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# Conformational States of the Nicotinic Acetylcholine Receptor from *Torpedo californica* Induced by the Binding of Agonists, Antagonists, and Local Anesthetics. Equilibrium Measurements Using Tritium–Hydrogen Exchange†

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ABSTRACT: The tritium–hydrogen exchange kinetics of *Torpedo californica* AChR, in native membrane vesicles at pH 7.4 and 0 °C, have been analyzed in the presence of agonists, partial agonists, local anesthetics, and competitive antagonists. The agonists carbamylcholine (10 μM–1 mM) and suberyldicholine (10 μM) and the partial agonists decamethonium (25 μM and 1 mM) and hexamethonium (1 mM) have no effect on the exchange kinetics, although at lower concentration carbamylcholine may slightly accelerate exchange. Nondesensitizing local anesthetics do affect the exchange behavior, dependent on concentration. Procaine at 500 μM moderately retards exchange while procaine at 10 mM and tetracaine at 5 mM slightly accelerate exchange. The competitive antagonist α-bungarotoxin retards exchange significantly, as does *d*-tubocurarine although to a lesser extent. These results suggest that the resting and desensitized conformations of the AChR are very similar in overall solvent accessibility and that at lower concentrations noncompetitive blockers such as procaine may stabilize a less solvent-accessible state of the AChR. The competitive antagonists α-bungarotoxin and *d*-tubocurare also stabilize a dynamically restricted, less solvent-accessible conformation of the acetylcholine receptor, demonstrating that a large conformational change accompanies binding of these toxins. Any change in conformation which may accompany desensitization is very different from these effects.

Tritium–hydrogen exchange provides a powerful measure of any change in secondary structure or solvent accessibility that accompanies ligand binding, or other alteration in state of a macromolecule. In principle, the method is of very high resolution, once individual amides which undergo change are assigned. We report here the initial low-resolution use of this global probe applied to structural and functional changes of the acetylcholine receptor (AChR).[1] The nicotinic AChR

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is the primary mediator of signal transmission in a variety of neuronal and neuromuscular synapses. The receptor is a five-subunit ($\alpha_2\beta\gamma\delta$) transmembranous glycoprotein (290 000 daltons) whose behavior is modified by a broad range of naturally occurring and pharmacologically active molecules. The AChR assumes a minimum of four different conformations (although additional and intermediate states probably exist) called the resting, open, desensitized, and deeply desensitized states, depending on the type and extent of ligation [recent reviews include McCarthy et al. (1986), Stroud and Finer-Moore (1985), Popot and Changeux (1984), and Conti-Tronconi and Raftery (1982)].

Binding of agonists such as carbamylcholine (carb) and suberyldicholine (sub) to native AChR in the resting state induces transient openings of the ion channel [junctional receptors have an average open time of 1.0 ms (Dreyer et al., 1976)], followed by transition to the nonresponsive, desensitized state upon prolonged exposure to agonist (Katz & Thesleff, 1957). In vitro, this shift from the resting to the desensitized state parallels a shift from low affinity to high affinity for agonist, on a time scale of seconds to minutes, both in the native membrane (Weber et al., 1975; Weiland et al., 1977) and in reconstituted systems (Walker et al., 1982). The polymethonium derivatives occupy an ambiguous position. Decamethonium (deca) functions as a partial agonist, both inducing channel opening and acting, like some local anesthetics, as a voltage-dependent, noncompetitive blocker (Adams & Sakmann, 1978). Hexamethonium (hexa) has been observed to act as a competitive antagonist and also to induce the shift to high affinity for agonist (Weber et al., 1975; Quast et al., 1978), dependent upon the state of reduction of the AChR from *Electrophorus* (Prinz & Maelicke, 1984) and chick muscle (Rang & Ritter, 1971) but not from *Torpedo californica* (Walker et al., 1981). Competitive antagonists, such as $\alpha$-bungarotoxin (BgTx) and *d*-tubocurarine (curare), block agonist induction of channel opening by binding with very high affinity to some or all of the agonist binding site. Local anesthetics may function in at least two ways, both by accelerating the transition to and by stabilizing the duration of the desensitized state (Weiland et al., 1977; Blanchard et al., 1979a; Boyd & Cohen, 1984), or in the case of molecules such as the tertiary amines procaine (pro) and tetracaine (tet) by binding to the open form of the receptor and by some mechanism, perhaps involving direct occlusion of the channel itself, stopping ion flow (Koblin & Lester, 1979).

An impressive number of physical and chemical techniques have been directed at characterizing these conformational states of the AChR. While these methods measure to some extent the changes in conformation performed by the functioning AChR, few are sensitive to the global changes in conformational flexibility and the fluctuations in structure which allow proteins to shift from state to state. Analysis of proton exchange is particularly responsive to global alterations in structure, as it monitors changes both in the extent of hydrogen bonding and in solvent accessibility. The technique of tritium–hydrogen exchange, first developed by Linderstrom-Lang [reviewed by Englander and Kallenbach (1983)], allows the rates at which previously incorporated tritium exchanges with bulk solvent ($H_2O$) to be correlated with changes in secondary structure and conformational stability, which in turn reflect the overall conformation of the protein (Englander & Englander, 1972; Englander, 1975; Allewell, 1983). This technique has been used to measure the relatively gross conformational transitions of the histone subunits observed upon alteration of the ionic strength (McCarthy et al., 1984), as well as the more subtle allosteric transitions of hemoglobin (Englander & Mauel, 1972) and *Escherichia coli* aspartate transcarbamylase (Lennick & Allewell, 1981; Burz & Allewell, 1986). It has also proved a sensitive assay of conformational flexibility in such membrane proteins as rhodopsin (Osborne, 1976; Downer & Englander, 1977) and bacteriorhodopsin (Englander & Englander, 1977; Konishi & Packer, 1977).

We report here on the structural transitions of the AChR as detected by its tritium–hydrogen exchange behavior in its native membrane, in the presence of the agonists carbamylcholine and suberyldicholine, the partial agonists decamethonium and hexamethonium, the competitive antagonists $\alpha$-bungarotoxin and *d*-tubocurarine, and the local anesthetics procaine and tetracaine.

## Materials and Methods

*Chemicals.* Procaine hydrochloride, tetracaine hydrochloride, and decamethonium were purchased from ICN Pharmaceuticals, hexamethonium and carbamylcholine were purchased from Sigma, suberyldicholine dichloride was purchased from Aldrich, and *d*-tubocurarine was purchased from Calbiochem.

*Purification of $\alpha$-Bungarotoxin.* $\alpha$-Bungarotoxin was purified by using the protocols of Mebs et al. (1972) and Spencer (1978) from *Bungarus multicinctus* venom purchased from the Miami Serpentarium. Toxin was stored refrigerated as a lyophilized powder. Protein concentration was determined assuming $\epsilon = 10\,500$ (Blanchard et al., 1979b) and a molecular weight of 8100.

*Purification of Acetylcholine Receptors.* Purified AChR membrane vesicles were prepared from frozen *Torpedo californica* electric organs according to a modification of the procedures of Klymkowsky et al. (1980) and Kistler and Stroud (1981). Live fish were purchased from Marinus or the Bodega Bay Marine Station of the University of California. Sixty grams of dice-sized electric organ fragments was homogenized in 120 mL of chilled homogenization buffer (400 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM iodoacetamide, 10 mM PMSF in EtOH, 0.01% $NaN_3$, and 10 mM $NaH_2PO_4$, pH 7.4, filtered through a 0.2-$\mu$m pore size Nucleopore filter) in a Virtis Model 23 homogenizer at setting 10 for 4 min. The crude homogenate was centrifuged for 15 min at 6500 rpm in an SS-34 rotor, and the resulting supernatant was collected through eight layers of cheesecloth. Typically, two 60-g batches of tissue were treated as described above and combined at this step. The filtered supernatant was centrifuged for 55 min at 19 500 rpm in an SS-34 rotor, and the pelleted membrane fractions were suspended in K buffer (1 mM EDTA, 1 mM EGTA, and 10 mM PMSF in EtOH, 0.01% $NaN_3$, and 10 mM $NaH_2PO_4$, pH 7.8, filtered through a 0.2-$\mu$m pore size Nucleopore filter). The membrane suspension was resubjected to the low-speed/high-speed centrifugation cycle as described above and resuspended in distilled water. The pH was adjusted to 11.0, and the membrane solution was kept for 1 h at room temperature to allow for the alkaline extraction of extrinsic membrane proteins (Neubig et al., 1979). The membranes were pelleted by high-speed centrifugation and resuspended in K buffer containing 30% sucrose. Step gradients in K buffer (3 mL of K buffer/5.6 mL of membrane suspension in 30% sucrose/4.5 mL of 35% sucrose/1.9 mL of 50% sucrose) were poured in two 25 × 38 mm Beckman Quick-seal tubes, and

[1] Abbreviations: AChR, acetylcholine receptor; BgTx, $\alpha$-bungarotoxin; carb, carbamylcholine; sub, suberyldicholine; deca, decamethonium; hexa, hexamethonium; pro, procaine; tet, tetracaine; curare, *d*-tubocurarine; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PySa, pyrene-1-sulfonyl azide.

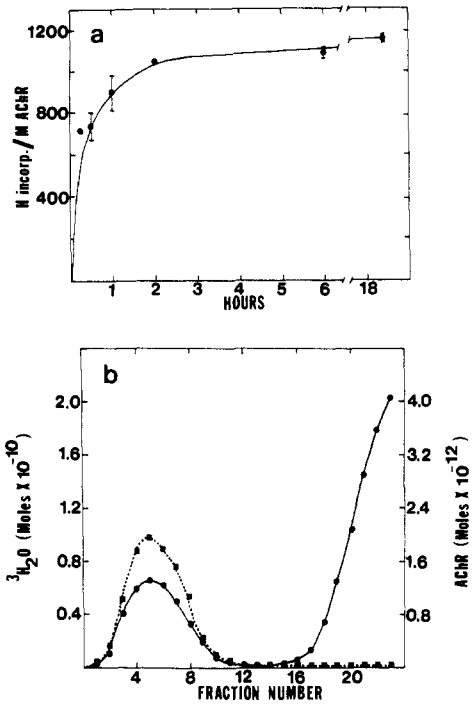


FIGURE 1: Analysis of experimental conditions. (a) Extent of labeled H incorporation at 22 °C with time. AChR, 1 mg/mL in K buffer, was incubated with 20 mCi of THO. Aliquots were removed at the times indicated, and free $^3H$ was separated from bound $^3H$ by using the two-column method of Englander and Englander (1972). (b) Separation of free and bound $^3H$ by gel filtration. AChR, 1 mg/mL in K buffer, was trace-labeled with [$^{14}$C]AChR and labeled with 20 mCi of THO at 22 °C for 18 h. Two drops of glycerol were added to the AChR/THO solution which was then applied to an SP-Sephadex G-25 column. Two-drop fractions were collected, and the amount of $^3H$ + $^{14}C$ present in each fraction was determined by liquid scintillation counting. (■) AChR; (●) $^3H_2O$.

the gradients were centrifuged for 60 min at 45 000 rpm in a VTi-50 reorienting rotor. Fractions containing 30–35% sucrose (reproducibly containing the peak of purified AChR membranes) were collected and assayed for purity by SDS gel electrophoresis and $^{125}$I-BgTx binding as described by Klymkowsky and Stroud (1979). The AChR constituted 80–90% of the protein component of the membrane vesicles (6.3–6.8 nM BgTx bound/mg of protein). AChR preparations showed, at both 4 and 22 °C, ion fluxing behavior typical of native AChR vesicle suspensions, using the $^{22}$Na flux assay of Walker et al. (1982). The phospholipid content of a typical membrane preparation was similar to that observed by other investigators (Gonzalez-Ros et al., 1982), with a molar phospholipid to AChR ratio of (174 ± 0.5)/1, assuming 7 mol of phosphate bound per mole of receptor. Phosphate was determined following the procedure of Ames (1966).

*Tritium–Hydrogen Exchange Experiments.* These experiments were performed according to the method of Englander and Englander (1972) as described earlier (McCarthy et al., 1984) using some of the modifications for membrane proteins suggested by Downer and Englander (1977). Typically, a 1 mg/mL solution of membrane-bound AChR was incubated for 18 h at room temperature (22–23 °C) with 20 mCi of THO. There was no proteolysis of AChR due to these incubation conditions, as shown by SDS gel electrophoresis. Under these conditions, the protein–lipid solution was maximally labeled, with essentially no further incorporation of tritium observed after 4 h of incubation, as seen in Figure 1a. Extension of the incubation period up to 92 h did not increase the level of tritiation. The exchange-out process was initiated by applying the tritiated protein sample atop an iced, 0.9 × 8 cm C25 SP-Sephadex column and separating the excess THO from the labeled protein under mild pressure (~3 psi) generated by a hand pump. As seen in Figure 1b, this method separated free THO from tritiated AChR quite effectively. The tritiated receptor was collected in a chilled test tube, and this exchange-out solution was kept at 0 °C for the remainder of the experiment. In experiments where the effects of bound ligands upon the exchange behavior of the AChR were studied, the AChR vesicle solution was collected into the ligand of choice upon elution from the first column and brought up to volume. All experiments were performed in 10 mM $NaH_2PO_4$, 1 mM EDTA, and 1 mM EGTA, pH 7.8.

Aliquots of the exchange-out solution were taken at various times, and the protein was separated from the tritium which had subsequently exchanged-out by passage over a second SP-Sephadex column. Fractions were collected and brought up to 200 μL in $H_2O$, and the amounts of tritium and protein present were determined in a liquid scintillation counter using 5 mL of Aquasol (New England Nuclear) as scintillant. Protein concentration was measured using trace amounts (~5%) of $^{14}$C-labeled AChR vesicles present in the exchange-out mixture, as described by Lennick and Allewell (1981). $^{14}$C-Labeled AChR vesicles were generated by labeling the receptor with H$^{14}$CHO, followed by $NaB_3HCN$ reduction (Jentoft & Dearborn, 1979). The ratio of $^{14}$C cpm/protein concentration was determined for each exchange-in solution. Protein concentration was determined according to the method devised by Markwell et al. (1978) for membrane proteins. The $^{14}$C-labeled receptor vesicles coeluted perfectly with bulk, unlabeled AChR vesicles. Corrections for crossover between the $^3H$ and $^{14}C$ channels in the liquid scintillation counter were performed according to Kobayashi and Maudsley (1974).

Analysis of the data was performed, following the approach of Englander and Englander (1972), by using the nonlinear least-squares program of Bevington (1969) fit to eq 1 assuming

$$H_{rem} = \sum N_i e^{-k_i t} \quad (1)$$

two or more kinetic classes, where $N_i$ is the number of exchangeable groups in the $i$th class, $K_i$ is their rate constant, $t$ is time in hours, and $H_{rem}$ is the number of exchanged groups at any one time. Data are reported as $H_{rem}$ (moles of H bound per AChR) rather than moles of tritium following a correction for the different rates of exchange for these isotopes from peptide amides (Englander & Englander, 1972).

## Results

*Characterization of Resting State.* At 4.7 min after the initiation of exchange-out (the earliest time observed, using the two-column separation method), there are 1200 labeled hydrogens remaining per AChR, in the resting state. As shown in Figure 2, after 8 h at 0 °C, only about 520 labeled hydrogens remain specifically bound to the AChR. This exchange-out behavior is most simply ascribed to two kinetic classes (although more undoubtedly exist) denoted fast and slow. When fit to eq 1, the size and rate of exchange of each kinetic class can be quantified, as shown in Table I. Including more than two kinetic classes or changing the number of iterations did not improve the quality of these fits. The variance in some of the parameters is somewhat high, particularly for the fast class, but these variance values are no higher than those reported in earlier tritium–hydrogen exchange studies (Lennick & Allewell, 1981; McCarthy et al., 1984), when errors are reported at all.

*Effects of Agonists.* Upon binding the agonist carb, the AChR is transiently activated and then enters the desensitized state. As seen in Figure 2, however, the addition of carb at

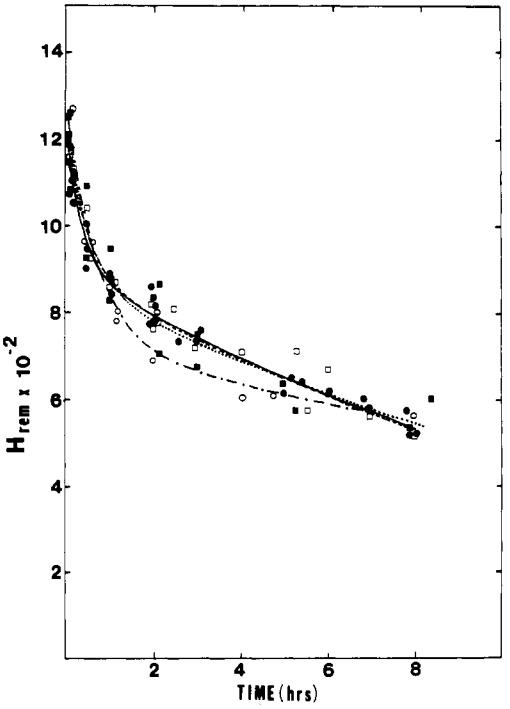


FIGURE 2: Effects of carb on the tritium–hydrogen exchange kinetics of the AChR. Tritiated AChR vesicles were collected into carb after separation of free and bound $^3H$ by filtration on the first column. Aliquots of the AChR/carb solution were applied to a second column, at the times indicated, and $^3H$ which had subsequently exchanged-out from the AChR was separated from bound $^3H$ by gel filtration. (●—●) No ligands present; (○–·–○) 10 μM carb; (□- - -□) 100 μM carb; (■···■) 1 mM carb. Ligand concentrations listed are the final concentration in the carb/AChR solutions. All curves were calculated by using the values for the parameters of eq 1 listed in Table I.

Table I

| exptl conditions | $N_1$ | $k_1$ ($h^{-1}$) | $N_2$ | $K_2$ ($h^{-1}$) |
|---|---|---|---|---|
| no ligands present | 312 (155)[a] | 2.58 (1.45) | 906 (81) | 0.07 (0.02) |
| 10 μM carb | 578 (242) | 1.42 (0.99) | 733 (93) | 0.04 (0.04) |
| 100 μM carb | 329 (182) | 2.09 (2.25) | 901 (68) | 0.07 (0.04) |
| 1 mM carb | 411 (172) | 1.77 (1.64) | 848 (75) | 0.06 (0.03) |
| 10 μM sub | 448 (227) | 1.32 (1.30) | 850 (99) | 0.03 (0.03) |
| 25 μM + 1 mM deca | 483 (187) | 1.98 (1.63) | 893 (78) | 0.06 (0.03) |
| 1 mM hexa | 476 (169) | 0.80 (0.60) | 748 (77) | 0.04 (0.03) |
| 0.5 mM pro | 320 (160) | 1.21 (1.26) | 981 (81) | 0.07 (0.03) |
| 10 mM pro | 284 (174) | 1.91 (2.05) | 852 (65) | 0.07 (0.02) |
| 5 mM tet | 398 (191) | 2.08 (1.91) | 811 (71) | 0.06 (0.02) |
| 0.5 mM curare | 394 (245) | 2.54 (2.83) | 1010 (88) | 0.06 (0.02) |
| 120 μM BgTx | 493 (53) | 0.72 (0.17) | 966 (27) | 0.03 (0.01) |

[a] Numbers in parentheses = variance.

higher concentrations did not significantly alter the exchange behavior of the AChR. This response, or lack of one, is not dependent upon agonist concentration greater than 100 μM, as varying the final carb concentration between 0.1 and 1.0 mM, and even up to 10 mM carb (data not shown), yielded largely indistinguishable exchange-out curves. The slightly variant behavior of AChR in the presence of 10 μM carb could conceivably be a genuine response induced by low concentrations of agonists, perhaps even channel opening. If so, this would indicate that the exchange behavior of approximately 50 exchangeable groups is accelerated upon transition to the open state. Another agonist analogue, sub at 10 μM, slightly retarded exchange from the slow class (Figure 3), but this effect may not be significant.

The partial agonists/antagonists hexa (1 mM) and deca (25 μM and 1 mM), like carb and sub, did not modify the exchange-out behavior of the AChR relative to the resting state (Figure 3). While there is some indication that deca slightly retards exchange-out at the earliest stages, it is not significantly above the experimental error, as reflected in the variance of the fits in Table I. Also, as a number of investigators have found different roles for hexa (agonist, partial agonist, antagonist), it is worth pointing out that in our experiments hexa acted in the same manner as carb, sub, and deca, suggesting that its desensitizing activity was predominant.

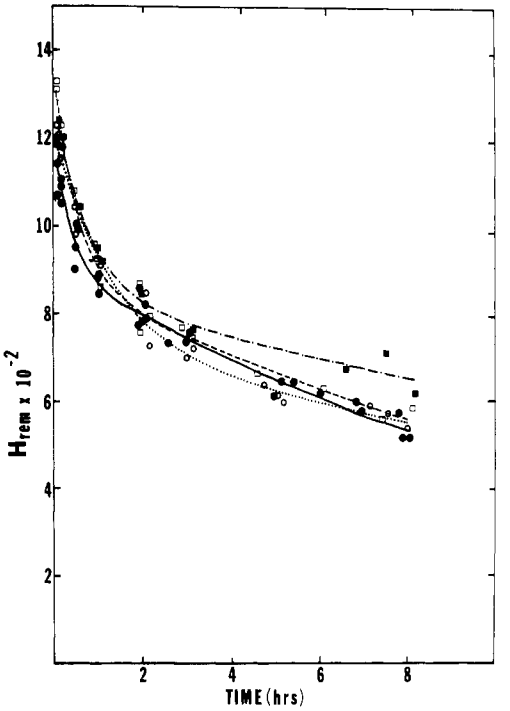


FIGURE 3: Effects of agonists and partial agonists on the tritium–hydrogen exchange kinetics of AChR. Experimental protocols are described in the legend to Figure 2 and under Materials and Methods. (●—●) No ligands present; (■–·–■) 10 μM sub; (□- - -□) 25 μM and 1 mM deca; (○···○) 1 mM hexa.

No significant alteration of exchange behavior at greater than 20 μM agonist concentration was observed for any of the agonists and partial agonists. This implies that the desensitized state of the AChR, induced by long-term exposure to agonists and partial agonists in equilibrium measurements such as these, is identical in overall conformation to the resting state, as measured by tritium–hydrogen exchange kinetics.

*Effects of Local Anesthetics.* The responses of membrane-bound AChR to the binding of the local anesthetics pro and tet are described in Figure 4. At a final concentration of 0.5 mM, pro induces a small but probably significant retardation of exchange. While exchange from the fast class is slowed mildly, the rate of exchange from the slow class is not affected, although the size of the slow class increases approximately 8%. However, increasing the pro concentration 20-fold to 10 mM, or adding 5 mM tet, produced a small acceleration of exchange. As with pro at 0.5 mM, it is the size of the slow class which is affected, not the exchange rate. This decrease in the number of exchanging groups is small (3–8%) but reproducible and may reflect the detergent-like solubilizing effect observed with high concentrations of amphiphilic local anesthetics (Seeman, 1972).

*Effects of Antagonists.* In the presence of excess BgTx, the exchange kinetics of the AChR are substantially altered, as seen in Figure 5. The initial, rapid exchange is slowed to the extent that there is a 20% increase in the number of groups whose exchange behavior is detectable, and the exchange rates

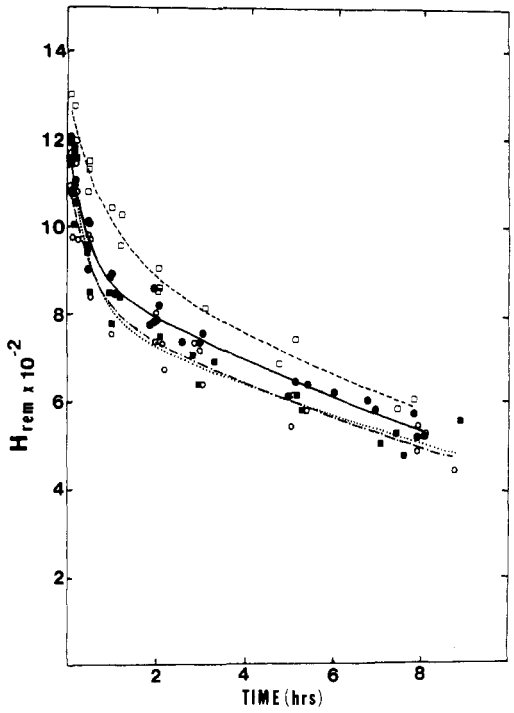


FIGURE 4: Effects of pro and tet on the tritium–hydrogen exchange kinetics of the AChR. Experimental protocols are described in the legend to Figure 2 and under Materials and Methods. (●—●) No ligands present; (□- - -□) 500 μM pro; (○–·–○) 10 mM pro; (■···■) 5 mM tet.

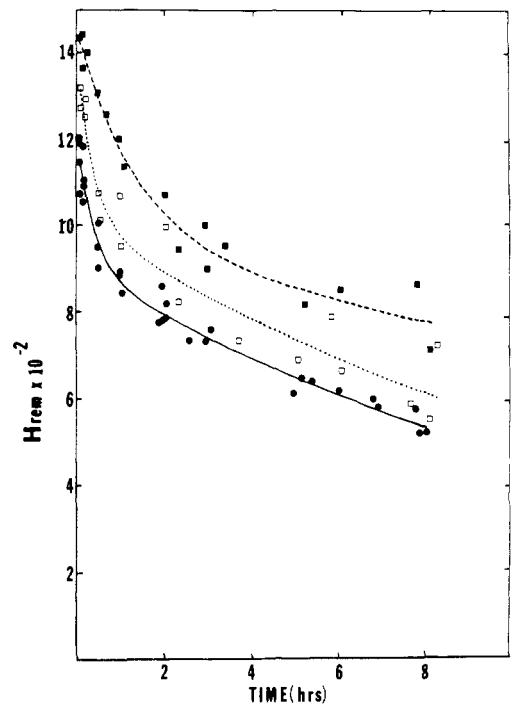


FIGURE 5: Effects of competitive antagonists on the tritium–hydrogen exchange kinetics of the AChR. Experimental protocols are described in the legend to Figure 2 and under Materials and Methods. (●—●) No ligands present; (■- - -■) 120 μM BgTx; (□···□) 500 μM curare.

of both the fast and slow classes are retarded. To test whether this effect is the result of a specific conformational change in the receptor (either induction of additional hydrogen-bonded structure or the damping of breathing modes) or merely due to reduced solvent accessibility upon "blanketing" the AChR with a relatively large ($M_r$ 8100) protein, we studied the effects of curare, a much smaller ($M_r$ 612) molecule, which should act as a competitive antagonist under our experimental conditions. As shown in Figure 5, curare does slow exchange-out from the receptor, but to a lesser extent. This implies that competitive antagonists do induce a conformational change in the AChR upon binding. The intermediate effect of curare may be due to its partial agonnist or local anesthetic behavior, or may indicate that some of the retardation of exchange observed with BgTx is due to a simple steric restriction of solvent accessibility.

## Discussion

The technique of tritium–hydrogen exchange is particularly sensitive to the degree of hydrogen bond formation and to the accessibility of solvent to the interior of a protein, which in turn reflects the overall conformation of the protein, in terms of both the degree of secondary structure and the compactness of folding, and of conformational dynamics. In these studies, we have used this technique to measure the extent of the structural transitions described by the functioning AChR in response to the binding of a variety of ligands.

*Resting State AChR.* The initial value of $H_{rem}$ (the number of labeled protons remaining some 4.5 min after initiation of exchange-out) for AChR largely in the resting state is 1200. This number is equal to 55% of the 2200 AChR peptide amide hydrogens (subtracting proline residues and amino termini) which we can expect to label and is essentially a measure of those amide protons which are involved in hydrogen bonding structure. Those amide protons which are not hydrogen bonded are presumed to exchange rapidly and account for the other 1000 peptide amides whose exchange is too rapid for detection by tritium–hydrogen exchange. This estimate of 55% of the number of amides which are hydrogen bonded agrees well with spectral assessments of the total degree of AChR secondary structure in which amides are hydrogen bonded. Circular dichroism studies of *Torpedo nobiliani* AChR predicted a structure composed of 34% α-helix and 29% β-structure including turns (63% total) (Moore et al., 1974), and recent studies with *T. californica* AChR indicated a structure that is about 15% α-helix and 50% β-sheet (65% total) (Mielke et al., 1986). Similarly, resonance Raman analysis of AChR from *Torpedo marmorata* suggested a structure which was 25% α-helix (plus 14% disordered α-helical ends) and 34% β-sheet (73% total) (Chang et al., 1983). The secondary structure prediction of Finer-Moore and Stroud (1984) suggested a structure which was 44% α-helix and 27% β-sheet (71% total).

Our hydrogen exchange studies indicate slightly smaller values for secondary structure, as expected, than the above spectral and predictive studies, as our first determinations were made 4–5 min after the initiation of exchange-out, and some of the labeled hydrogens involved in weakly hydrogen-bonded structure would have already been lost. Also, as clearly demonstrated by earlier work (Osborne, 1976; Downer & Englander, 1977; Englander et al., 1982), specific quantitation of the extent of secondary structure in membrane proteins based on tritium–hydrogen exchange experiments should be approached with caution, as exchange from side chains buried in the bilayer may add to the overall exchange-out profile.

*Effects of Agonists.* As indicated in Figures 2 and 3, there is little alteration in the exchange behavior of the AChR upon incubation with agonists. Since these are equilibrium measurements, we are not able to describe the conformational events which occur upon channel opening and closure. The various agonist analogues used, instead, drive the receptor into the desensitized state, and it is these two states we compare in the presence and absence of larger concentrations of agonists. The overall solvent accessibility to tritium–hydrogen

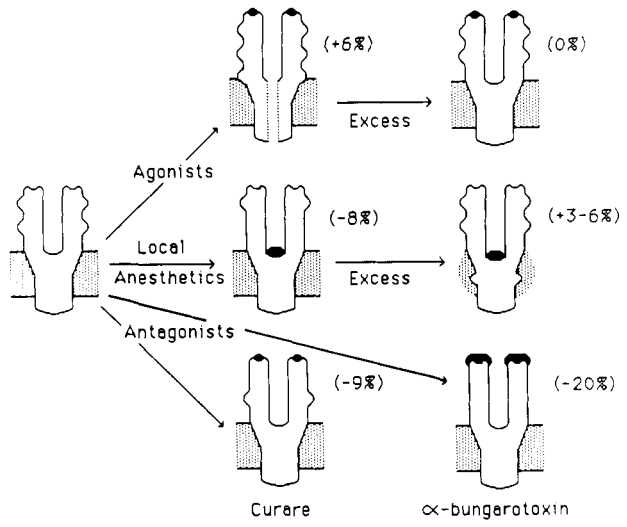


FIGURE 6: Model of the conformational effects of ligand binding to the AChR. Resting state AChR is indicated on the left. The numbers in parentheses approximate the magnitude and type (acceleration or retardation) of effect the binding of the indicated ligands have on the global conformation of the AChR. The sites of ligand binding are only best estimates.

exchange of the AChR in the resting and desensitized states is identical. If, despite the strongly desensitizing conditions, excursions to the open form occurred during our measurements [cf. Sakmann et al. (1980)], their effect was not detectable, suggesting either that their occurrence was very rare and brief or that the conformational transitions involved in channel opening are small. Consistent with the second possibility, it is possible that the ion channel remains water-filled even in the closed state, as suggested by the presence of terbium binding sites within the bilayer-spanning region of the AChR (Fairclough et al., 1986), and the dense staining of the channel by uranyl ions, seen in the electron microscope (Kistler et al., 1982), which would certainly diminish the measurable difference between the open and closed forms of the AChR in a tritium–hydrogen exchange experiment.

A model is suggested by these tritium–hydrogen exchange experiments which is paralleled by earlier work. First, the membrane-spanning regions of the AChR are not responsive to conformational changes occurring upon agonist binding, at least not between the resting and desensitized states (Gonzalez-Ros et al., 1983; Giradaut et al., 1985). Second, while local changes in conformation are detected in the hydrophilic portions of the AChR, they are often opposed in effect (i.e., an increase or decrease in fluorescence or chemical reactivity) depending upon the specific assay [cf. Bonner et al. (1976), Dunn et al. (1980), Weiland et al. (1979), Oswald and Changeux (1981), Witzemann and Raftery (1978), and Otero and Hamilton (1984)]. This suggests that there is not a large shift to a dramatically different conformation in the presence of agonists such as is observed with bovine histones upon shifting the ionic strength (McCarthy et al., 1984), but rather small compensatory changes which differ in local conformation. Third, those techniques which appear able to sample some of the open form of the receptor such as release of bound ions (Chang & Neumann, 1976; Rubsanem et al., 1978), or rapid labeling techniques (Heidmann & Changeux, 1984; Muhn et al., 1984a,b), suggest a less compact, more solvent-exposed structure relative to the resting and desensitized forms of the AChR. This model is schematized on the top of Figure 6.

In our tritium–hydrogen exchange experiments, we cannot measure the open state, and small, opposed changes in the extramembranous regions combined with no changes in the membrane-spanning regions would be expected to yield indistinguishable exchange behavior for both the resting and desensitized AChR. The slow rates and relatively small effects on overall receptor conformation of desensitization suggest that this conformational transition, at least the slow form, may involve *cis–trans*-proline isomerization(s). For example, proline isomerization rates ranging from 1 to 3 min, depending on the state of the protein, have been identified for a specific proline residue in RNase (Lin & Brandts, 1983), which is on the same time scale as slow desensitization. Good candidates for proline residues which may be involved in desensitization are the two conserved prolines found near the paired cysteines implicated in the agonist binding site (Kao et al., 1984). The sequence CCPXTP is conserved in all nicotinic AChR $\alpha$-subunits sequenced thus far (Stroud & Finer-Moore, 1985), and one or both of these prolines may act as a trigger for desensitization.

*Effects of Procaine and Tetracaine.* Interpretation of the effects of pro and tet is more clear-cut, although their local anesthetic behavior upon the AChR is perhaps not as well-defined as it is with the sodium channel (Ohki et al., 1978). While many of the amine local anesthetics enhance and accelerate the transition to the high-affinity desensitized form of the AChR, pro has little or no effect, and tet actually inhibits the shift to the desensitized state in vitro (Blanchard et al., 1979a; Boyd & Cohen, 1984), although Weiland et al. (1977) did see an enhancement of the carb-induced transition in the presence of 200–500 $\mu$M pro and tet. However, in electrophysiological experiments, pro and tet function as noncompetitive inhibitors which act specifically to block the open form of the AChR in a voltage-dependent manner in both frog neuromuscular junction (Adams, 1977) and *Electrophorus* electroplaque (Koblin & Lester, 1979), classifying these local anesthetics as noncompetitive blockers and not desensitizing agents. Most biochemical assays detect little effect of these ligands in studies of AChR conformation. However, Muhn et al. (1984b) did see inhibition of TPMP labeling of $\beta$- and $\delta$-subunits in the presence of 100 $\mu$M pro and tet, and pro at micromolar concentrations caused an increase in the fluorescence of noncovalently bound ethidium in *T. californica* AChR, while tet at all concentrations and pro at greater than millimolar concentration decreased ethidium fluorescence (Schimerlik et al., 1979). The decrease in ethidium fluorescence observed at high concentrations of pro might reflect dissociation of ethidium. The covalently attached hydrophobic probe PySa showed no change upon addition of 1 $\mu$M tet, but fluorescence lifetimes decreased to the same extent in the presence of 1 mM tet or upon solubilization in 0.1% Triton-X-100 (Gonzalez-Ros et al., 1983). These results are consistent with our results. First, neither pro nor tet shifts the AChR to the desensitized state, and the exchange behavior of the AChR in their presence is different from that in the presence of agonists. Instead, pro at 500 $\mu$M may be acting as a noncompetitive blocker, stabilizing a different conformation of the AChR as indicated by the retardation of exchange in our experiments and the enhancement of ethidium fluorescence (Schimerlik et al., 1979) and inhibition of TPMP labeling (Muhn et al., 1984b) with micromolar pro observed by other workers. At concentrations above millimolar, pro and tet may act to solubilize the membrane-bound AChR, as suggested by their slight acceleration of exchange and by the observation that 1 mM tet or 0.1% Triton X-100 (but not 1 $\mu$M tet) reduced the fluorescence lifetime of PySa (Gonzalez-Ros et al., 1983). Solubilization by high concentrations

of amphiphilic local anesthetics has been observed in other systems as well (Seeman, 1972). The observation that noncompetitive blockers retard exchange at lower concentrations correlates with their proposed mechanism of action, suggesting that procaine may be acting to exlude solvent from some or all of the channel region in its role as an open channel blocker. The effects of low and high concentrations of local anesthetics are schematized in Figure 6.

*Effects of Antagonists.* Our experiments with BgTx and curare suggest that competitive inhibitors modify the conformation of the AChR more than any other ligand studied here. BgTx retards exchange-out significantly. In most other studies, BgTx and curare are seen to have similar effects on AChR conformation. This probably reflects their common binding site, as their binding is competitively inhibited by agonists and blocked by covalent modifiers such as MBTA ([4-(*n*-maleimido)benzyl]trimethylammonium iodide) (Damle & Karlin, 1978) and bromoacetylcholine (Damle et al., 1978). Neither affected the fluorescence lifetimes of the hydrophobic probe PySa (Gonzalez-Ros et al., 1983) although BgTx did enhance quenching by nitromethane while carb diminished it, nor did curare or *Naja naja* $\alpha$-toxin alter the fluorescence of bound quinacrine (Grunhagen & Changeux, 1976). Thus, the local changes assayed by these probes did not detect the change in conformation induced by these antagonists. Some assays are sensitive to the conformational change observed upon antagonist binding; however, BgTx binding caused the uptake of four to six Ca ions in *T. californica* AChR (Chang & Neumann, 1976), inhibited [$^3$H]ethidium binding by the $\alpha$-subunit (Witzemann & Raftery, 1978), and displaced bound ethidium (Schimerlik et al., 1979), and *Naja naja* $\alpha$-toxin was found to protect all four subunits, to varying degrees, to labeling with [(4-azido-2-nitro)benzyl]trimethylammonium fluoroborate (Hucho et al., 1976). Also, 0.01–1 $\mu$M curare was found to increase the fluorescence of noncovalently attached ethidium (Schimerlik et al. 1979), and 100 $\mu$M curare was seen to enhance the labeling of $\beta$- and $\delta$-subunits with triphenylmethylphosphonium (Muhn et al., 1984b; Fahr et al., 1985). So, for both BgTx and curare, only certain probes are sensitive to the global transition induced by antagonists. The effects of curare are not as straightforward to interpret, however, as curare is seen to induce the transition to the desensitized form of the receptor (Quast et al., 1979) while binding with the same affinity to both the resting and desensitized states (Weber et al., 1975; Weiland & Taylor, 1979).

The retardation of exchange we observe upon BgTx binding may be explained by at least two mechanisms. In general, the combination of two proteins results in slowed exchange-out kinetics (Schreier & Baldwin, 1977; Lennick & Allewell, 1981), probably due to the damping of vibrational modes and shielding, although protein interactions have been observed to accelerate exchange in the case of the histone subunits (McCarthy et al., 1984). BgTx, with 78 amides and a molecular weight of 8100, has been estimated to cover 20 × 30 Å of the AChR surface upon binding (Low, 1979; Kistler et al., 1982). Two bound toxins, each in $\beta$-sheet contact with the AChR, would involve at most 50% of the total BgTx peptide carbonyls involved in sheet contact. These could at best account for 78 protected peptide amides on the AChR. In addition, exchange is also retarded by the much smaller competitive antagonist curare (612 daltons), although to a lesser extent, implying that shielding cannot be the only explanation for retardation of tritium hydrogen exchange by antagonists. Rather, it seems that both antagonists stabilize a dynamically restricted form of the AChR which is markedly less accessible to solvent and that the differences between their effects may reflect a greater damping of breathing modes and unfolding reactions of the AChR by BgTx, or conversely contributions from the different forms of the AChR induced by curare (inhibited, desensitized, blocked). The relative effects of curare and BgTx on the tritium–hydrogen exchange behavior of the AChR are modeled in Figure 6.

In tritium–hydrogen exchange experiments with other protein systems, a range of responses (acceleration, retardation, and no change) has been observed upon addition of ligands. The largest conformation response of the AChR was the 20% increase in the number of detectably exchanging groups induced by BgTx. In hemoglobin, the best studied example, oxygenation accelerates the exchange-out of about 33% of the detectably exchanging peptide amide hydrogens, which have been grouped into different, allosterically sensitive kinetic classes (Malin & Englander, 1980). However, the AChR is about 6 times larger than hemoglobin, and some 250 exchanging groups are affected by the binding of BgTx to the AChR, compared to the roughly 108 peptide amides affected by oxygenation of hemoglobin. In general, the responses in hemoglobin are large compared to most other proteins. Exchange-out is retarded, to a much smaller extent, both in *Escherichia coli* aspartate transcarbamylase upon addition of substrate analogues (approximately 11%) (Lennick & Allewell, 1980) and in oat phytochrome upon photoisomerization (approximately 7%) (Hahn et al., 1984) and is accelerated upon addition of pyruvate to yeast pyruvate decarboxylase (approximately 14%) (Prinz & Gounaris, 1972). These transitions are on a scale similar to those induced by the binding of micromolar pro to AChR (exchange retarded approximately 8%). In some cases, addition of ligands caused no change in exchange kinetics. Bovine liver glutamate dehydrogenase showed no alteration in exchange behavior upon addition of NADPH, ADP, ATP, or L-glutamate (Stryker & Parker, 1970), and there was no change in the exchange behavior of aspartate transcarbamylase upon addition of the allosteric inhibitor CTP or the activator ATP (Lennick & Allewell, 1981). In the latter case, other physical assays (circular dichroism, protease susceptibility, sedimentation rates, sulfhydryl reactivity, etc.) do detect changes upon addition of ATP or CTP (Jacobson & Stark, 1973) which are presumably either local or opposing in nature at different sites in the molecule, and thus not manifested in global changes in tritium–hydrogen exchange rates. This is similar to our observations of the AChR in the presence and absence of high concentrations of agonist.

The exchange data we report here assess the overall change in conformation rather than the change at any particular probe site, and all three types of response to ligation described above are observed. The largest conformational change upon agonist binding (the slight acceleration of exchange observed at 10 $\mu$M carb) is small, affecting less than 50 protonic sites in the entire AChR. If this reflects channel opening, then a gating mechanism in which the entire AChR quaternary structure is changed in global fashion is not indicated. The mechanism is probably more subtle, even though the sites of agonist binding and the most likely location of the gate are separated by at least 60 Å. Models such as that proposed for gap junction, which involves large-scale, concerted movements of the subunits, do not seem likely for the AChR. Rather, a mechanism which focuses its effect into the channel region is more consistent. One possibility involves an electrostatic scheme in which gating is provided by fine-tuning of electrostrictive sites within the channel.

The large change induced by BgTx is consistent with a mechanism in which the secondary structure around its binding site, predicted to be a β-barrel structure (Finer-Moore & Stroud, 1984), is undone to re-form β-sheet with the toxin chain. This is in agreement with the observed structure of BgTx from high-resolution crystallography (Love & Stroud, 1986). Effects of chemical modifications and studies of alterations in sequence show that a 20 × 30 Å toxin surface is involved, and most recently, proton NMR studies show that the toxin is highly flexible in solution (Basus et al., 1988), as suggested by previous crystal studies. At most, 2 bound BgTx could directly retard the exchange of 78 amide protons on the AChR by forming hydrogen bonds. The additional 220 protonic groups whose exchange is retarded upon BgTx binding suggest that BgTx binding induces a radically different conformation of the AChR. The effects of curare, while smaller, are equally significant and may also indicate a restructuring of part of the extracellular components. The large change evoked by curare also cannot be localized to the antagonist binding site and indicates that curare binding induces a conformational change which is disseminated throughout the AChR.

Together these studies support electrostrictive mechanisms for channel opening, gating, and desensitization (Fairclough et al., 1986) while arguing against proposals suggesting global conformational changes, such as subunit rearrangements, and also provide the initial evidence for large conformational effects for blockade by toxin or curare.

**Registry No.** BgTx, 11032-79-4; carb, 462-58-8; sub, 7262-79-5; deca, 156-74-1; hexa, 60-26-4; pro, 59-46-1; tet, 94-24-6; *d*-tubocurarine, 57-95-4.

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