Effects of Agonists and Antagonists on the Reactivity of the Binding Site Disulfide in Acetylcholine Receptor from *Torpedo californica*†

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ABSTRACT: The disulfide bond previously shown to be at the periphery of the acetylcholine binding site in nicotinic acetylcholine receptors is readily reduced by dithiothreitol. In receptor-rich membrane from *Torpedo californica* electric tissue, the second-order rate constant for the reduction at pH 8.0 and 25 °C is 640 L mol^{-1} min^{-1}. In the presence of saturating concentrations of acetylcholine, *n*-butyrylcholine, succinyldicholine, carbamoylcholine, phenyltrimethylammonium, and tetramethylammonium, the rates of reduction are from 80-fold to 15-fold smaller than in the absence of these agonists. The decrease in the rate of reduction in the presence of these agonists is correlated with their effectiveness in promoting the influx of Rb^+ in *Torpedo* membrane vesicles. Choline and decamethonium, which do not act as agonists in *Torpedo* vesicles, but are agonists under other conditions, decrease the rate of reduction maximally about fourfold. The competitive inhibitors, hexamethonium, gallamine, diallyltoxiferine, and *d*-tubocurarine, decrease the rate by twofold or less. Various local anesthetics have no effect on the rate of reduction. Carbamoylcholine protects the binding site disulfide both in the active and in the desensitized states of the membrane-bound receptor; the disulfide of receptor in detergent solution is also protected. In each case, the concentration of carbamoylcholine providing half-maximal protection is comparable to the equilibrium dissociation constant characteristic of the state of the receptor. The protection is not due to steric hindrance by the agonist but rather to a local conformational change induced by the binding of agonist which persists in the desensitized state. This change is rapidly reversed upon dissociation of the ligand. The magnitude of the local conformational change that protects the disulfide against reduction increases as the potency of the agonist increases. It is hypothesized that the magnitude of the local conformational change determines the rates of the opening and closing of the receptor-associated channel. The local conformational change is propagated to the channel in the transition from the resting to the active state. Although the local change persists in the desensitized state of the receptor, it is not coupled in this state to the channel.

The binding of acetylcholine and other agonists to the nicotinic acetylcholine receptor induces a rapid (millisecond time scale) activation resulting in the opening of a channel and in addition a slower (seconds to minutes time scale) transition to a desensitized state (reviewed in Heidmann & Changeux, 1978; Karlin, 1980; Steinbach, 1980). The binding of agonists can be positively cooperative, and the binding of local anesthetics, possibly to the region of the channel, affects the binding of agonists. It is axiomatic that the interactions between agonist binding sites, between agonist sites and the channel, and between agonist sites and local anesthetic sites are mediated by conformational changes. More or less successful attempts have been made to correlate, in terms of pharmacological specificity and kinetics, the physiologically observed transitions of the receptor with changes in the affinity of agonists (Weber et al., 1975; Colquhoun & Rang, 1976; Quast et al., 1978; Sine & Taylor, 1979; Cohen & Boyd, 1979), in the fluorescence of intrinsic fluorescent amino acid residues (Barrantes, 1978) and of extrinsic fluorescent probes (Grunhagen & Changeux, 1976; Heidmann & Changeux, 1979; Schimerlik & Raftery, 1976; Quast et al., 1979), in the binding of calcium ion (Neumann & Chang, 1976), and in the reactivity of sulfhydryl groups (Suarez-Isla & Hucho, 1977). In the present paper, we consider the effects of the binding of agonists and antagonists on the rate of reduction of a disulfide bond located in the vicinity of the acetylcholine binding site.

Considerable evidence has been obtained that nicotinic receptors characteristically contain a readily reducible disulfide bond at the periphery of at least one acetylcholine binding site (reviewed in Karlin, 1980). Following reduction of this bond, the resulting sulfhydryls are susceptible to affinity alkylation. The affinity alkylation by quaternary ammonium alkylating agents is blocked both by agonists and antagonists by what appears to be competition for the binding site. By contrast, physiological evidence has been obtained that cleavage of the disulfide bond is retarded in the presence of agonists but not antagonists. In central neurons of the mollusc, *Limnaea stagnalis*, the relative effectiveness of a ligand as an agonist is correlated with its ability to protect the receptor against inactivation by dithiothreitol; antagonists give no protection (Bregestovski et al., 1977). In frog muscle, sodium bisulfite causes an increase in the sensitivity of the receptor to acetylcholine, presumably subsequent to its cleavage of the binding site disulfide with S-sulfonation of one of the sulfurs; this reaction is retarded in the presence of acetylcholine (Steinacker, 1979).

The effects of various receptor ligands on the reduction of the binding site disulfide can be determined chemically by the use of the affinity alkylating agent, 4-(*N*-maleimido)benzyltrimethylammonium (MBTA),[1] which reacts, on the average, 1000 times faster with the reduced disulfide of the binding site than with other protein sulfhydryls (Karlin, 1969; Karlin & Cowburn, 1973; Weill et al., 1974). Furthermore, the reaction of MBTA with the reduced binding site disulfide is uniquely

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[1] Abbreviations used: MBTA, 4-(*N*-maleimido)benzyltrimethylammonium; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; dTC, *d*-tubocurarine; toxin, principal curarimimetic toxin from venom of *Naja naja siamensis*.

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blocked by agonists and antagonists, including the curarimimetic snake toxins. It is thus possible to use MBTA to monitor the extent of reduction of the binding site disulfide.

The receptor-rich membrane isolated from *Torpedo* electric tissue is well-suited for an analysis of the influence of ligands on disulfide bond reduction, since the receptor from this source is biochemically characterized (Heidmann & Changeux, 1978; Karlin, 1980), the site of reaction of MBTA is determined (Weill et al., 1974), and the activation of the receptor in the membrane can be monitored as a change in cation permeability (Popot et al., 1976). Thus, the effectiveness of agents in protecting the binding site disulfide and in activating the receptor may be quantitated and compared.

Experimental Procedures

Materials

Solutions frequently used were as follows: NP50, 50 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, and 3 mM NaN_3 (pH 7.0); NP150, same as NP50 except for the addition of 150 mM NaCl; NT150, same as NP150, except 20 mM Tris buffer (pH 8.3) is used instead of $NaPO_4$; TNP50, TNP150, and TNT150, 0.2% Triton X-100 in NP50, NP150, and NT150; a 1:1 mixture of NP50 (or TNP50) and NT150 (or TNT150) contains 100 mM NaCl and is pH 8.0.

Receptor-rich membrane was isolated from the electric tissue of *T. californica* by a slight modification of the method of Hamilton et al. (1979). Membrane was resuspended in 358 mM sucrose and 10 mM $NaPO_4$, pH 7.0, and stored in liquid nitrogen. Typically, it contained 4 to 5 mg of protein per mL of suspension, and 0.7 to 1.3 nmol of toxin binding sites per mg of protein. Purified receptor was prepared essentially as described before (Karlin et al., 1976b), except that the affinity gel was derivatized with bromoacetylcholine. Purified receptor was extensively dialyzed against TNP50 to remove carbamoylcholine used to elute it from the affinity gel.

The principal curarimimetic toxin of the venom of *Naja naja siamensis* was purified (Karlsson et al., 1971) and radioactively labeled as previously described (Damle & Karlin, 1978). Synthesis of [^{3}H]MBTA has been described before (Karlin, 1977).

Drugs were obtained from the following sources: butyrylcholine iodide, succinyldicholine chloride carbamoylcholine chloride, and decamethonium bromide (K & K); acetylcholine bromide (recrystallized), tetramethylammonium bromide, and phenyltrimethylammonium iodide (Eastman Organic); *d*-tubocurarine chloride and hexamethonium chloride (Mann Research); gallamine triethiodide, choline chloride (recrystallized), and eserine sulfate (Sigma); 1-adamantanamine hydrochloride and 2-adamantanamine hydrochloride (Aldrich); procaine hydrochloride (Pfaltz and Bauer).

Methods

Affinity labeling assay of the receptor with [^{3}H]MBTA, both in membrane fractions and in solution, was performed as described previously (Karlin et al., 1976a; Karlin, 1977), except that all volumes were halved (see below). Hence, it was found that instead of the previous extensive washing (Karlin et al., 1976a), the shorter filtration and washing procedure used in the [^{3}H]bromoacetylcholine assay (Damle et al., 1978) was sufficient. Also, it was found that prior filtration of the stock [^{3}H]MBTA in CH_3CN through a short column of glass wool significantly lowers background counts on glass fiber filters. Effects of various ligands and reagents on reduction of the disulfide bond by dithiothreitol were studied in one of the following ways. Conditions for all experiments were 25 °C and 100 mM NaCl, pH 8.0, at the reduction stage.

Method A. The simplest and most straightforward method is to add the ligand either before or after dithiothreitol during the usual [^{3}H]MBTA assay (Karlin, 1977; filtration as in Damle et al., 1978) and to determine the effect on specific labeling. The assay of the receptor is briefly as follows.

To quadruplicate samples (10–15 pmol of toxin binding sites) in 25 μL of TNP50 (or NP50) in 7-mL capacity (13 × 85) stoppered plastic tubes (Sarstedt) was added 25 μL of dithiothreitol in TNT150 (or NT150) to give 0.2 mM dithiothreitol at pH 8.0; the mixtures were incubated for 20–30 min at room temperature, during which time the disulfide bond was normally completely reduced. The mixtures in duplicate were diluted to 250 μL, brought to pH 7.0 by the addition of TNP150 (or NP150) buffer containing additional $NaPO_4$ to 20 mM and either with or without 1 μM toxin, and incubated for 15–20 min at room temperature. Toxin blocks all receptor binding sites, whether reduced or not. [^{3}H]MBTA (250 μL; 2 μM) was added, and, after several minutes, the labeling reaction was terminated by the addition of a large excess of 2-mercaptoethanol. Succinylated lysozyme carrier (100 μg) was added, and the mixtures were processed as described in detail elsewhere (Karlin, 1977; Damle et al., 1978) with the modifications stated above. Specific labeling is the difference in labeling obtained for samples without and with toxin, each of which is the mean of duplicate determinations.

To test the effects of ligand binding on reduction, receptor samples in TNP50 (or NP50) were preincubated with the desired concentration of ligand before addition of dithiothreitol. In controls, the same concentration of ligand was added 30 min after the addition of dithiothreitol. Thus, any difference in the specific labeling by [^{3}H]MBTA must be due to the effect of ligand on the reduction, since the concentration of ligand present during reaction with [^{3}H]MBTA is the same. The concentration of ligand at the moment of reaction with 1 μM MBTA is about one-tenth its value at the dithiothreitol reduction stage, whether added before or after, and below a concentration which depends on the ligand, there is no direct effect on the specific [^{3}H]MBTA labeling. Higher concentrations of ligand can be used only if the receptor is separated from all other reagents, e.g., by centrifugation of the receptor in the membrane or by gel filtration of the receptor in solution.

Method B. To 50 (or 75) μL of membrane in NP50 containing 100–150 pmol of toxin binding sites (2–4% of which are labeled with [^{3}H]methyl-labeled toxin as a marker for recovery) was added 10 μL of ligand in water and 50 (or 75) μL of dithiothreitol in NT150 to give the required ligand and dithiothreitol concentrations. (With ligands subject to hydrolysis by esterase, 100 μM eserine was added first to the membrane.) After the mixture was incubated at room temperature for a specified length of time (10 s to 30 min), 25–75 μL of 25–100 mM *N*-ethylmaleimide in water was added, and the mixture was kept on ice for 30 min. *N*-Ethylmaleimide rapidly reacts with dithiothreitol and with all of the protein sulfhydryls, including those formed by reduction of the binding site and other disulfides. The rate constants for the *N*-ethylmaleimide reaction at 25 °C, pH 7.0, are 1660 L mol^{-1} s^{-1} for dithiothreitol sulfhydryls and 60 L mol^{-1} s^{-1} for the sulfhydryls of the reduced receptor (Karlin & Winnik, 1968; Karlin, 1969; Karlin & Cowburn, 1973). The mixture was centrifuged in a Beckman airfuge at 160000*g* for 3 min, the supernatant was discarded, and the pellet was resuspended in 150 μL of NP50. Centrifugation and resuspension in 150 μL of NP50 were repeated once more. After the final centrifugation, the pellet was resuspended in 250 μL of TNP50. Duplicate 50-μL aliquots mixed with 5 mL of Scintisol (Isolab)

were counted to determine ^{3}H-labeled toxin and hence the recovery of receptor sites. Four 25-μL aliquots were assayed with [^{3}H]MBTA as described above under method A (i.e., after further reduction with 0.2 mM dithiothreitol) to determine the number of binding site disulfides which were not reduced by dithiothreitol and hence not alkylated by *N*-ethylmaleimide in the preceding steps.

In method B, we directly determine the binding site disulfides protected against dithiothreitol reduction. In the controls, ligand is added to receptor after the first reduction by dithiothreitol. The advantages of this method are that nonspecific labeling by [^{3}H]MBTA is very low, since *N*-ethylmaleimide has already alkylated all the protein sulfhydryls, including those formed during the first dithiothreitol reduction. The reduction reaction can be stopped with excess *N*-ethylmaleimide at any time precisely, so that kinetic studies are feasible. It is possible to use high concentrations of dithiothreitol and ligands, since they all are removed by repeated pelleting and suspension before the second reduction and reaction with [^{3}H]MBTA.

All of the experiments reported here except those in Figure 5 were performed by method B. Further details of the experimental conditions in each case are given in the text below, in tables, and in figure legends. In all cases, ligand and dithiothreitol concentrations and times of reaction refer to the initial reduction, i.e., to the mixture receptor + ligand + dithiothreitol, irrespective of the order of addition.

Influx Experiments. ^{86}Rb (5–10 mCi/mg) was purchased from New England Nuclear. The solution in 0.5 M HCl was lyophilized and the residue was redissolved in water. Buffers used were 358 mM sucrose and 10 mM $NaPO_4$, pH 7.0 (the buffer also used for resuspension and storage of membrane in liquid nitrogen), and the isotonic buffer containing 218 mM NaCl and 10 mM $NaPO_4$, pH 7.0. These two were mixed in various proportions. Receptor activation by various ligands was investigated by monitoring ^{86}Rb uptake by receptor-rich membrane vesicles (Popot et al., 1976; Miller et al., 1978; Hess et al., 1979) as follows.

The membrane was diluted in a "flux" buffer containing 50–100 mM NaCl, 270–180 mM sucrose, and 10 mM $NaPO_4$, pH 7.0. Between 2 and 4% of the total toxin sites were labeled with [^{3}H]methyl-labeled toxin as a marker for recovery. The membrane suspension was initially incubated at 25 to 37 °C for 0.5 h and then put on ice. ^{86}Rb was diluted in the "flux" buffer to give 1.5×10^6 cpm in 25 μL. To 25 μL of ^{86}Rb solution on ice was added 10 μL of carbamoylcholine or other effector solution in water and 25 μL of membrane (50 μg total protein); the solution was mixed well. After the desired time interval (usually 10 s), 1 mL of "stop" buffer containing 218 mM NaCl, 10 mM $NaPO_4$, pH 7.0, 25–50 μM *d*-tubocurarine, and 2 mM 2-adamantanamine was added to arrest the flux, and the mixture was rapidly filtered under low vacuum (150 mmHg) through Millipore filters (HATF 02500, 0.45 μm), presoaked in stop buffer. The filters were washed with 3×3 mL of stop buffer and transferred to counting vials. Water (100 μL) was added, followed by 5 mL of Scintisol (Isolab). After 1 h at room temperature the ^{3}H and ^{86}Rb radioactivities were counted in a liquid scintillation counter.

Calculations. Under these conditions, recovery of membrane was about 90%, and ^{86}Rb trapped on filters was 3–6 times the background, i.e., ^{86}Rb trapped in the absence of membrane. ^{3}H counts per minute were corrected for the instrument crossover from ^{86}Rb to ^{3}H channel (about 4–6%). ^{86}Rb counts per minute were then corrected for background and normalized with respect to membrane recovery, in terms of corrected ^{3}H counts per minute.

Table I: Effect of Agonists and Antagonists on Dithiothreitol Reduction of the Receptor Binding Site Disulfide Bond in *Torpedo* Membrane[a]

ligand	concn[b] (mM)	protection[c] (%)	k[d] (L mol^{-1}) min^{-1})	influx[e] (%)
acetylcholine	0.1	96 ± 3	8 ± 6	100 ± 5
n-butyrylcholine	0.1	95 ± 5	10 ± 11	96 ± 5
succinyldicholine	0.1	93 ± 5	15 ± 10	106 ± 17
carbamoylcholine	0.1	87 ± 2	30 ± 3	97 ± 4
phenyltrimethylammonium	1.0	81 ± 3	42 ± 5	73 ± 14
tetramethylammonium	1.0	78 ± 2	50 ± 5	47 ± 8
decamethonium	1.0	45 ± 3	160 ± 15	8 ± 6
choline	1.0	43 ± 4	170 ± 20	7 ± 5
gallamine	0.1	17 ± 3	350 ± 40	13 ± 8
d-tubocurarine	0.1	11 ± 3	360 ± 30	2 ± 2
diallyltoxiferine	0.1	10 ± 10	470 ± 100	8 ± 6
hexamethonium	1.0	4 ± 2	650 ± 80	2 ± 2
none		7 ± 4	640 ± 40	0

[a] Reduction of disulfide determined by method B given under Experimental Procedures with 1 mM dithiothreitol reacted for 5 min, at 25 °C, pH 8.0. [b] Concentration of ligand during the reduction. Ligands were incubated with membrane 20 to 30 min before addition of dithiothreitol. In experiments involving ligands subject to hydrolysis by esterase, the membrane was preincubated for 30 min with 100 μM eserine, which was also present during incubation with the ligand. [c] The fraction of total binding site disulfides that are not reduced. Mean of duplicate experiments on four different membrane preparations. [d] Second-order rate constant calculated from the data in the table using eq 1. Rates listed for carbamoylcholine, *d*-tubocurarine, and control, however, are calculated from the more extensive data of Figure 3. Similar values are obtained from the data in the table, viz., 28 ± 5, 440 ± 60, and 530 ± 120, respectively. [e] Influx of ^{86}Rb was determined at the indicated concentration and is expressed as the percent of the specific influx obtained with acetylcholine. Membrane was preincubated with 100 μM eserine, which was also present during the influx.

Results

Effect of Agonists and Antagonists on Reduction of the Disulfide Bond in Membrane. The presence of cholinomimetics, which unambiguously open the nicotinic acetylcholine receptor channel both in cells and in membrane vesicles, protects the binding site disulfide against reduction by dithiothreitol (Table I). For acetylcholine, carbamoylcholine, *n*-butyrylcholine, succinyldicholine, phenyltrimethylammonium, and tetramethylammonium, the protection at saturating ligand concentrations, under the conditions specified in Table I, is maximally 80 to 95%. For choline and decamethonium, which under certain conditions are partial agonists, but which do not detectably increase the cation permeability of *Torpedo* vesicles (Table I and Popot et al., 1976), the protection obtained is maximally about 45%. The classical competitive inhibitors, *d*-tubocurarine, diallyltoxiferine, gallamine, and hexamethonium, afford little, if any, protection.

Controls demonstrate that the cholinomimetics are inhibiting the initial reduction of the disulfide by dithiothreitol and not the alkylation by *N*-ethylmaleimide or by MBTA. When the ligands are added 5 min after dithiothreitol, reduction is complete, and there is no inhibition of the reaction of the sulfhydryls with *N*-ethylmaleimide under the conditions of this experiment. (When *N*-ethylmaleimide is reacted at 1 mM for only 1 min, as opposed to 10 mM for 30 min in the preceding experiments, we do observe a small difference in the extent of alkylation of the reduced binding site disulfide in the presence and absence of 100 μM carbamoylcholine.) Additional control experiments performed with 100 μM carba-

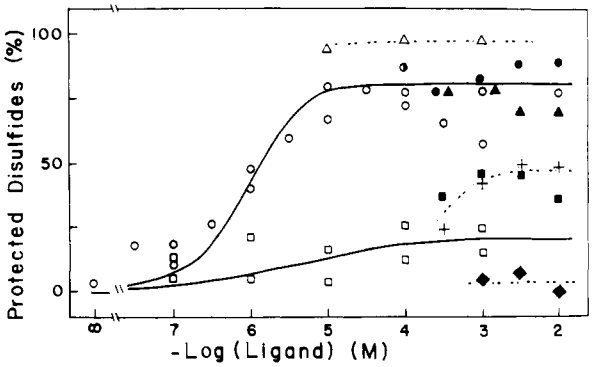


FIGURE 1: Fraction of *Torpedo* receptor binding site disulfides protected against dithiothreitol reduction in membrane vs. concentration of the protecting ligand. Protection was determined by method B (see Experimental Procedures). The abscissa refers to ligand concentration at the time of the initial reduction. Receptor concentration was 1 μM in terms of toxin binding sites. Membrane was incubated with ligand for 30 min prior to addition of dithiothreitol. Data shown are for four different membrane preparations. Solid lines (0.2 mM dithiothreitol and 30-min incubation time): (□) *d*-tubocurarine; (○) carbamoylcholine. Dashed lines (1 mM dithiothreitol and 5-min incubation time): (△) acetylcholine; (◑) carbamoylcholine; (●) phenyltrimethylammonium; (▲) tetramethylammonium; (■) decamethonium; (+) choline; (♦) hexamethonium.

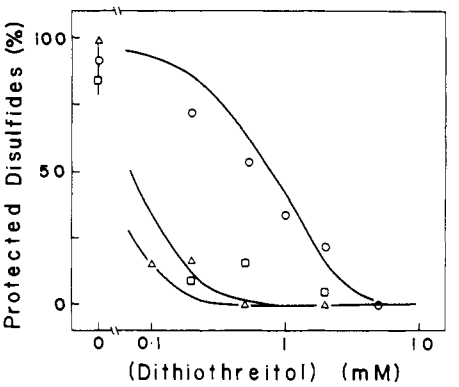


FIGURE 2: The fraction of binding site disulfides remaining unreduced as a function of dithiothreitol concentration for *Torpedo* receptor in membrane. Method B was applied with dithiothreitol concentrations as shown on the abscissa, and incubation time was 30 min. The concentration of receptor in terms of toxin binding sites was 1.2 μM. Membrane was preincubated with ligands for 30 min prior to dithiothreitol addition. Ligand concentration was 100 μM at the reduction stage: (△) no ligand; (□) *d*-tubocurarine; (○) carbamoylcholine. Solid lines are theoretical curves computed from eq 1 as described in the text.

moylcholine and 100 μM *d*-tubocurarine show that when membrane was incubated with (i) no reagents, (ii) ligands only, (iii) *N*-ethylmaleimide only, (iv) ligands + *N*-ethylmaleimide, or (v) excess *N*-ethylmaleimide + dithiothreitol (in that order), equivalent specific labeling is obtained with [^{3}H]MBTA in the subsequent assay, indicating that all reagents are washed off during repeated pelleting and resuspension, and that, for (v), excess *N*-ethylmaleimide completely alkylates dithiothreitol before the latter reduces the receptor to any significant extent. Similarly, when membrane was incubated with (vi) dithiothreitol only, (vii) dithiothreitol, then ligands, or (viii) ligands, than dithiothreitol, equal labeling was obtained. In the last case, agonists do block the reduction in membrane; however, all of the unreduced disulfides are completely reduced and labeled during the subsequent reduction and [^{3}H]MBTA reaction. Thus, there is no residual ligand or *N*-ethylmaleimide in the final resuspension in TNP50 that may interfere with the [^{3}H]MBTA assay. Only when we add excess *N*-ethylmaleimide after the dithiothreitol incubation in membrane do we get the results listed in Table I. In this case, the sulfhydryls, including those formed by dithiothreitol reduction of disulfides, are irreversibly alkylated by *N*-ethylmaleimide and hence are not labeled by [^{3}H]MBTA. It is the disulfides, which were not reduced during the first dithiothreitol incubation, that are labeled specifically during the subsequent assay by [^{3}H]-MBTA.

It should be mentioned that if an excess of 5,5′-dithiobis-(2-nitrobenzoate) is added after dithiothreitol but before *N*-ethylmaleimide, complete [^{3}H]MBTA labeling is obtained during the subsequent assay. Either 5,5′-dithiobis(2-nitrobenzoate) reoxidizes the reduced sulfhydryls to original disulfides (Karlin & Bartels, 1966), or it may form mixed disulfides with them; in either case, these sulfhydryls are protected against reaction with *N*-ethylmaleimide. During the subsequent assay either type of disulfide is reduced and labeled.

Effect of Varying Ligand Concentration. Variation of the extent of protection of the disulfide against dithiothreitol reduction in the membrane was investigated as a function of ligand concentration (Figure 1). Reduction of the disulfide bond by dithiothreitol and its alkylation with *N*-ethylmaleimide were seen to be complete in the absence of ligands. With increasing carbamoylcholine concentration, the fraction of disulfides protected against reduction by 0.2 mM dithiothreitol during 30 min increases to a maximum of about 80% of the total number of disulfides present. With *d*-tubocurarine, on the other hand, there is only a small increase in protection with increasing concentration to a maximum of about 20%. Half-maximal protection is obtained at about 1 μM carbamoylcholine, total concentration; since the binding site concentration is also about 1 μM, the actual free concentration of carbamoylcholine is less than 1 μM. The extent of protection afforded by several other ligands is maximal at a concentration of 1 mM or less (Figure 1).

Effect of Increasing Dithiothreitol Concentration. The effect of increasing dithiothreitol concentration is to overcome the protection offered by the agonist (Figure 2). The membrane was preincubated with carbamoylcholine or *d*-tubocurarine. The final ligand concentration was 100 μM at the time of dithiothreitol incubation. At this concentration, the sites are presumably all occupied, and the extent of protection has reached a plateau (Figure 1). With increasing dithiothreitol concentration the extent of protection decreases, and the reduction of the disulfide is complete in 30 min at a dithiothreitol concentration of 5 mM or higher for membrane incubated with carbamoylcholine, while for *d*-tubocurarine or control the reduction is complete at much lower dithiothreitol concentrations (Figure 2). Thus, the protection afforded by an agonist is not absolute. It is possible to reduce the disulfide bond of the membrane-bound receptor whether the binding site is free, occupied by an antagonist, or by an agonist. Only the rates of reduction seem to be different.

Determination of Rates of Reduction. The effect of ligands was quantitated by determining the rates of reduction of the disulfide bond by dithiothreitol (Figure 3). The rates were determined at three different dithiothreitol concentrations: 0.2, 1.0, and 5.0 mM. Three different membrane preparations were used, and the receptor concentration was 1 to 1.5 μM in terms of toxin binding sites. Since dithiothreitol is in large excess, its concentration remains practically constant during the reduction, which is therefore a pseudo-first-order reaction. Hence, we have

$$\ln (S/S_0) = -kct \qquad (1)$$

where S/S_0 is the fraction of disulfides which are unreduced

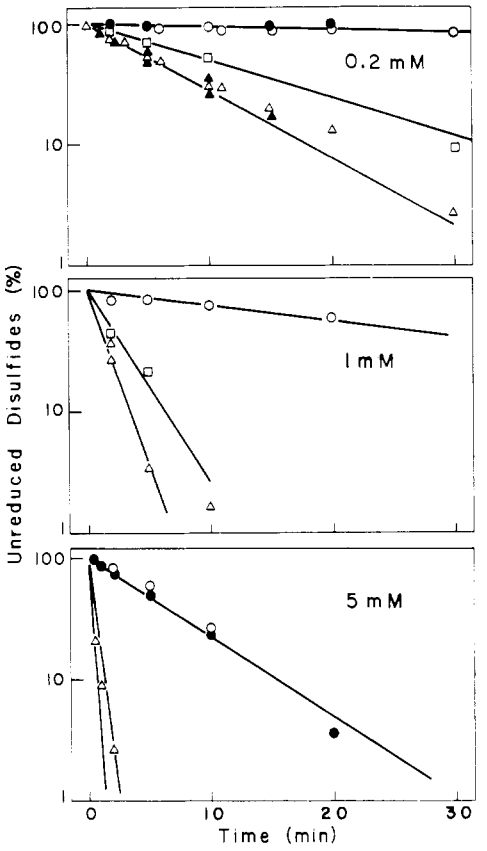
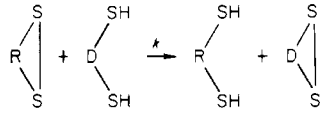


FIGURE 3: Kinetics of reduction of the binding site disulfide bond by dithiothreitol at 25 °C and 100 mM NaCl buffer, pH 8.0. Data are for three different membrane preparations and experiments were performed by method B. The receptor concentration was 1 μM in terms of toxin binding sites and dithiothreitol concentration as shown, both at the reduction stage. Membrane was incubated with ligand (when present) for 30 min before addition of dithiothreitol (zero time). Reduction was stopped at times indicated on the abscissa by adding excess *N*-ethylmaleimide for all except ▲. Open symbols (ligand concentration was 100 μM at the reduction state): (△) control (no ligand); (□) *d*-tubocurarine; (○) carbamoylcholine. Closed symbols: (▲) at zero time dithiothreitol was added to membrane and mixtures were incubated at 25 °C; at indicated times carbamoylcholine was added to make them 100 μM in carbamoylcholine; incubation was continued for a total of 30 min before addition of excess *N*-ethylmaleimide to terminate reduction reaction; (●) dithiothreitol + carbamoylcholine added together to membrane to give 100 μM carbamoylcholine and dithiothreitol concentration as shown. Straight lines are theoretical, expected for a pseudo-first-order reaction (eq 1) computed with the following values of rate constants: 640, 360, and 30, respectively, all in units of L mol^{-1} min^{-1}, for receptor alone, receptor + 100 μM *d*-tubocurarine, and receptor + 100 μM carbamoylcholine.

at time t, c is the total dithiothreitol concentration, and k is the second-order rate constant for the reduction reaction:



(2)

The data fit eq 1 for all three dithiothreitol concentrations and over t ranging from 0 to 30 min (Figure 3). Mean values obtained for the rate constants are 640 ± 40, 360 ± 30, and 30 ± 3, all in units of liters mole^{-1} min^{-1}, in the control, in the presence of 100 μM *d*-tubocurarine, and in the presence of 100 μM carbamoylcholine, respectively. The straight lines in Figure 3 are theoretical, computed from eq 1, using these values of rate constants and the appropriate c. Also, taking t = 30 min and the appropriate values for k, we have used eq 1 to compute the fraction of disulfides remaining unreduced for various c. The resulting theoretical curves are shown in Figure 2 superimposed upon experimental date.

Carbamoylcholine decreases the rate constant by more than a factor of 20, while *d*-tubocurarine decreases it by a factor of about 2. We have also used eq 1 to estimated the rate constants for the ligands listed in Table I from the data shown in the table, and these values are also listed in Table I. It can be seen that acetylcholine, *n*-butyrylcholine, succinyldicholine, tetramethylammonium, and phenylthimethylammonium cause a decrease in reactivity similar to carbamoylcholine, while choline and decamethonium have a smaller effect. Although decamethonium causes about a fourfold decrease in the rate, hexamethonium, also a bisquaternary ligand, has no effect. Table I shows the extent of protection by each ligand at a concentration that gives the maximum effect (Figure 1) and that by competition with [^{3}H]acetylcholine binding has been shown to saturate the binding sites (data not shown).

Effect of Noncompetitive Inhibitors. Three agents which block the receptor-mediated increase in cation permeability in *Torpedo* vesicles were tested for their effect on disulfide reduction. Two of these, procaine (Weber & Changeux, 1974a,b) and 1-adamantanamine (Tsai et al., 1978), act as local anesthetics in this system. Another, 2-adamantanamine, blocks the permeability response at 2 mM concentration without affecting the maximum binding of acetylcholine (unpublished observations). None of these agents, at 2 mM concentration, has any effect on the reduction of the binding site disulfide bond by dithiothreitol. Furthermore, carbamoylcholine is as effective in protecting the disulfide against reduction in the presence as in the absence of these agents.

Time Dependence of the Protection by Agonists. In the previous experiments the membrane was incubated with agonist for 20 to 30 min before the addition of dithiothreitol, with which the membrane was reacted for up to 30 min. Under these conditions, the receptor was presumably mostly in the desensitized (high affinity, uncoupled) state, and the dependence of protection on agonist concentration might be expected to reflect this state (Figure 1). The binding site disulfide can be 90% reduced by 25 mM dithiothreitol in 10 s. Comparison of the concentration dependence of protection against reduction by 25 mM dithiothreitol in 10 s by agonist, either added together with the dithiothreitol or added 30 min before dithiothreitol, shows that the concentration of carbamoylcholine providing 50% protection is about 60-fold higher in the former case (Figure 4). The concentrations giving 50% protection are ~50 μM with simultaneous addition and 0.7 μM with 30-min preincubation. At high concentrations of carbamoylcholine (>100 μM), there is little difference in the extent of protection, whether the agonist is preincubated with the membrane or it is added simultaneously with dithiothreitol (see closed circles, Figure 3).

Mutual Displacement of Carbamoylcholine and d-Tubocurarine. Ligand binding and dissociation are very fast. It is known from direct binding studies that a bound agonist is displaced by an excess of antagonist and vice versa (Kasai & Changeux, 1971a,b; Eldefrawi et al., 1971; Weber & Changeux, 1974a,b), and these displacement reactions are probably also quite fast. In order to test whether the changes in the reactivity of the disulfide when a bound agonist is displaced by an antagonist or vise versa are faster than the onset of or recovery from desensitization, we performed experiments with 10 mM dithiothreitol and 1-min incubation, conditions under which the disulfide bond is practically completely reduced in free receptor and in receptor bathing *d*-tubocurarine, while only about 25% reduction is expected for receptor occupied

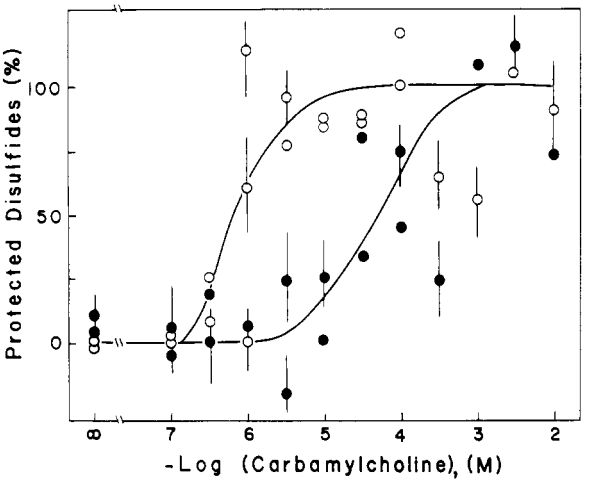


FIGURE 4: Conversion of membrane-bound *Torpedo* receptor from low to high affinity form after preincubation with carbamoylcholine, as monitored by the extent of protection of the binding site disulfide against dithiothreitol reduction; experiments performed as per method B. The concentration of dithiothreitol at the reduction stage was 25 mM, and that of carbamoylcholine as shown on the abscissa. Reduction was stopped after 10 s by adding excess *N*-ethylmaleimide. Data shown are for two different membrane preparations: (○) membrane was preincubated with carbamoylcholine for 30 min prior to addition of dithiothreitol; (●) dithiothreitol + carbamoylcholine were added together to membrane. For this figure only, the ordinate is normalized with respect to the difference in protection obtained at low and high carbamoylcholine concentrations.

Table II: Displacement of a Bound Ligand by an Excess of Another Ligand Monitored by the Extent of Protection of the Binding Site Disulfide[a]

first ligand[b]	concn (mM)	second ligand	concn (mM)	protection after incubation with second ligand for[c]: 30 min (%)	0 min (%)
none		none		3	0
none		carb[d]	0.01	59 ± 1	29 ± 1
none		carb	1.0	69 ± 4	58 ± 3
none		curare	0.01	0 ± 2	8 ± 3
none		curare	1.0	0 ± 1	3 ± 4
carb	0.01	curare	1.0	3 ± 1	9 ± 2
curare	0.01	carb	1.0	48 ± 4	52 ± 2

[a] Determined by method B (see Experimental Procedures) with 10 mM dithiothreitol for 1 min. All ligand concentrations are those during reduction. [b] First ligand incubated with membrane for 30 min before addition of second ligand. [c] Second ligand either was incubated with membrane 30 min before addition of dithiothreitol or was added together with dithiothreitol. [d] carb = carbamoylcholine; curare = *d*-tubocurarine.

by carbamoylcholine. Experiments were done in two fashions: The receptor was incubated for 30 min with a first ligand, then either (i) a second ligand was added, the mixture was incubated for 30 min, and finally dithiothreitol was added, or (ii) a second ligand and dithiothreitol were added together. From the extent of the protection of the disulfide, it appears that 10 μM carbamoylcholine is displaced by 1 mM *d*-tubocurarine, and similarly 10 μM *d*-tubocurarine is displaced by 1 mM carbamoylcholine, and that the corresponding changes in the disulfide reactivity are complete with 1 min (Table II). In addition, in the latter case, receptor activation as measured by the flux of Rb^+ in vesicles is complete within 10 s (data not shown). It may be noted that, for 10 μM carbamoylcholine alone, there is a considerable difference in the extent of protection depending upon whether the membrane was preincubated with carbamoylcholine or carbamoylcholine and dithiothreitol were added together, while only a small difference is found for 1 mM carbamoylcholine (Table II; also see Figure 4.

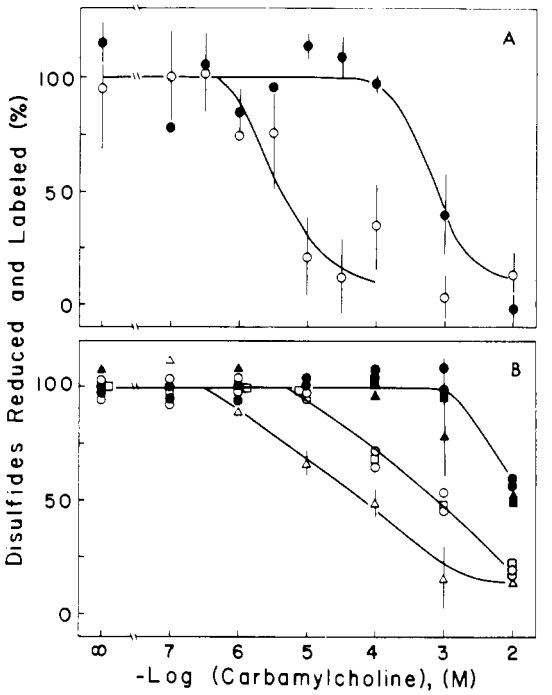


FIGURE 5: Fraction of binding site disulfides reduced and labeled as a function of carbamoylcholine concentration. Experiments performed as per method A: 100 mM NaCl, pH 8.0, 25 °C. Open symbols: protection curves; receptor was incubated with carbamoylcholine for 30 min, then dithiothreitol was added and mixtures were incubated for an addition 30 min. At this stage, dithiothreitol was 0.2 mM, and carbamoylcholine concentration was as shown on the abscissa. They are reduced by a factor of 10 at the time of labeling reaction. Closed symbols: control curves; receptor was first reduced with 0.2 mM dithiothreitol for 30 min, then carbamoylcholine was added to give the indicated concentrations and incubated for an additional 30 min. (A) Receptor in membrane (top figure); (B) receptor in 0.2% Triton X-100 solution (bottom figure); (△, ▲) membrane solubilized in TNP50; (□, ■) membrane solubilized in TNP50 + 1% Triton, incubated for 1 h, and centrifuged at 160000*g* for 3 min to remove insoluble material; clear supernatant, which contained all of the toxin binding activity, was used; (○, ●) purified receptor in TNP50, dialyzed extensively. Data are for two different preparations.

Protection in Solution. *Torpedo* receptor can be reduced and affinity alkylated in detergent solution as well as in membrane. We have determined the effect of carbamoylcholine on the reduction using method A (see Experimental Procedures), which is convenient for solution. In this method, the carbamoylcholine present during the reduction is diluted only 10-fold at the time of the affinity alkylation; hence, it is necessary to examine the effect of carbamoylcholine on the alkylation. When carbamoylcholine is added after the reduction is complete, there is no dimunition in the extent of specific alkylation up to an initial concentration of 100 μM in membrane and 1 mM in solution (Figure 5, closed symbols). When the agonist is added 30 min before dithiothreitol, the protection against reduction is 85% at 100 μM carbamoylcholine in membrane (Figure 5A, open symbols; cf. Figures 1 and 4) and is between 50 and 80% at 1 mM carbamoylcholine in solution (Figure 5B, open symbols). Thus, even in detergent solution, the reduction of the binding site disulfide is considerably retarded in the presence of an agonist. There is a difference, however, in the concentration dependence of the protection between membrane to which Triton X-100 has been added, on the one hand, and either the supernatant from such solubilized membrane or purified receptor, on the other

hand (Figure 5B). Approximate total concentrations affording 50% protection in these experiments are 3 μM in membrane, 70 μM in membrane plus Triton X-100, and 1 mM in the supernatant obtained by centrifuging the membrane solubilized in Triton X-100 or in purified receptor. All receptor was recovered in the supernatant and hence was solubilized under the conditions used.

Discussion

Consistent with physiological experiments on *Limnaea* neurons (Bregestovski et al., 1977) and on frog muscle (Steinacker, 1979), agonists protect the binding site disulfide in the acetylcholine receptor of *Torpedo californica* against reduction by dithiothreitol (Table I). The protection is not absolute; rather the rate constant for the reaction of dithiothreitol with the binding site disulfide is considerably diminished when the site is occupied by an agonist. There is a gradation in the protection, measured as the ratio of the rate constants in the absence and presence of a saturating concentration of each agonist (Table I). For the agonists tested, the order is acetylcholine ($\sim$80) $\geq$ *n*-butyrylcholine ($\sim$64) $\geq$ succinyldicholine ($\sim$43) $>$ carbamoylcholine ($\sim$21) $>$ phenyltrimethylammonium ($\sim$15) $\approx$ tetramethylammonium ($\sim$13). This order roughly parallels the order of effectiveness of these agonists in promoting the integrated influx over 10 s of ^{86}Rb in *Torpedo* membrane vesicles (Table I; also see Popot et al., 1976).

Choline and decamethonium, which elicit no significant increase in the uptake of Rb by the vesicles, cause nevertheless a fourfold decrease in the rate of reduction of the disulfide. Although decamethonium fails to cause a detectable increase in cation permeability in vesicles from *T. californica* (Table I) and *T. marmorata* (Popot et al., 1976), it depolarizes the intact electroplax from *T. marmorata* (Moreau & Changeux, 1976). Decamethonium also acts as an agonist on intact electroplax and vesicles derived from *Electrophorus* (Changeux & Podleski, 1968; Karlin, 1969; Kasai & Changeux, 1971a,b). In frog muscle, also, decamethonium activates the receptor, but, in addition, it apparently binds to and blocks the open receptor channel (Adams & Sakmann, 1978). Choline has very weak agonist properties on *Electrophorus* electroplax (Webb & Mautner, 1966), on which it is four orders of magnitude less potent than decamethonium. Choline also acts as a weak agonist on frog muscle (Adams, 1975). The response of *Torpedo* electroplax to choline has not been determined.

Hexamethonium, gallamine, diallyltoxiferine, and *d*-tubocurarine are competitive antagonists in *Torpedo* electroplax (Moreau & Changeux, 1976) and in subcellular membrane vesicles from this cell (Popot et al., 1976), and also in electroplax and vesicles from *Electrophous* (Higman et al., 1963; Karlin & Winnik, 1968; Kasai & Changeux, 1971a,b). (It is obvious that the overall effect of a ligand is not invariant. This is exemplified by the different effects of decamethonium on intact *Torpedo* electroplax and the derived vesicles.) In addition, hexamethonium is an activator of the reduced receptor in *Electrophorus* (Karlin & Winnik, 1968), and *d*-tubocurarine activates receptor in embryonic rat muscle (Ziskind & Dennis, 1978). Tubocurarine, gallamine, and diallyltoxiferine are even less effective than choline in retarding reduction of the binding site disulfide, and hexamethonium has no effect on the reduction (Table I). Since there is a correlation of the protection of the disulfide provided by a ligand and its potency as an agonist, and since, except for the large antagonists, there is little difference in bulk among the ligands tested, the major contribution to the protection of the disulfide cannot be steric hindrance by the ligand. (Steric hindrance might play a role, however, in the small protection afforded by the large antagonists.) Also, phenyltrimethylammonium, which is an agonist and protects the disulfide of the *Torpedo* receptor, is an antagonist and fails to protect the disulfide of the *Limnaea* receptor (Bregestovski et al., 1977). We conclude that the retardation of the rate of reduction of the disulfide by ligands is due to a change in conformation of the receptor. This change may either restrict access to the disulfide, or change its local environment, or relieve strain in the bond.

That the binding site disulfide bond may be exposed to solvent is suggested by a comparison of its rate of reduction by dithiothreitol with that of other protein disulfides. For example, in receptor, the rate of reduction of the binding site disulfide by dithiothreitol at 25 °C and pH 8.0, with the site unoccupied, is 640 L mol^{-1} min^{-1}, while that of the disulfide between δ chains in the dimer form of receptor is 115 L mol^{-1} min^{-1} (calculated from the data of Hamilton et al., 1979). We have calculated from published data the rates of reduction of disulfides in several other proteins by dithiothreitol and have extrapolated these rates to pH 8.0 and 25 °C. Our estimates, in units of liters mole^{-1} minute^{-1}, are α-lactalbumin in 6 M urea, 100 (Iyer & Klee, 1973), bovine serum albumin in 6 mM urea, 50 (Iyer & Klee, 1973), ribonuclease in 6 M urea, 20 (Iyer & Klee, 1973), lysozyme in 6 M urea, 3 (Iyer & Klee, 1973), trypsinogen, 70 (Sondack & Light, 1971), and chymotrypsinogen, 3 (Sondack & Light, 1971). In all cases, the disulfides are reduced at a lower rate than the receptor binding site disulfide, in fact, closer to the rate of reduction of the protected binding site disulfide. By contrast, a disulfide known to be exposed to solvent in pancreatic trypsin inhibitor is reduced by dithiothreitol at a rate (extrapolated to our conditions) of approximately 560 L mol^{-1} min^{-1}, which is consistent with the expected relationship between the pK_a of the SH formed and the rate constant (Creighton, 1975). Applying the same relationship to the binding site disulfide, we calculate a pK_a for the SH formed of 8.6, close to that of SH groups found in the active sites of several enzymes (Torchinskii, 1974). It seems likely that the conformational change that protects the disulfide does so by transferring it to a less accessible, less polar region of the protein.

Since the maximum extents of protection afforded by the different agonists at saturating concentrations are graded, the magnitudes of the conformational change induced by the binding of the ligands are likely also to be graded. This conformational change does not necessarily lead to the opening of the channel, since choline and decamethonium do not detectably open the channel in the membrane and yet provide significant protection against reduction. Furthermore, the channel has only two states, open or closed; only the rates of transition between these states vary (Neher & Sakmann, 1976; reviewed in Steinbach, 1980). It is likely, moreover, that the graded change leading to protection of the disulfide is confined to the vicinity of the binding site. We suggest that when this *local* change is sufficiently large, it triggers a *global* change in receptor conformation, which propagates to the channel and opens it. To the extent that disulfide protection measures the amplitude of the local conformational change, a local change that diminishes the rate of disulfide reduction at least 15-fold is sufficiently large to open the channel in *Torpedo* receptor (Table I). A graded local change in conformation had previously been hypothesized to account for the increasing depolarization of the *Electrophorus* electroplax resulting from the reaction of the reduced receptor binding site with affinity

labels of decreasing length (Karlin, 1969).

In the desensitized state of the receptor, binding of agonist is uncoupled from the channel. It is clear, however, that the disulfide is protected in the desensitized state of the receptor, since in most of the experiments described above protecting ligand was incubated with the receptor for 10 to 30 min before the addition of dithiothreitol, sufficient time for most of the receptor to equilibrate to the desensitized state (Weiland et al., 1977; Cohen & Boyd, 1979). Consistent with the receptor being in the high-affinity, desensitized state, the concentration of carbamoylcholine that provides 50% of maximum protection is less than 1 μM after 30 min of incubation (Figures 1 and 4). The dissociating constant for the binding of carbamoylcholine to receptor, in *Torpedo* membrane, after 30 min of incubation, is about 0.5 μM (Weiland et al., 1977; Weber & Changeux, 1974a,b). In contrast, when carbamoylcholine is added to the membrane simultaneously with dithiothreitol and the reaction is terminated in 10 s, the concentration of carbamoylcholine affording half-maximal protection is about 50 μM (Figure 4). This concentration is comparable to that aliciting a half-maximal response in a *Torpedo* electroplax (Moreau & Changeux, 1976) and vesicles (Popot et al., 1976; Miller et al., 1978), *Electrophorus* electroplax (Karlin, 1967) and vesicles (Kasai & Changeux, 1971a,b), and BC3H-1 muscle cells (Sine & Taylor, 1979). Furthermore, this concentration corresponds approximately to the dissociation constant for the binding of carbamoylcholine within 10 s or so of addition, both in *Torpedo* membrane (Weiland et al., 1977; Quast et al., 1978) and in BC3H-1 cells (Sine & Taylor, 1979), and is characteristic of binding to a low-affinity, non-desensitized state of the receptor.

Desensitization, itself, cannot account for the protection of the disulfide taking place during the 10-s reaction with 25 mM dithiothreitol (Figure 4). The half-time for the reduction of the disulfide at this concentration of dithiothreitol is 2.6 s in the absence of protecting ligand (Table I). The half-time for the transition of the fully occupied receptor to the desensitized state was found to be about 1 min in the case of carbamoylcholine and *T. californica* membranes (Weiland et al., 1977) and about 4 s in the case of acetylcholine and *T. maramorata* membranes (Cohen & Boyd, 1979). Even if the latter half-time applied, at the highest concentration of carbamoylcholine the disulfide would be more than 50% reduced by the time the receptor were desensitized, contrary to the nearly complete protection obtained (Figure 4). Also, the half-time for the recovery from desensitization upon removal of agonist is 3 to 4 min in *Torpedo* membrane (Weiland et al., 1977; Cohen & Boyd, 1979); however, immediately upon the displacement of carbamoylcholine from the desensitized receptor by excess *d*-tubocurarine, the disulfide is no longer significantly protected (Table II). Similarly, in *Limnaea*, following the washout of acetylcholine, loss of protection occurred before recovery from desensitization (Bregestovski et al., 1977). The binding site disulfide is protected so long as the site is occupied by a suitable ligand both initially in the resting or active state and finally in the desensitized state.

The binding site disulfide of receptor in detergent solution is also protected by carbamoylcholine (Figure 5). The concentration giving half-maximal protection in a solution prepared by adding sufficient Triton X-100 to membrane to solubilize the receptor, without further processing, is about 70 μM, similar to the equilibrium dissociation constants obtained for the binding of carbamoylcholine to detergent-solubilized and purified receptor from *T. californica* (Raftery et al., 1975) and to detergent-solubilized receptor from *T. marmorata* (Moody et al., 1973; Eldefrawi & Eldefrawi, 1973). Half-maximal protection of the disulfide in receptor that has been solubilized and further processed, either centrifuged or purified by affinity chromatography, is considerably higher, about 1 mM. This lowering of the apparent affinity for carbamoylcholine is consistent with the deleterious effects on receptor functionality of replacing all phospholipid with detergent (Briley & Changeux, 1978; Epstein & Racker, 1978; Chang & Bock, 1979). It is clear, nevertheless, that the local conformational change responsible for protection of the disulfide is occurring even in detergent solution. The rate of reduction of the disulfide of the unoccupied binding site of purified receptor in detergent solution is, incidentally, the same as that of receptor in membrane (computed from data of Karlin et al. (1976a) to be $\sim$640 L mol^{-1} min^{-1}).

The pharmacological specificity of the disulfide protection in *Torpedo* membrane is somewhat similar to that of the fluorescent changes observed with the extrinsic probes, quinacrine (Grunhagen et al., 1977) and ethidium (Quast et al., 1979). For example, the fluorescent changes induced by choline are similar to those induced by full agonists of the receptor in *Torpedo* vesicles, just as choline protects the binding site disulfide, albeit to a lesser extent than full agonists. It would be more difficult than it has been with the fluorescent probes to use the disulfide protection to investigate the millisecond and submillisecond events that underlie receptor activation. The local change that results in disulfide protection may be, however, an early event in activation which persists even after desensitization. It is likely to be the coupling of this local change to channel opening that is transitory and which is finally absent in the desensitized state.

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