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Effects of Reduction and Alkylation on Ligand Binding and Cation Transport by *Torpedo californica* Acetylcholine Receptor†

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ABSTRACT: The effects of sulfhydryl group modification on ligand binding and functional properties of the membrane-bound acetylcholine receptor from *Torpedo californica* have been investigated. Agonist binding kinetics were monitored by changes in fluorescence of the probe 5-(iodoacetamido)-salicylic acid which was covalently bound to the receptor after reduction of a reactive disulfide bond(s) by low concentrations of dithiothreitol. These labeling procedures did not affect either the equilibrium binding constant for [^{3}H]acetylcholine or the number of high-affinity binding sites measured in centrifugation experiments. Further reduction of these labeled receptor preparations by higher concentrations of dithiothreitol and subsequent alkylation by excess iodoacetamide resulted in a more than 10-fold decrease in the affinity of the receptor for [^{3}H]acetylcholine. This reduction and alkylation did not, however, radically alter the observed kinetics of acetylcholine binding. The fluorescence signal change on binding consisted of at least three phases similar to those observed for the control preparations, and the ligand concentration dependencies of the measured rate constants could be described by the same kinetic mechanism involving sequential binding of two ligand molecules and three conformational changes. Variation in the values of some of the kinetic parameters describing the formation of the monoliganded complex adequately accounted for the measured decrease in affinity for [^{3}H]acetylcholine. Stopped-flow fluorescence experiments showed that extensive reduction and alkylation resulted in an apparent loss of the ability of the acetylcholine receptor to mediate agonist-induced cation flux. These results show that reduction of disulfide bonds by high concentrations of dithiothreitol followed by alkylation with iodoacetamide seriously perturbs receptor function although the receptor can still undergo its characteristic conformational changes on the binding of acetylcholine but with altered concentration dependence accounting for the reduced affinity for agonist.

Chemical modification of sulfhydryl groups of the nicotinic acetylcholine receptor results in altered pharmacological responses and ligand binding properties. Treatment of *Electrophorus* electroplax with dithiothreitol (DTT)[1] inhibited the permeability response of the postsynaptic membrane to applied acetylcholine, and this inhibition was rendered irreversible by subsequent reaction with *N*-ethylmaleimide (Karlin & Bartels, 1966). After DTT reduction, the dose-response curves showed a decrease both in the affinity for agonist and in the apparent cooperativity of the response (Karlin, 1969). It has also been suggested from noise analysis experiments using the frog neuromuscular junction that DTT causes a reduction in the lifetime and conductance of single channels (Ben-Haim et al., 1975).

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[1] Abbreviations: AcCh, acetylcholine; AcChR, acetylcholine receptor; ANTS, 8-amino-1,3,6-naphthalenetrisulfonic acid; α-BuTx, α-bungarotoxin; Carb, carbamylcholine; DTT, dithiothreitol; HTX, histrionicotoxin; IAS, 5-(iodoacetamido)salicylic acid; MBTA, [4-(*N*-maleimido)-benzyl]trimethylammonium diiodide; NEM, *N*-ethylmaleimide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MPTA, [4-(*N*-maleimido)phenyl]trimethylammonium.

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Affinity labeling experiments have demonstrated that nicotinic acetylcholine receptors from several species including *Torpedo*, *Electrophorus*, and vertebrate muscle contain a reactive disulfide bond in the vicinity of an agonist binding site located on the subunit of molecular weight approximately 40 000. In purified receptor preparations, this bond can be readily reduced by low concentrations of DTT and then covalently labeled by alkylating affinity reagents such as MBTA (Weill et al., 1974; Karlin et al., 1975) and bromoacetylcholine (Chang et al., 1977; Damle et al., 1978; Moore & Raftery, 1979a; Lyddiatt et al., 1979; Wolosin et al., 1980).

Disulfide bond formation is also involved in dimerization of the acetylcholine receptor. The *Torpedo* receptor exists as both a 9S monomer and a 13S dimer (Raftery et al., 1972) in which two monomers are linked by an intermolecular disulfide bond(s) between the 65 000-dalton subunits (Chang & Bock, 1977; Suarez-Isla & Hucho, 1978; Hamilton et al., 1979; Witzemann & Raftery, 1978). In the membrane-bound state, the predominant form is the dimeric species, but the two forms are interconvertible by oxidation and reduction reactions of the thiol groups involved in the linkage.

Investigation of the effects of sulfhydryl group modification on the ligand binding properties of receptor-enriched membrane fragments from *Torpedo* has indicated that reduction causes a decreased affinity of the AcChR for agonists (Schiebler et al., 1977; Moore & Raftery, 1979b; Barrantes, 1980; Walker et al., 1981). In its membrane-bound state, the acetylcholine receptor undergoes a conformational change on exposure to agonists leading to a form having higher affinity for these ligands (Weber et al., 1975; Weiland et al., 1976, 1977; Lee et al., 1977; Quast et al., 1978). This in vitro ligand-induced affinity change has been correlated with the in vivo phenomenon of desensitization (Katz & Thesleff, 1957), and it has been suggested that one of the effects of disulfide bond reduction is inhibition of this ligand-mediated transition (Moore & Raftery, 1979b; Barrantes, 1980).

Schiebler et al. (1977) showed that treatment of *Torpedo* AcChR with either β-mercaptoethanol or dithioerythritol resulted in a decreased $^{22}Na^+$ efflux response to the addition of acetylcholine which was apparently not due to loss of the ion permeability capability of the receptor but rather was correlated with the decreased affinity for agonist . Similar results were reported by Walker et al. (1981) for the effect of DTT reduction on the $^{22}Na^+$ permeability increase induced by carbamylcholine. Subsequent alkylation of these reduced membranes by NEM, however, completely inhibited the flux response.

In this paper, we decribe the effects of reduction and alkylation on the ligand binding and functional properties of AcChR-enriched membrane fragments from *Torpedo californica*. Agonist binding kinetics were monitored by changes in the fluorescence of the covalently bound probe 5-(iodoacetamido)salicylic acid which we have used in previous studies of ligand binding (Dunn et al., 1980, 1981). Extensive reduction and alkylation decreased the receptor's affinity for acetylcholine without seriously affecting the kinetic binding mechanism. Quanitative analysis of the kinetics of cation flux indicated that receptor function is, however, dramatically altered by sulfhydryl group modification.

Materials and Methods

AcChR-enriched membrane fragments were prepared from *Torpedo californica* electric organ as described by Elliott et al. (1980), and the buffer used in the final stages of purification was Ca^{2+}-free *Torpedo* Ringers (20 mM Hepes, 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 0.02% NaN_3, pH 7.4). The concentration of α-BuTx sites was measured by the DEAE disc assay of Schmidt & Raftery (1973) by using [^{125}I]-α-BuTx prepared as described by Blanchard et al. (1979). Protein concentrations were determined by the method of Lowry et al. (1951), and the specific activities of the membrane preparations were 1–2 nmol of α-BuTx sites/mg of protein.

Covalent labeling of the membrane-bound receptor by IAS (Molecular Probes Inc.) was carried out by using the procedures of Dunn et al. (1980) in which membrane fragments were reduced with 50 μM DTT (Sigma Chemical Co.) for 1 h at 4 °C, alkylated by reaction with 250 μM IAS for 2 h at 4 °C, and centrifuged to remove free reagents. Final resuspension of the membrane pellet was in Ca^{2+}-free Ringers to give a concentration of about 10 μM in α-BuTx sites.

Further chemical modification of the IAS-labeled membrane fragments was accomplished by a modification of the method of Moore & Raftery (1979b). Membrane fragments were diluted to $\sim$1 μM in α-BuTx sites in Ca^{2+}-free Ringers, and 1 mM DTT was added. The vials were flushed with argon, and reduction was allowed to proceed for 1 h at 4 °C. Following addition of 3 mM iodoacetamide, incubation was continued for a further hour at 4 °C. The membrane fragments were then pelleted, washed, and resuspended in Ca^{2+}-free Ringers.

Equilibrium and kinetic experiments of AcCh binding were carried out using membrane fragments in which acetylcholine esterase activity was inhibited by prior incubation with diethyl nitrophenyl phosphate. These preparations were devoid of esterase activity as measured by the method of Ellman et al. (1961) with acetylthiocholine as substrate. The membranes were diluted into Ringers containing 4 mM $CaCl_2$ before use.

[^{3}H]AcCh (New England Nuclear) binding was measured by centrifugation assay using an Eppendorf microfuge and the protocol described for [^{3}H]Carb binding by Dunn et al. (1980). Nonspecific binding was estimated from the results of parallel experiments in which an excess of nonradioactive AcCh was included in the incubation mixture.

Kinetic data were obtained by using the stopped-flow instrumentation and data collection systems previously described (Dunn et al., 1980, 1981).

The kinetics of agonist-mediated cation translocation were measured by the Tl^+ fluorescence quenching method of Moore & Raftery (1980) using membrane vesicles which had not undergone preliminary labeling by IAS. In these experiments, both the unmodified preparations and those which had been reduced by 1 mM DTT and reacted with iodoacetamide as described above were equilibrated in 10 mM Hepes and 35 mM $NaNO_3$, pH 7.4, before the vesicles were loaded with the fluorescent probe ANTS (Chemical Service).

Stopped-flow data were analyzed by using nonlinear regression programs written in Fortran for a MINC computer (Digital Equipment Corp). Kinetic traces recorded for AcCh binding to IAS-labeled membrane fragments were analyzed by using either a single-exponential equation or a sum of two exponentials (Dunn et al., 1980):

$$F(t) = A_0 + A_1\,[\exp(-k_1 t)] + A_2\,[\exp(-k_2 t)] + k_0 t$$

in which $F(t)$ is the fluorescence level at time t, A_0 is the equilibrium fluorescence, k_0 is the slope of the base line due to photolytic reactions linear with time, k_1 and k_2 are the rate constants of the two exponential processes, and A_1 and A_2 are the corresponding amplitudes.

Tl^+ flux data were fitted by

$$F(t) = A_0 + A_1/[1 + KT_\infty(1 - e^{-k_1 t})] + k_0 t$$

in which the term KT_∞ was fixed by using the known final

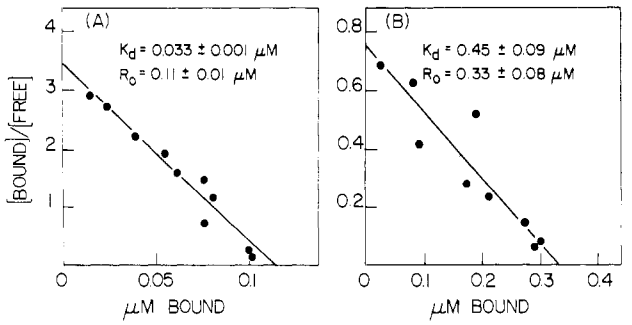
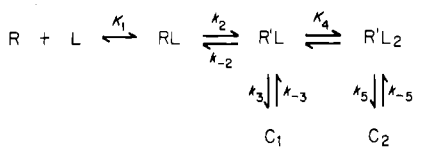


FIGURE 1: Scatchard plots of [^{3}H]AcCh binding to IAS-labeled membrane fragments before (A) and after (B) reduction by 1 mM DTT and alkylation by 3 mM $IAcNH_2$. The AcChR concentration was 0.10 μM in α-BuTx sites in (A) and 0.35 μM in (B). Solid lines are least-squares fits of the data.

Scheme I



concentration of Tl^+ (T_∞) of 17 mM and the Stern–Volmer constant for quenching (K) of 96 M^{-1} (Moore & Raftery, 1980).

Results

Effects of Sulfhydryl Group Modification on the Equilibrium Binding of Acetylcholine. Covalent labeling of AcChR-enriched membrane fragments by the fluorescent probe IAS after reduction of the receptor with 50 μM DTT gives rise to a receptor preparation which responds to the binding of agonists with an enhancement of the fluorescence of the bound fluorophore. It has previously been shown that incorporation of IAS inhibits the covalent labeling by bromoacetylcholine (Dunn et al., 1980), suggesting that the IAS-modified disulfide which gives the signal change on ligand binding is located near this agonist binding site on the subunit of molecular weight 40K.

The equilibrium dissociation constant for [^{3}H]AcCh binding to membrane fragments was measured in centrifugation experiments, and Scatchard plots of the data are shown in Figure 1. The IAS-labeled preparation bound [^{3}H]AcCH with a K_d of 33 nM, which was not significantly different from that of unmodified preparations. The number of high-affinity binding sites and the AcCh/α-BuTx binding stoichiometry were also unaffected by IAS labeling. Further reduction of the AcChR by 1 mM DTT followed by alkylation with iodoacetamide resulted in a decrease in affinity for AcCh, the complex being characterized by a K_d of 0.4 μM (Figure 1B). There was, however, no change in the measured number of ligand binding sites, and no evidence for binding site heterogeneity was found. Similar results were found for membrane preparations which had not undergone the preliminary IAS modification.

Effect of Reduction and Alkylation on the Kinetics of AcCh Binding. When AcCh was rapidly mixed with IAS-labeled membrane fragments and the fluorescence of the bound probe was monitored by using excitation via energy transfer from the protein, the reaction traces consisted of a multiphasic fluorescence enhancement. By recording traces over several different time scales at each AcCh concentration, we could obtain reliable kinetic constants for three apparently first-order processes which had rates of approximately 5, 1, and 0.03 s^{-1} at a ligand concentration of 10 μM. Figure 2 shows the ligand

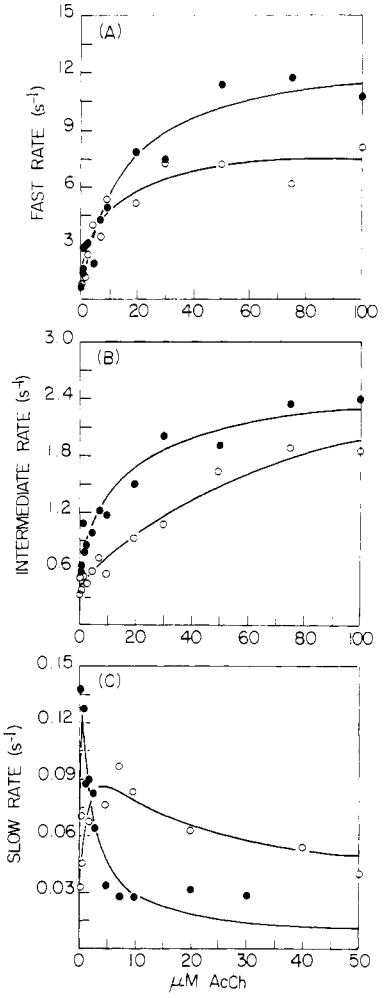


FIGURE 2: Effect of receptor reduction and alkylation on the kinetics of AcCh binding to IAS-labeled membrane fragments. The rates of the fast, intermediate, and slow phases for control (●) and DTT/$IAcNH_2$-treated (○) membrane preparations are plotted as a function of AcCh concentration. Solid lines are those calculated by using the following parameters from fitting the data to Scheme I as described by Dunn et al. (1980). (A) Fast phase: control, $k_{-2}K_4$ = 2.1 μM

$$k_{fast} = \frac{k_{-2}K_4}{K_4 + [L]} + \frac{k_2[L]}{K_1 + [L]} \quad (A)$$

s^{-1}, K_4 = 17.4 μM, k_2 = 13.1 s^{-1}, and K_1 = 14.6 μM; DTT/$IAcNH_2$, $k_{-2}K_4$ = 90 μM s^{-1}, K_4 = 67.7 μM, k_2 = 7.9 s^{-1}, and K_1 = 13.1 μM. (B) Intermediate phase: control, k_{-5} = 0.60 s^{-1}, $k_5/(K_1K_2K_4)$ = 0.88

$$k_{inter} = k_{-5} + \frac{k_5[L^2]/(K_1K_2K_4)}{1 + [L]/(K_1K_2) + [L^2]/(K_1K_2K_4)} \quad (B)$$

s^{-1} μM^{-2}, K_1K_2 = 0.134 μM, and $K_1K_2K_4$ = 2.25 μM^2; DTT/$IAcNH_2$, k_{-5} = 0.41 s^{-1}, $k_5/(K_1K_2K_4)$ = 0.018 s^{-1} μM^{-2}, K_1K_2 = 2.16 μM, and $K_1K_2K_4$ = 147 μM^2. (C) Slow phase: control, k_{-3} = 0.006 s^{-1},

$$k_{slow} = k_{-3} + \frac{k_3[L]/(K_1K_2)}{1 + [L]/(K_1K_2) + [L^2]/(K_1K_2K_4K_5)} \quad (C)$$

$k_3/(K_1K_2)$ = 1.49 s^{-1} μM^{-1}, K_1K_2 = 0.134 μM, and $K_1K_2K_4K_5$ = 0.175 μM^2; DTT/$IAcNH_2$, k_{-3} = 0.030 s^{-1}, $k_3/(K_1K_2)$ = 0.049 s^{-1} μM^{-1}, K_1K_2 = 2.16 μM, and $K_1K_2K_4K_5$ = 21.9 μM^2.

concentration dependence of the kinetic constants measured for each phase. Qualitatively similar behavior has been reported for Carb binding (Dunn et al., 1980), and the mechanism previously proposed (see Scheme I) was consistent also with the AcCh binding kinetics.

According to this mechanism, the fast phase arises from R'L formation, the intermediate phase from C_2 formation, and the

Table I: Effect of Reduction and Alkylation on the Kinetics of AcCh Binding to IAS-Labeled AcChR[a]

parameter	control	DTT/$IAcNH_2$[b] treated
K_1 (μM)	14.6	13.1
K_4 (μM)	17.4	67.7
k_2 (s^{-1})	13.1	7.9
k_{-2} (s^{-1})	0.12	1.33
k_3 (s^{-1})	0.20	0.11
k_{-3} (s^{-1})	0.006	0.030
k_5 (s^{-1})	1.98	2.60
k_{-5} (s^{-1})	0.60	0.41
K_2	0.0092	0.167
K_3	0.03	0.286
K_5	0.30	0.16
$K_1K_2K_3$ (nM)	4.0	619.0

[a] Parameters were derived from a fit of the data shown in Figure 2 to Scheme I. [b] $IAcNH_2$ is iodoacetamide.

slow phase from C_1 formation. This slow phase decreases in both rate and amplitude at high ligand concentration as a consequence of the binding of a second ligand to R'L which favors the formation of C_2.

Conversion of the AcChR to a form having lower affinity for AcCh by extensive reduction and alkylation did not have a dramatic effect on the kinetics of AcCh binding, and the three phases measured were of similar rate to those characterizing the control preparations. The ligand concentration dependence of each phase was however slightly different for the two membrane preparations as shown in Figure 2. In this figure, the solid lines are those calculated from nonlinear regression fitting of the data to the appropriate equations describing Scheme I which are given in the figure legend and were previously derived by Dunn et al. (1980). According to Scheme I, the rate of the slow phase reaches a maximum at a ligand concentration $L_{max} \approx (K_1K_2K_4K_5)^{1/2}$ (Quast et al., 1979; Dunn et al., 1980). In the case of the control preparation, this maximum is reached at a much lower AcCh concentration (0.8 μM, see Table I) than for the reduced and alkylated AcChR (4.9 μM). The curves describing the concentration dependence of the rate of this slow phase (Figure 2C) are therefore qualitatively different although both are consistent with the predictions of Scheme I. Comparison of the kinetic parameters obtained from the fitting procedures (Table I) indicates that the major effect of reduction and alkylation was a more than 10-fold increase in the value of k_{-2} with also a significant, though slightly lower, increase in the fitted value of k_{-3}.

Effect of Reduction and Alkylation on Cation Flux. The ability of AcChR to mediate agonist-induced cation flux before and after reduction and alkylation was measured by the Tl^+ fluorescence quenching method of Moore & Raftery (1980). This method in which Tl^+, in the presence of agonist, is rapidly mixed with membrane vesicles loaded with the fluorophore, ANTS, is sensitive to relatively minor perturbations of receptor function since the rate of Tl^+ transport into the vesicles is directly proportional to the number of ion channels opened in response to agonist binding.

In the absence of agonist, the signal change occuring when control membrane vesicles were mixed with Tl^+ was a quench having a half-time of about 10 s due to the slow leakage of Tl^+ across the membrane. Inclusion of AcCh in the buffer containing Tl^+ greatly enhanced the rate of flux as shown in Figure 3. The rate of Tl^+ transport increased with the increasing agonist concentration, and at AcCh concentratons greater than 25 μM, the rate of quench exceeded the capability of the stopped-flow technique. At high ligand concentrations,

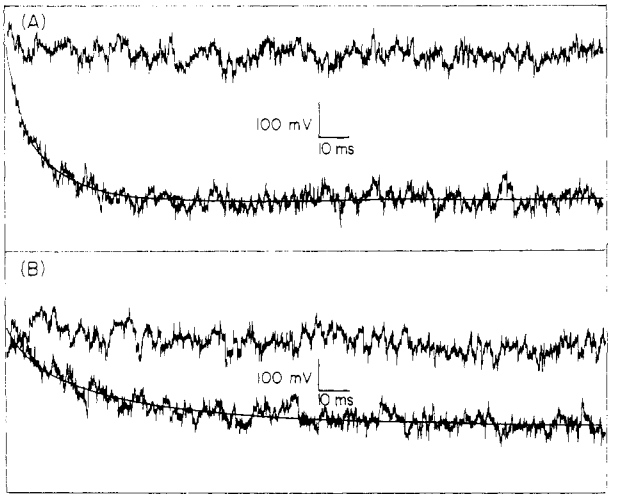


FIGURE 3: Kinetics of AcCh-induced Tl^+ flux. (A) Control membrane vesicles. (Upper trace) ANTS-loaded vesicles were mixed with 10 mM Hepes and 17 mM $TlNO_3$, pH 7.4 (final concentrations after mixing); (lower trace) kinetics of Tl^+ flux in the presence of 10 μM AcCh. The solid line was calculated by using the best-fit parameters A_1 = 910 mV and k_1 = 54.9 s^{-1}. (B) Membrane vesicles reduced by 1 mM DTT and alkylated with 3 mM iodoacetamide. (Upper trace) Vesicles were mixed with buffer containing 17 mM $TlNO_3$ and 10 μM AcCh; (lower trace) Tl^+ flux induced by 250 μM AcCh. The solid line was calculated by using A_1 = 484 mV and k_1 = 20.7 s^{-1}.

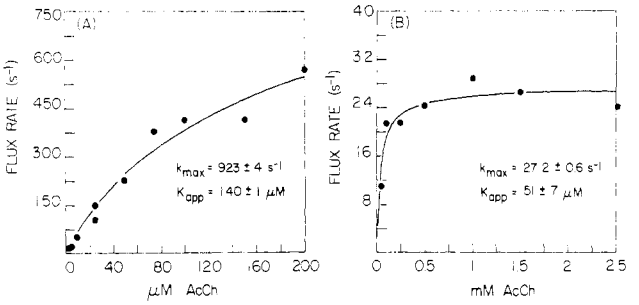


FIGURE 4: Effect of acetylcholine concentration on Tl^+ flux rate. (A) Control membrane vesicles: At concentrations of AcCh greater than 25 μM, the flux rate was extrapolated from the apparent rate constant in the presence of HTX as described in the text. The data were fitted to the equation $k_{app} = k_{max}[L]/(K_d + [L])$, giving k_{max} = 923 ± 4 s^{-1} and K_d = 140 ± 1 μM. (B) DTT/$IAcNH_2$-treated membrane vesicles: data were fitted to the same equation as in (A), giving k_{max} = 27.2 ± 0.6 s^{-1} and K_d = 50 ± 7 μM.

Tl^+ flux was therefore measured by using receptor preparations which were partially inactivated by incubation with 1–3 μM HTX to reduce the number of channels capable of activation and therefore also the rate of ion transport (Moore & Raftery, 1980). The true flux rate was then calculated by normalization to the results obtained for fully active preparations. Figure 4A shows the results of these experiments, and nonlinear regression fitting of the data, assuming that a single ligand binding leads to channel opening, gave estimates of 923 s^{-1} for the maximal Tl^+ transport and 140 μM for the effective dissociation constant.

Membrane fragments which had been reduced and alkylated prior to being loaded with ANTS displayed quite a different flux response. In the absence of agonist, the Tl^+ leak rate was not significantly different from that measured for control vesicle preparations. However, at AcCh concentrations less than ~50 μM, the amplitude of the agonist-induced enhancement of flux, if any, was too small relative to the large Tl^+ leak signal to be accurately measured. When the AcCH concentration was increased above 50 μM, some agonist-in-

duced increase in the cation transport rate was apparent (Figure 3B), but this rate reached a saturating level of only $\sim 27\ s^{-1}$ at high ligand concentration.

Discussion

Covalent labeling of the membrane-bound acetylcholine receptor by IAS did not affect either its affinity for [^{3}H]AcCh or the number of high-affinity binding sites measured in equilibrium experiments. It has previously been demonstrated that the specific fluorescence changes occurring on ligand binding arise from IAS labeling of a reduced disulfide and not a free receptor sulfhydryl group (Dunn et al., 1980). The concentration of DTT required for effective IAS modification is similar to that known to reduce a reactive disulfide bond in the vicinity of an agonist binding site on the subunit of molecular weight ~40 000 (Weill et al., 1974; Chang et al., 1977; Damle et al., 1978; Moore & Raftery, 1979a; Lyddiatt et al., 1979). In addition, IAS modification inhibited subsequent covalent binding of bromo[^{3}H]acetylcholine (Dunn et al., 1980). These observations indicate that IAS labels the same disulfide, close to this ligand binding site, which is labeled by the affinity alkylating reagents. The finding that IAS modification did not alter the equilibrium binding properties of the receptor therefore supports the notion that an intact disulfide bond in this position is not required for correct ligand discrimination as was previously suggested by Barrantes (1980) from his indirect study of the effects of MPTA labeling on the direction of changes in the receptor intrinsic fluorescence induced by ligand binding.

Reduction of IAS-labeled membrane fragments by higher concentrations of DTT (1 mM) followed by alkylation with excess iodoacetamide resulted in an increase in the dissociation constant for [^{3}H]AcCh binding from 0.033 to 0.45 μM (Figure 1). This decrease in ligand affinity is in agreement with results previously obtained from direct [^{3}H]AcCh binding measurements (Schiebler et al., 1977) and indirectly from the effect of incubation with Carb on the rate of α-toxin binding (Moore & Raftery, 1979a,b; Barrantes, 1980; Walker et al., 1981). In contrast to the results of Schiebler et al. (1977), who found that DTT treatment caused a decrease in the Hill coefficient for AcCh binding from 1.7 to 1.0, the Scatchard plots of Figure 1 show no curvature at low ligand concentrations and thus give no evidence of positive cooperativity for either the control or the treated preparations.

Incubation of AcChR-enriched membrane fragments with agonists leads to a time-dependent increase in affinity which is thought to be the parallel of in vivo desensitization (Weber et al., 1975; Weiland et al., 1976, 1977; Lee et al., 1977; Quast et al., 1978). The effects of sulfhydryl group modification on this affinity state transition have been studied by measuring the decrease in the initial rate of radiolabeled α-toxin binding with time of exposure to agonist. Using this approach, Moore & Raftery (1979b) showed that after extensive reduction and alkylation no measurable affinity change was induced by incubation of AcChR with 1 μM Carb. However, when higher agonist concentrations were used to counteract the decreased ligand affinity of the receptor, some agonist-mediated transitions could be measured although both the initial and final states had reduced affinity for Carb compared to control values (Barrantes, 1980; Walker et al., 1981).

Measurement of the kinetics of radiolabeled α-toxin binding is an indirect method of studying ligand-induced affinity changes and is subject to ambiguity and misinterpretation of results as a consequence of the limited resolution of the technique and the complexity of the system under study. Direct monitoring of the kinetics of interaction of AcCh and receptor-enriched membrane fragments has given a quantitative measurement of the effects of sulfhydryl group modification on the conformational changes occurring on ligand binding.

Extensive reduction and alkylation did not radically alter the kinetics of AcCh binding, and the observed behavior was consistent with the mechanism (see Scheme I) previously proposed to account for Carb binding kinetics (Dunn et al., 1980). In this model, the $R \rightarrow C_1$ pathway can be equated with the ligand binding properties measured in equilibrium experiments by using radioactive ligand (Figure 1) since the pathway for binding of the second ligand, $R'L \rightarrow C_2$, becomes important, and indeed dominant, only at high ligand concentrations as a consequence of the relatively high value of the dissociation constant K_4. The calculated dissociation constant for the first ligand binding, $K_1K_2K_3$, was significantly increased, from 4 nM to 0.62 μM, as a result of reduction and alkylation (Table I), and these estimates are in reasonable agreement with the values obtained independently from the Scatchard plots in Figure 1. The decrease in affinity may be attributed primarily to an increase in the reverse rate constant k_{-2}, characterizing the $RL \rightleftharpoons R'L$ transition. The value of k_{-3} was also increased after such thiol modification, but this parameter is rather less reliable than k_{-2} since the nonlinear least-squares technique used to fit the kinetic parameter of the slow phase requires that an initial estimate for k_{-3} be derived from the intercept of the plot of the slow rate constant against ligand concentration and, as is readily apparent in Figure 3, this is subject to large error, particularly for the control preparations.

Measurements of AcCh binding kinetics therefore show directly that reduced and alkylated receptor preparations retain the ability to undergo a time-dependent increase in affinity upon exposure to agonist although this is shifted to higher ligand concentrations as a reflection of the overall reduced affinity. According to Scheme I, when AcCh and AcChR are mixed, RL is formed in a rapid equilibration reaction, and this first complex isomerizes to R'L on a fairly fast time scale. The conformational change leading to C_1 occurs on a much slower time scale and, at a ligand concentration of 1 μM, has a half-time of about 10 s. This transition does, however, lead to a tighter binding of the agonist, the dissociation constant of the complex decreasing by the factor of K_3 (<1, Table I). These results are consistent with the previous observations that, after reduction, the receptor can still undergo "quantitatively diminished" state transitions as measured by the inhibition of the initial rate of α-toxin binding with Carb as agonist (Barrantes, 1980; Walker et al., 1981).

Electrophysiological experiments have shown that the postsynaptic responses of isolated eel electroplax were dramatically decrased by treatment with DTT (Karlin & Bartels, 1966). The effects of reduction and alkylation on the functional responses of AcChR-enriched membrane vesicles have been investigated by using in vitro $^{22}Na^+$ flux assays developed by Kasai & Changeux (1971); by measuring $^{22}Na^+$ efflux, Schiebler et al. (1977) found that an apparent decrease in the ability of the receptor to mediate agonist-induced cation translocation after treatment with dithioerythritol could be counteracted by increasing the concentration of agonist. It was concluded that reduction did not affect the ion flux properties of the receptor although it did reduce the affinity for agonist. Similar results were recently obtained by Walker et al. (1981), who further showed that subsequent alkylation of the reduced membranes by NEM resulted in complete inhibition of cation flux.

The $^{22}Na^+$ flux filtration method gives only a qualitative

measure of receptor function since the limited time resolution of the technique allows only integrated responses to be measured, giving an estimate of the flux amplitude with no information on the rate of ion transport. The recent development of a stopped-flow technique for measuring influx of Tl^+ ions (Moore & Raftery, 1980) allows a quantitative measure of the effects of chemical modification on the ability of the receptor to mediate cation flux. When unmodified membrane vesicle preparations were used, the rate of ion transport increased with increasing AcCh concentration, and an apparent K_d of 140 μM for the AcCh-induced enhancement was measured, which is in good agreement with previous results obtained by using a reconstituted receptor system (Wu et al., 1981). After reduction and alkylation, the Tl^+ flux response was radically altered, and the agonist-mediated rate was much slower than that of the control. This rate reached a limiting value of $\sim 27\ s^{-1}$ at AcCh concentrations greater than ~ 0.5 mM which suggests that the decreased response was not due to a drastic increase in the effective K_d for AcCh. There are two obvious possible interpretations of this behavior; one is that the number of ions transported per channel was significantly decreased by disulfide modification, perhaps due to alkylation of sulfhydryl groups directly associated with the ion channel; however, a simpler explanation is that a few receptor molecules were unmodified by the DTT and iodoactamide treatment. Comparison of the maximal flux rates displayed by the treated and control membrane preparations would suggest that the fraction remaining which was capable of activation was less than 3% of the total, which is not an unreasonable estimate of the extent of reaction. The concentration of AcCh eliciting half-maximal flux rate for the modified preparation was ~ 50 μM which is similar to that measured for the control membrane vesicles (Figure 4). Although the experimental error in this estimated value is large, as a consequence of the relatively small range of measured rate constants, this finding is consistent with the occurrence of residual unreacted AcChR.

It has previously been reported that IAS-labeled membrane preparations retain the ability to mediate cation translocation (Dunn et al., 1981) which shows that the relatively mild reducing conditions (50 μM DTT) used for this modification are not sufficient to alter receptor function. At the much higher concentrations of DTT used in the above experiments, it may be assumed that the disulfide bond(s) between the 65-kdalton subunits which cause receptor dimerization was (were) also reduced (Chang & Bock, 1977). It is, however, unlikely that the observed inhibition of the flux response was due to modification of this bond alone since it has been shown that monomers reconstituted into membrane vesicles (Wu & Raftery, 1981) and monomers produced by trypsin treatment of native membranes (Conti-Tronconi et al., 1982) can still elicit a full Tl^+ flux response.

In summary, the results presented here show that extensive reduction and alkylation of the membrane-bound receptor decrease the affinity of AcCh for its high-affinity binding site by more than 10-fold. However, this is unlikely to be the direct result of modification of the readily reducible disulfide bond known to exist near the site since it was unaffected by the presence of the bound fluorophore, IAS. The kinetic mechanism for AcCh binding was not dramatically affected by reduction and alkylation of the AcChR, and, following the initial binding step, the receptor–ligand complex underwent conformational transitions similar to those characterizing the control preparations but with variations in the values of some of the kinetic parameters as a result of the overall reduced affinity. Although AcCh could still bind to the reduced and alkylated receptor, the functional response of cation translocation was seriously perturbed, and the observed slow rate of agonist-mediated flux could be adequately explained by the existence of a few, unmodified receptors.

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Multiple Binding Sites for Agonists on *Torpedo californica* Acetylcholine Receptor†

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ABSTRACT: The equilibrium and kinetic properties of agonist binding to the membrane-bound acetylcholine receptor from *Torpedo californica* have been measured by the fluorescence changes of a probe, 4-[[(iodoacetoxy)ethyl]methylamino]-7-nitro-2,1,3-benzoxadiazole, which was covalently bound to the receptor protein. Dissociation constants for the binding of several agonists have been measured in fluorescence titration experiments, and these are in good agreement with apparent equilibrium constants obtained from the concentration dependence of the cation flux response measured in quantitative in vitro kinetic experiments. These results provide evidence for the existence of a low-affinity binding site for agonists which is likely to be a functionally important site for channel activation. The kinetics of carbamylcholine and acetylcholine binding to this site have been measured in stopped-flow fluorescence experiments. Kinetic traces were recorded over a wide range of agonist concentrations, and all could be fit by a single exponential process whose rate and amplitude increased hyperbolically with the concentration of ligand. The observed signal change has been ascribed to a conformational transition of the receptor-ligand complex, and this occurs on a millisecond time scale at saturating ligand concentrations which is sufficiently fast to suggest a role for this binding site in the process of channel activation. These results indicate that in the *Torpedo* AcChR activation and desensitization may be parallel processes which are mediated by agonist association with different receptor binding sites.

The binding of acetylcholine to its nicotinic receptor at the neuromuscular junction results in the transient opening of cation-selective channels. Electrophysiological studies have shown that channel activation occurs within a few milliseconds of transmitter release, but, after prolonged exposure to agonist, the process of desensitization (Katz & Thesleff, 1957) leads to channel closing and a loss of the permeability response over a time scale of several seconds.

Much information on the ligand binding properties of the acetylcholine receptor (AcChR)[1] has come from studies of membrane-bound preparations purified from the electric organs of *Torpedo* species. *Torpedo* AcChR undergoes an agonist-induced conformational change to a state having higher affinity for these ligands, and this has been correlated with the process of pharmacological desensitization (Weber et al., 1975; Weiland et al., 1976, 1977; Lee et al., 1977; Quast et al., 1978a). Apparent dissociation constants for Carb binding to the resting and induced high-affinity states have been estimated from the time-dependent increase in Carb inhibition of the rate of α-neurotoxin binding to be $\sim$30 and 0.1 μM, respectively (Weiland et al., 1977; Quast et al., 1978a). This in vitro transition also occurs on a relatively slow time scale, and therefore to gain information on the ligand binding events leading to the functional response of channel opening, it is necessary to monitor the ligand-AcChR interaction on rapid time scales.

A variety of fluorescence techniques have recently been used for measuring the kinetics of binding of agonists to the membrane-bound AcChR. These include the monitoring of changes in the intrinsic fluorescence of the receptor protein (Bonner et al., 1976; Barrantes, 1976), the use of extrinsic probes, both noncovalent (Grunhagen & Changeux, 1976; Grunhagen et al., 1976, 1977; Schimerlik et al., 1979; Quast et al., 1978b, 1979) and covalent (Dunn et al., 1980), and the use of fluorescent analogues of acetylcholine (Heidmann & Changeux, 1979, 1980; Jürss et al., 1979). In all of these studies, conformational changes were observed, and a variety of

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[1] Abbreviations: AcChR, acetylcholine receptor; Carb, carbamylcholine; MBTA, [4-(*N*-maleimido)benzyl]trimethylammonium diiodide; IANBD, 4-[[(iodoacetoxy)ethyl]methylamino]-7-nitro-2,1,3-benzoxadiazole; α-BuTx, α-bungarotoxin; DEAE, diethylaminoethyl; ANTS, 8-amino-1,3,6-naphthalenetrisulfonate; HTX, histrionicotoxin; DTT, dithiothreitol; PTA, phenyltrimethylammonium chloride; AcCh, acetylcholine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; NBD, 7-nitro-2,1,3-benzoxadiazole.

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