al., 1980), polypeptide chains that comprise the

AcChR protein has been debated during the past years. Affinity reagents which are analogues of AcCh labeled the $\alpha$ chain [for reviews, see Karlin (1980) and Changeux (1980)], but the roles of the $\beta$, $\gamma$, and $\delta$ chains, tightly associated with the $\alpha$ chain, were unknown. The present results show that the covalent labeling of the $\delta$ subunit by an azido derivative of trimethisoquin, 5A[$^3$H]T, is similar in several respects to the reversible equilibrium binding of the noncompetitive blocker [$^3$H]phencyclidine.

This resemblance is striking even though all of the kinetic parameters of 5A[$^3$H]T labeling have not yet been estimated in a quantitative manner. A series of noncompetitive blockers interfere with both 5A[$^3$H]T labeling and [$^3$H]phencyclidine equilibrium binding with the same rank order, they are submitted to the same regulation by cholinergic agonists and antagonists, and the number of binding sites are similar for both compounds. Thus, 5A[$^3$H]T labels the high-affinity binding site for noncompetitive blockers, and the $\delta$ subunit carries at least part of this site.

A significant labeling of the $\alpha$ chain by 5A[$^3$H]T also takes place in the same range of 5A[$^3$H]T concentration as the $\delta$ chain. Reversible binding of aminated local anesthetics to the AcCh binding sites has been reported (Weber & Changeux, 1974) but occurs, in general, in a range of concentrations higher than for the high-affinity site for noncompetitive blockers. Despite major differences in structural specificity, the AcCh binding site on the $\alpha$ chain and the site for noncompetitive blockers on the $\delta$ chain share common features. An attractive hypothesis (Changeux, 1980) is that these structural similarities are related to the sequence homologies noticed between the different chains of the receptor (Tzartos & Lindstrom, 1980; Raftery et al., 1980). In other words, the site for noncompetitive blockers on the $\delta$ chain would derive from, or even still be, a homologous AcCh binding site. The "endogenous ligand" for the site for noncompetitive blockers thus could be acetylcholine itself.

The AcChR in its membrane-bound and detergent-extracted forms (Heidmann et al., 1980) may undergo conformational transitions between distinct structural states which differ by their relative affinities for cholinergic ligands and noncompetitive blockers and by the state of opening of the ion channel. The rapid kinetic data available are accounted for by a minimal functional model which postulates four interconvertible conformations of the receptor: resting (R), active (A), intermediate (I), and desensitized (D) (Walker et al., 1981; Neubig & Cohen, 1980; Heidmann & Changeux, 1980). The ion channel is open only in the A state. In the absence of agonist, about 80% of the AcChR molecules are in the R conformation, and nearly all receptor molecules are in the D form after equilibration with an agonist (Heidmann & Changeux, 1979; Boyd & Cohen, 1980). The A and I states are present in significant quantities only transiently following the addition of agonists. In the present study, because of the relatively long periods of incubation, only the binding properties of the R and D states of the AcChR have been investigated. Since carbamoylcholine causes an increase of affinity of the AcChR for 5A[$^3$H]T and [$^3$H]phencyclidine, these two noncompetitive blockers not only bind preferentially to the D state of the receptor but also exhibit a significant affinity for their site in the R state. In other words, they behave as "nonexclusive" ligands (Rubin & Changeux, 1966) to both the R and D states. An allosteric increase of affinity of the noncompetitive blockers [$^{14}$C]meproadifen (Krodel et al., 1979), [$^3$H]trimethisoquin (Sobel et al., 1980), and [$^3$H]-perhydrohistrionicotoxin (Eldefrawi et al., 1980a; Cohen et al., 1980a) for their high-affinity site has been reported after equilibration with agonists. Consistent with the observations of Grünhagen & Changeux (1976) and Krodel et al. (1979), some competitive antagonists also cause an increase of affinity for 5A[$^3$H]T and [$^3$H]phencyclidine. These compounds may preferentially, though nonexclusively, bind to the D state, an interpretation consistent with the known in vivo acceleration of desensitization to agonists by "metaphilic" antagonists (Rang & Ritter, 1970).

The effect of snake $\alpha$-neurotoxins differs. In the presence of erabutoxin B, with or without carbamoylcholine, the affinity of 5A[$^3$H]T for the $\delta$ subunit and of [$^3$H]phencyclidine for the AcChR-rich membranes decreases. This effect is not due simply to a steric hindrance of the binding site for noncompetitive blockers by erabutoxin B because (1) the maximal level of 5A[$^3$H]T incorporation or [$^3$H]phencyclidine binding is the same as in the absence of erabutoxin B and (2) histrionicotoxin remains an effective blocker of the binding even in the presence of erabutoxin B. Alternative explanations for this phenomenon are the following: (1) [$^3$H]Phencyclidine and 5A[$^3$H]T interact with the AcCh sites in the absence of agonist and, as a consequence, increase the affinity of the site for noncompetitive blockers (but to a smaller extent as that observed with agonists). Erabutoxin B binding to the AcChR site blocks this effect by binding preferentially to the resting state. (2) As shown by Heidmann & Changeux (1979), the AcChR exists, in the absence of agonists, in an equilibrium between the R and D states. The affinity for 5A[$^3$H]T and [$^3$H]phencyclidine observed in the absence of effector is thus an average of the affinity for the R and D states. If one assumes that erabutoxin B binds preferentially to the R state, in its presence the fraction of AcChR in the D state would decrease and, as a consequence, the "average" affinity for noncompetitive blockers would decrease as well. The observation that 5A[$^3$H]T binds to the AcCh site on the $\alpha$ subunit is consistent with the first hypothesis. On the other hand, phencyclidine at the concentrations tested does not significantly interact with the $\alpha$-neurotoxin binding site (Oswald & Changeux, 1981; Albuquerque et al., 1980a; Kloog et al., 1980), rendering the first hypothesis less plausible, at least for phencyclidine.

In a final experiment designed to explore the relationships between the incorporation of 5A[$^3$H]T into the $\delta$ chain and the binding of [$^3$H]phencyclidine to the AcChR-rich membranes, prior labeling by 5AT was shown to decrease the number of phencyclidine binding sites without changing the affinity for the remaining ones. The loss of phencyclidine sites paralleled the loss of sites available for subsequent 5A[$^3$H]T labeling. These observations support the conclusion that 5A-[$^3$H]T and phencyclidine bind to the same site and that, in agreement with UV irradiation experiments (Oswald & Changeux, 1981), this site is, at least partially, carried by the $\delta$ chain.

A number of electrophysiological studies have suggested that local anesthetics (Adams, 1977; Neher & Steinbach, 1978), histrionicotoxin and perhydrohistrionicotoxin (Eldefrawi et al., 1980a), phencyclidine (Eldefrawi et al., 1980b; Albuquerque et al., 1980a,b), adamantine (Tsai et al., 1978), and TEA (Eldefrawi et al., 1977; Adler et al., 1979) may interact directly with the ion channel associated with the AcChR protein and, as a consequence, exert their noncompetitive inhibition. In most cases, the results were interpreted on the basis of the preferential binding of the noncompetitive blocker to the open channel (Neher & Steinbach, 1978), although mixed effects have been reported (Adler et al., 1979; Albuquerque et al., 1980a). Alternatively, the noncompetitive blocking of the

permeability response may result from the stabilization by the effector of a conformation of the receptor where the ion channel is shut but different from the resting state (i.e., the I or the D state). According to this last interpretation (Oswald et al., 1981), the binding site for noncompetitive blockers would not be part of the ion channel. An allosteric interaction would mediate the blocking of the ion channel by the noncompetitive effector.

Kinetic experiments with [$^3$H]perhydrohistrionicotoxin (Eldefrawi et al., 1980a) and [$^{14}$C]meproadifen (Cohen et al., 1980a), as well as preliminary studies with 5A[$^3$H]T (R. E. Oswald, unpublished experiments), suggest that noncompetitive blockers interact preferentially with a state existing transiently after the addition of agonists but before the stabilization of the high-affinity state. According to the four-state model of the AcChR, this could be either the A or I states. Reversible binding of noncompetitive blockers and covalent labeling by derivatives of noncompetitive blockers (such as 5A[$^3$H]T) in an adequate time scale might lead to a distinction between these alternatives and potentially to the identification of the subunit(s) and peptide sequences which comprise the ion channel.

Acknowledgments

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# Selective Labeling of the Hydrophobic Core of Membranes with 3-(Trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine, a Carbene-Generating Reagent†

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ABSTRACT: The synthesis of a new photoactivatable probe, 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine ([$^{125}$I]-TID), with a high specific radioactivity (10 Ci mmol$^{-1}$) is described. It was tested as a probe for the hydrophobic core of membranes. TID partitions strongly in favor of the lipid phase of membranes, and the photogenerated carbene labels intrinsic membrane proteins in a highly selective manner. This conclusion was reached from the distribution of radioactivity among the proteins of [$^{125}$I]TID-labeled human erythrocyte membranes. By far the most heavily labeled protein is band 3 [nomenclature of Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617] while the labeling of glycophorin is approximately 5 times less than that of band 3. There is little or no labeling of known extrinsic proteins.

The principal goal of hydrophobic membrane labeling is the identification of those domains of proteins that penetrate the lipid core. When those polypeptide segments which are in contact with the fatty acyl chains of the membrane lipids are selectively modified, this method represents a valuable complement to existing surface labeling techniques.

Most of the reagents which are in current use for hydrophobic membrane labeling are photoactivatable precursors of aryl nitrenes (Klip & Gitler, 1974; Bercovici & Gitler, 1978; Kahane & Gitler, 1978; Wells & Findlay, 1979a,b; Cerletti & Schatz, 1979) or of carbenes (Bayley & Knowles, 1978b; Gupta et al., 1979; Brunner & Richards, 1980). These precursors are hydrophobic or amphipathic and partition to a high extent into the lipid core of membranes. Unlike classical protein modifying reagents, nitrenes and carbenes are very reactive and exhibit less chemical selectivity. In model membrane systems prepared from saturated phospholipids, carbenes have been shown to react to some extent even with aliphatic residues. Therefore, carbenes are particularly attractive candidates for the chemical modification of the hydrophobic and chemically predominantly inert polypeptide side chains. Aryl nitrenes are less reactive than corresponding carbenes, and C–H insertion does not substantially, if at all, contribute to the overall labeling process (Bayley & Knowles, 1978a; Gupta et al., 1979; Brunner & Richards, 1980).

Nitrenes and carbenes are susceptible to intramolecular rearrangements which result in the transient appearance of second intermediates which not only are less reactive than nitrenes and carbenes but also may be more polar than the precursors. Since these species are assumed to be relatively long-lived (as compared to nitrenes and carbenes), the probability is increased that reactive amino acid residues are modified that are located on the surface area of proteins which faces the aqueous medium. That labeling of proteins may be dominated by group-specific reactions involving such intermediates has been proposed recently (Hu & Wisnieski, 1979).

[$^{3}$H]Adamantanyldiazirine generates a highly reactive carbene and labels intrinsic membrane proteins far more heavily than the extrinsic proteins (Goldman et al., 1979; Bayley & Knowles, 1980). As suggested by these investigators, the reaction of extrinsic proteins may well be reduced or even eliminated if reagents more hydrophobic than adamantanyldiazirine were available.

When [$^{3}$H]adamantanyldiazirine in native membranes is used the labeling of various proteins was found to occur predominantly in those polypeptide segments which presumably span the hydrophobic layer. In the case of glycophorin, however, the extent of labeling was reduced by the presence of glutathione in the water phase (Bayley & Knowles, 1980). This showed that some reaction did take place at sites which were accessible to the water-soluble thiol. As the authors suggested, these sites exposed to water might have reacted not with the carbene but with another intermediate (for example,

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