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Ligand Interactions with Cholinergic Receptor-Enriched Membranes from *Torpedo*: Influence of Agonist Exposure on Receptor Properties

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SUMMARY

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Carbamylcholine and a nitrogen-substituted mononitroxide congener of decamethonium (I) show an inhibitory capacity toward the binding of cobra α-toxin to membranes enriched in cholinergic receptor that is dependent on the duration of exposure of the quaternary ligand prior to adding toxin. This behavior is characterized by (a) inhibition of the initial rate of toxin binding, which depends on the duration of ligand conditioning, (b) depression of the equilibrium binding of toxin, which at short exposure intervals cannot be accounted for by the decrease in association rate for toxin binding, and (c) a slow change in receptor state, in which the affinity for ligand is increased. The last can be demonstrated directly by electron spin resonance measurements of the free and bound resonance peaks of the spin-labeled bisquaternary ligand. The slow increase in affinity measured by ESR appears to be slightly smaller than the increased affinity calculated from conditioning effects of ligand exposure on the initial rate of toxin binding. The spin-labeled ligand is completely dissociated by excess toxin and binds to one site per toxin binding site. Conditioning effects of the ligands are lost upon solubilization of the receptor with Triton X-100. In contrast to the above ligands, the antagonist *d*-tubocurarine does not show time-dependent inhibition of toxin binding, and its interaction with the receptor-enriched membranes appears to be competitive with toxin binding.

INTRODUCTION

A wide variety of studies have been undertaken on the competitive interaction between the binding of cholinergic agonists or antagonists and the binding of neurotoxins on isolated nicotinic receptors (1-8), and these studies have provided supportive evidence that the isolated toxin binding protein is derived from the cholinergic receptor. Changes in ligand specificity for the receptor induced by various detergents have also been documented, and it appears that agonist binding affinities are influenced more by

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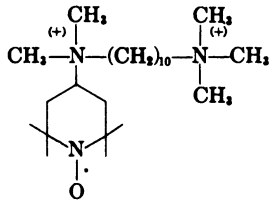
detergent solubilization than those of antagonists (5–8). More recently Weber *et al.* (9) reported that the apparent competition between cholinergic agonists and cobra α-toxin is dependent on the duration of agonist exposure. We had seen similar behavior and decided to examine this slow conversion of receptor state in more detail. In contrast to the previous study, our experiments have been carried out with toxin in excess and measure the entire time course for approach to equilibrium. Although the kinetics was consistent with a slow conversion between receptor states, the results reveal complexities which were not apparent when only initial rates were monitored with receptor in excess. With a spin-labeled ligand, we have attempted to correlate the conditioning effects of the ligand on toxin binding with the slow increase of affinity for the ligand as measured by electron spin resonance titrations. Some of our findings have been presented previously in abstract form (10).

MATERIALS AND METHODS

Materials. The spin-labeled analogue of decamethonium, 4-[dimethyl-10-(trimethylammonio)decylammonio]-2,2,6,6-tetramethyl-1-piperidinyloxyl diiodide (I),



I

was synthesized as previously described (11). Carbamylcholine chloride and *d*-tubocurarine were products of Sigma Chemical Company. ^{125}I was obtained from New England Nuclear Corporation (NEZ 033-H). All other chemicals were of reagent grade or the highest purity available.

Preparation of iodinated toxin. Cobra α-toxin (*Naja naja siamensis*) was purified to apparent homogeneity (12) and iodinated under conditions such that stoichiometry approached 1:1 as described previously (13). The iodinated toxin was then electrofocused at 4° on a 110-ml LKB column, using pH 9.0–11.0 Ampholine. Focusing was carried out for 48–72 hr with a potential difference of 400 V. The major radioactivity peak corresponded to a pH of 10.65 and the ^{125}I was incorporated only as monoiodotyrosyl residues. A smaller peak, at pH 10.37, contained 2–21% of the total radioactivity in the various preparations. In a separate experiment uniodinated toxin, when mixed with iodinated toxin, migrated to a pH of 10.77 and only partially overlapped the major iodinated peak. The electrofocusing procedure resulted in a 24–30% increase in bound radioactivity when the unfocused and focused toxins in stoichiometric excess were assayed with equivalent quantities of receptor. Since monoiodotoxin is the predominant toxin species following iodination, the additional enhancement of bound radioactivity presumably reflects separation from residual uniodinated toxin. The material comprising the major peak was dialyzed against 0.01 M sodium phosphate, pH 7.0, for 5–6 hr, and then stored on ice in the presence of 0.02% NaN_3. Toxin was prepared routinely on a monthly basis, and each new preparation was assayed in comparison with the previous batch for receptor binding activity.

Membranes enriched in cholinergic receptor were isolated by the sequential differential centrifugation, density gradient centrifugation, and affinity partitioning steps outlined by Flanagan *et al.* (13). To increase yields for the ESR studies, a discontinuous gradient consisting of 10 ml of 55%, 15 ml of 45%, and 15 ml of 35% sucrose and 10 ml of resuspended membranes replaced the continuous sucrose gradients, and the material was not subjected to the subsequent affinity partitioning step. Specific activities for the preparations ranged between 0.8 and 1.6 nmoles of bound toxin per milligram of protein for the discontinuous gradients, 1.2–2.2 nmoles of bound toxin per milligram of protein for the continuous gradient, and 2.2–3.5 nmoles of bound toxin per milligram of protein for the material recovered after affinity partitioning. Apparent changes in toxin binding kinetics

which are dependent on the duration of carbamylcholine exposure were comparable with each method of preparation. Acetylcholinesterase activity was measured routinely in the discontinuous gradient preparations. Since the turnover number and stoichiometry for ligand binding are known for this enzyme (14), the sites for bisquaternary ligands attributable to acetylcholinesterase were calculated to be between 1.8% and 2.4% of the toxin binding sites.

Kinetics of toxin binding. Rates of α-toxin binding were usually monitored with 8–10 nM toxin (16–41 Ci/mmole) and membranes that were 3–6 nM in receptor sites. Thirty minutes prior to initiating the reaction the membranes were diluted to the above concentration in 0.1 M NaCl–0.01 M sodium phosphate, pH 7.4, at 22°. Competing ligand was then added at the appropriate time to give specified exposure duration, and [^{125}I]α-toxin was subsequently added to initiate the toxin binding reaction. Triton X-100 (0.1%) was included in the reaction mixture only in the case of solubilized receptor preparations. Either 0.05- or 0.1-ml aliquots were removed from the 5-ml reaction vessel, spotted on DEAE-cellulose discs, washed for 30 min in 0.1% Triton-containing buffer, and counted as previously described (13, 15). The rate at which non-receptor-associated toxin washed off the discs was found to be the same when carbamylcholine was present in the reaction mixture or when incubations were carried out in the absence of receptor. Removal of unbound toxin from the discs was complete within 30 min.

Association of toxin and receptor was analyzed according to the second-order rate equation, whose integrated form is

$$\ln \frac{[T_0] - [RT]}{[R_0] - [RT]} - \ln \frac{[T_0]}{[R_0]} = ([T_0] - [R_0])k_T t$$

where T_0 and R_0 are the initial toxin and receptor concentrations and k_T is the bimolecular association rate constant. Calculations were done on a Tektronix model 31 computer, and the initial toxin and receptor concentrations, the associated radioactivity corresponding to RT, total toxin, and blanks were supplied to the program. The blanks obtained by application of toxin to the discs in the absence of receptor were the same as those found following a 2-hr incubation of toxin and receptor in the presence of 10 mM carbamylcholine. Slow adsorption of toxin to glass was apparent, so that total toxin concentrations were measured over the entire time course. k_T was determined from a least-squares fit for the slope of the above equation. Details of the computer program may be obtained on request from the authors.

Toxin dissociation rates were monitored by incubating receptor with a 1.2-fold molar excess of labeled toxin for 2 hr prior to the addition of a 1000-fold excess of unlabeled toxin, 1 mM carbamylcholine, or excess toxin in the presence of 30 μM carbamylcholine. At various times up to 8 hr, retention of labeled toxin was monitored by the filter binding assay described above.

Solubilization of receptor. On the day the toxin binding experiments were initiated, the membrane fragments described above were gently agitated with 0.1% Triton X-100 in 0.1 N NaCl and 0.01 M sodium phosphate, pH 7.4, at 4° for 30 min. The solubilized membranes were then centrifuged at 40,000 × g for 30 min at 4°, and the supernatant was employed in the binding measurements.

Electron spin resonance spectrometry. Electron spin resonance spectra were obtained at 22° with a Varian E-4 spectrometer operating at 9.5 GHz. Titrations were conducted by adding increments of spin label from a Hamilton microsyringe to the top of a quartz aqueous sample cell containing 0.5–1.0 ml of membranes suspended in 0.1 N NaCl–0.01 M sodium phosphate buffer, pH 7.4 (16). Power saturation studies were employed to ascertain allowable modulation and power settings (17). For the kinetic studies the concentration of unbound spin label was estimated from the amplitude of the low-field line (Fig. 5b, arrow 2). Amplitudes of the low-field lines arising from the free species were monitored from 30 sec after ad-

dition of ligand (the shortest time possible) to 1 hr. Amplitude was measured from peak-to-peak distances and related to concentration by calibration with known amounts of unbound ligand.

RESULTS

α-Toxin association and dissociation kinetics. Toxin association with the receptor-enriched membranes appears to follow bimolecular kinetics, indicating that toxin and the membrane-associated receptor behave operationally as a single species (Fig. 1a).[3] Dissociation was unimolecular for at least the first 8 hr and appeared to be the same whether reassociation of labeled toxin was prevented by excess unlabeled toxin, a high concentration of carbamylcholine, or excess toxin in the presence of carbamylcholine (Fig. 1b). A toxin dissociation constant of 1.7×10^{-11} M can be estimated from the association and dissociation kinetics of the iodinated derivative. Toxin dissociation in the absence of excess unlabeled toxin or competing ligand was slower, indicating that dissociation was not due to deterioration of the preparation.

Influence of carbamylcholine and d-tubocurarine on time course of toxin association. When α-toxin is added to the membrane-associated receptor following a 30-sec exposure to *d*-tubocurarine, a reduction of the toxin association rate is observed at concentrations where there is minimal depression of toxin binding at equilibrium (Fig. 2a). This behavior would be expected for a competitive inhibitor of toxin binding, since the free toxin concentrations at equilibrium greatly exceed the toxin-receptor dissociation constant. In contrast, toxin addition following a 30-sec carbamylcholine exposure results in reductions of initial velocity and equilibrium binding of toxin that are nearly equal in magnitude (Fig. 2b). The concentration dependence of the decrease in binding at equilibrium and of the apparent velocity of association of toxin in the presence of the two ligands is plotted in Fig. 3.

Influence of ligand exposure time on α-toxin binding. A comparison of α-toxin binding rates following 30 sec and 30 min of carbamylcholine exposure shows very different rates of toxin association; yet bound toxin at equilibrium is nearly equivalent in both cases (Fig. 4A and Table 1). After carbamylcholine exposure for 30 min, bimolecular association is evident, while at 30 sec substantial deviations from the second-order rate law are regularly observed (Fig. 4A, inset). In contrast with the 30-sec exposure, the further reduction in initial rate following the longer duration of carbamylcholine exposure would account for reduced toxin binding at equilibrium. Thus toxin binding kinetics following a 30-sec carbamylcholine exposure appears to reflect a transient condition, and a conversion of receptor state is evident during the course of toxin association.[4] The kinetics of association gives the appearance of more than one phase, and, despite the large difference in apparent association rates following 30 sec and 30 min of exposure, similar quantities of bound toxin are observed at equilibrium.

In contrast, *d*-tubocurarine inhibition of

[3] Deviations from bimolecular association kinetics were evident in early experiments, when the toxin was not subjected to electrofocusing. We attribute these deviations to the presence of more than a single toxin species when precautions were not taken to ensure that only monoiodotyrosyl toxin was present.

[4] If a conversion of receptor state is occurring simultaneously with toxin binding, the following scheme could be employed to describe the kinetics:

$$\begin{array}{ccc} T + R & \xrightarrow{k_T} & TR \\ & & \downarrow k_I \\ T + R' & \xrightarrow{k'_T} & TR' \end{array}$$

where k_T and k'_T are the observed rate constants for toxin binding to the receptor at short and long intervals of ligand exposure and k_I is the isomerization rate constant. Integration of this equation, where $R' = 0$ at $t = 0$ and $T \gg R$ yields

$$RT = R_0\left[1 - \frac{1}{k'_T(T) - k_T(T) - k_I}\right.$$
$$\left.((k'_T(T) - k_T(T))e^{-(k_T(T)+k_I)t} - k_I e^{-k'_T(T)t})\right]$$

This scheme predicts that the ordinate intercept from the slow phase of the reaction will be dependent on toxin concentration. Varying the toxin concentration would enable one to estimate the three kinetic constants from the toxin binding profiles.

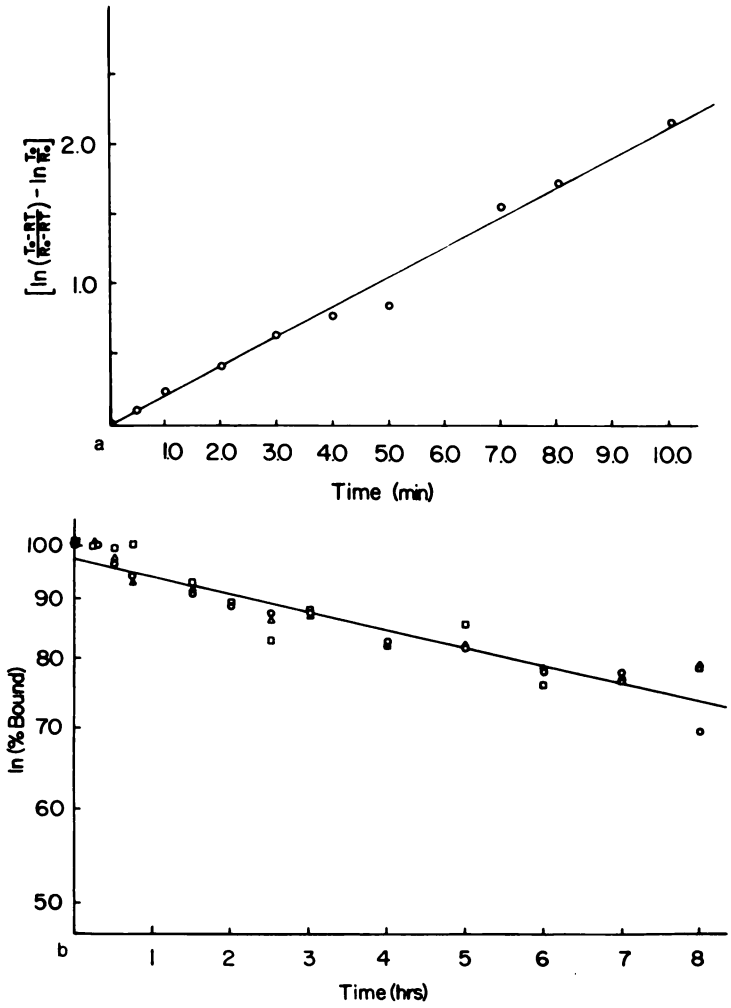


FIG. 1. *Kinetics of complexation between [^{125}I]α-toxin and receptor-enriched membranes*

a. Association kinetics. [^{125}I]α-Toxin (12 nM) and membranes 4 nM in toxin sites were incubated in 0.1 N NaCl–0.01 M sodium phosphate, pH 7.4, at 22°.

b. Dissociation kinetics. [^{125}I]α-Toxin (10 nM) and receptor (8 nM) were allowed to react for 2 hr prior to the addition of carbamylcholine or excess uniodinated α-toxin to prevent reassociation of labeled toxin. The time dependence of retention of labeled α-toxin was measured thereafter: ○, addition of 10 μM α-toxin; △, addition of 10 μM α-toxin and 30 μM carbamylcholine; □, addition of 1 mM carbamylcholine.

toxin binding to the membranes does not show a dependence on exposure duration (Table 1). The dependence on exposure time for carbamylcholine is also lost when the receptors are solubilized. Moreover, as noted previously (6–8), the apparent affinity of carbamylcholine ascertained by competition with α-toxin is reduced appreciably upon solubilization.

Behavior of the receptor-containing membranes following initial exposure to high concentrations of ligand with subsequent dilution is also illustrative of the slow, exposure-dependent effects of carbamylcholine on toxin binding. Exposure of receptor to 3 μM carbamylcholine for 29.5 min followed by dilution of ligand and receptor to 0.3 μM carbamylcholine 30 sec prior to toxin addition results in a slower rate of toxin association than if the receptor had been exposed to 0.3 μM carbamylcholine for 30 min (Table 2). If receptor exposed to 3 μM carbamylcholine is subsequently diluted to 3 nM carbamylcholine 30

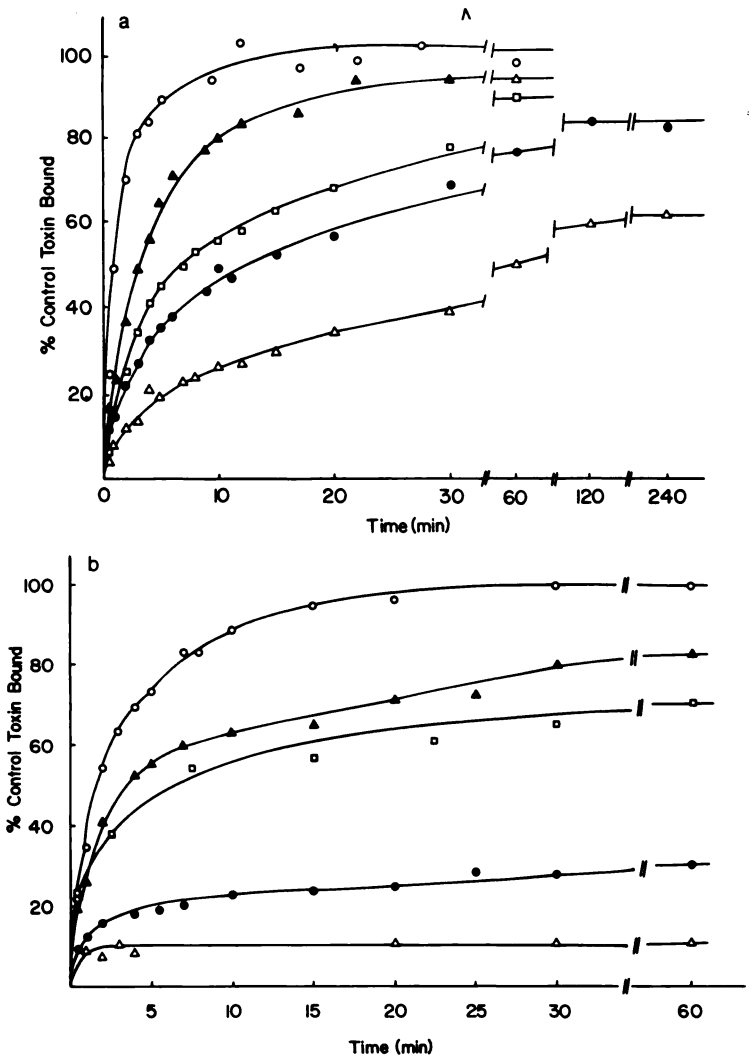


FIG. 2. *Kinetics of association of [^{125}I]α-toxin following a 30-sec exposure to d-tubocurarine or carbamylcholine*

In each case between 10 and 12 nM toxin and membranes which were 4–5 nM in toxin binding sites were employed. The procedure is described in MATERIALS AND METHODS. a. *d*-Tubocurarine inhibition: ○——○, no ligand; ▲——▲, 0.1 μM; □——□, 0.3 μM; ●——●, 1 μM; △——△, 3 μM. b. Carbamylcholine inhibition: ○——○, no ligand; ▲——▲, 1.5 μM; □——□, 3 μM; ●——●, 15 μM; △——△, 300 μM. The measurements shown in Fig. 2b were carried out for 4 hr. After 1 hr we did not observe further changes in toxin binding.

sec prior to adding toxin, the conditioning with 3 μM carbamylcholine no longer exerts an influence on toxin binding kinetics. Since, even with 30 min of exposure, 3 nM carbamylcholine is below the concentration of ligand required to inhibit toxin binding, the slow conversion of receptor state is only expressed by toxin binding in the presence of receptor-associated carbamylcholine. Thus the conversion is reflected in an increased affinity for carbamylcholine but not in an intrinsic change in toxin binding kinetics.

The possibility that carbamylcholine and α-toxin binding are competitive and that the affinity of the carbamylcholine-receptor complex increases slowly with time is consistent with the fall in rate of toxin binding as the duration of exposure to agonist is increased but is not entirely

consonant with the observed relationship between the rate of toxin binding and the bound toxin at equilibrium. In these experiments the excess toxin at equilibrium exceeds its dissociation constant by about two orders of magnitude. Thus, for a simple competitive mechanism to prevail, reduction by carbamylcholine of the amount of toxin bound at equilibrium would not be evident until the rate of toxin binding was reduced by almost two orders of magnitude, nor would one expect a similar reduction in equilibrium binding following 0.5 and 30 min of ligand exposure. The relationship between the reductions in toxin association rate and bound toxin at equilibrium suggests that binding of carbamylcholine and toxin may not be mutually exclusive and the carbamylcholine-receptor complex can also bind toxin.

Examination of the change in receptor state could be pursued more advantageously if a rapid and direct measure of ligand association were employed in conjunction with measurements of ligand competition with toxin. Electron spin resonance spectroscopy offers a particularly attractive approach, since the free and bound species exhibit separately observable signals and one is not reliant on a composite signal arising from a free and a bound species. Furthermore, the magnetic resonance technique obviates problems of light scattering intrinsic to optical monitoring of membrane-associated receptors.

Inhibition of toxin binding by spin-labeled bisquaternary ligands. As found with carbamylcholine, inhibition of toxin binding rate by the bisquaternary spin label analogue of decamethonium ($C_{10}A$) is dependent on the duration of ligand exposure (Table 1 and Fig. 4B). However, the magnitude of the time-dependent component of inhibitory capacity in relation to the total inhibition of rate appears to be somewhat smaller for the bisquaternary ligand when compared with carbamylcholine. Thus the spin-labeled ligand may lie somewhere between *d*-tubocurarine and carbamylcholine, where the former appears to inhibit toxin binding competitively and the latter primarily effects a slow alteration in receptor state. Nevertheless, the apparent competition between toxin and $C_{10}A$ is distinctly dependent on the exposure duration of the spin label, and the apparent degree of competition with toxin binding can be correlated with the fraction of bound ligand.

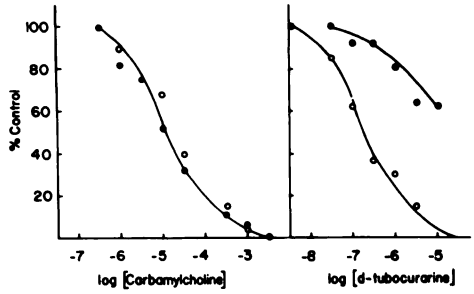


FIG. 3. *Concentration dependence of d-tubocurarine and carbamylcholine inhibition of toxin binding*

α-Toxin bound at equilibrium and initial velocities of α-toxin binding are plotted as a function of *d*-tubocurarine (right) and carbamylcholine (left) concentration. ●, [^{125}I]toxin bound at equilibrium; ○, estimated initial velocity for binding. The membranes were exposed to the respective ligand for 30 sec prior to the addition of toxin. Following 30 sec of exposure to carbamylcholine, deviations from bimolecular reaction kinetics were observed (see Fig. 4).

Electron spin resonance studies. The spin label ($C_{10}A$) possessed a sufficiently high affinity for the receptor to enable one to detect free and bound species using receptor concentrations between 0.5 and 10 μM in toxin binding sites. In the spectrum (Fig. 5a and b) the sharp resonances of the low- and high-field extrema that arise from unbound ligand ($2a_0$ = 32 G) are clearly separated from the broad low- and high-field resonances of the bound species ($2T_{\parallel}$ = 70 G). The broadened peaks with increased coupling constant suggest that the bound nitroxide is highly immobilized on the receptor surface. This high degree of immobilization is also reminiscent of the spectrum observed with binding of this ligand to an 11 S form of acetylcholinesterase (16). The temporal dependence of the amplitude of the low-field peak of the free species was measured following addition of spin label to the membrane at less than stoichiometric equivalence to toxin binding sites (Fig. 6). A time-dependent decrease in the relative population of free

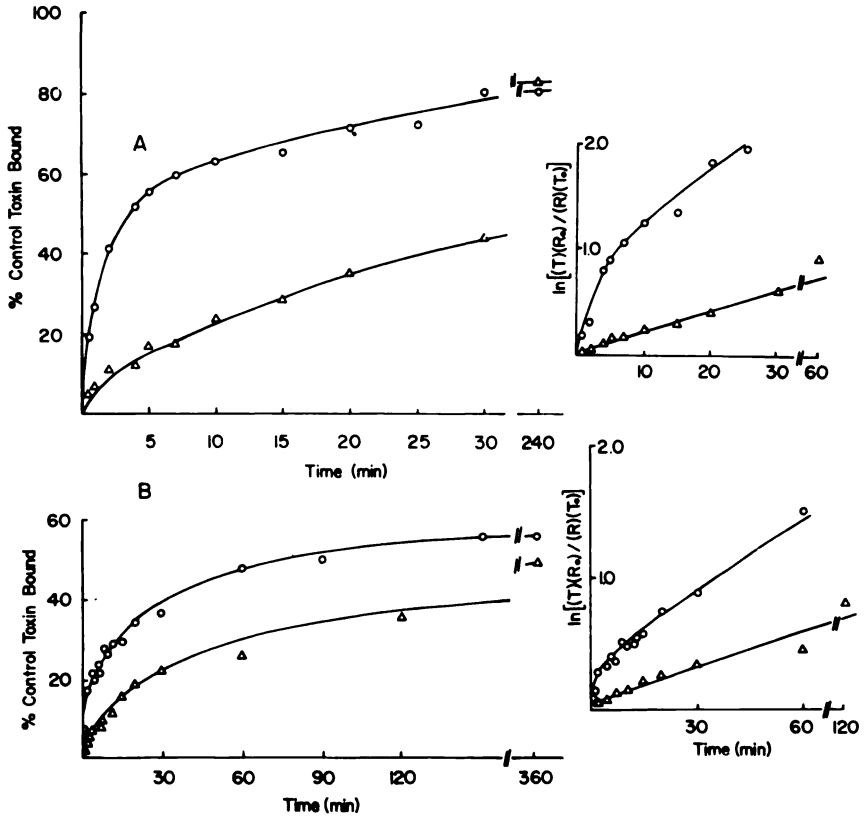


FIG. 4. *Kinetics of toxin binding following 30 sec and 30 min of exposure to carbamylcholine or mononitroxide analogue of decamethonium (I)*

Receptor-enriched membranes (4 nM in binding sites) were incubated with ligand for either 30 sec (○) or 30 min (△) prior to initiating the toxin binding reaction. The α-toxin concentration was 10 nM. Details of the procedure are given in MATERIALS AND METHODS. In the insets the same data are plotted according to the second-order rate equation described in MATERIALS AND METHODS. A. Carbamylcholine, 1.5 μM. B. Bisquaternary mononitroxide (I), 10 μM. T_0 and R_0 represent the total toxin and receptor site concentrations.

ligand was clearly evident in each of the kinetic studies carried out. Although the time dependence of the decrease in free ligand species essentially parallels the increase in inhibitory capacity for toxin binding, the decrease in dissociation constant does not always appear sufficient to account for the time-dependent increase in inhibition of toxin binding (Table 3). The slow change in signal amplitude for the unbound ligand does not appear to be due to chemical conversion of the nitroxide with a loss of spin intensity. As is evident in Fig. 5b, the decrease in free ligand signal is accompanied by an increase in signal for the bound ligand. Moreover, addition of a 3-fold molar excess of sodium dichromate to the receptor preparation does not alter the intensity of the ESR spectrum, suggesting that reduction of the nitroxide group does not occur during ligand binding.

The bound spin label appears to be specifically localized at the receptor, since addition of toxin in a stoichiometric excess results in a sharp, three-line spectrum characteristic of isotropic motion for the unbound ligand. The signal intensity also becomes equivalent to that found for an equivalent concentration of spin label in the absence of receptor and toxin. This behavior contrasts with the monoquaternary spin label, 8-doxylpalmitoylcholine, which appears to bind to the receptor site

as well as partition into the lipid membrane (18). The bisquaternary structure of $C_{10}A$ minimizes the hydrophobic contribution of the methylene bridge, thus eliminating nonspecific partitioning of the spin label into the membrane bilayer.

Titration of binding sites by ESR shows approximately one spin label site for each toxin binding site (Fig. 7). The dissociation constant obtained from a Scatchard analysis, $K = 1.7 \times 10^{-6}$ M, is in reasonable agreement with those estimated following a 30-min exposure to a single concentration of spin label (see Table 3). Measurement of the slow increase in binding affinity at a spin label concentration slightly

TABLE 1

Influence of ligand exposure on inhibition of toxin binding[a]

Ligand	Concentration	Exposure duration	k_T	Equilibrium ratio[b]
	μM	*min*	$M^{-1}\ sec^{-1}$	
Membrane-associated receptor				
None			$7.6 \pm 0.6 \times 10^5$ (13)[c]	1.0
d-Tubocurarine	1	0.5	2.8×10^4	0.88
		30.0	4.0×10^4	0.88
	10	0.5	2.2×10^4	0.68
		30.0	2.4×10^4	0.63
Carbamylcholine	0.3	0.5	6.7×10^5[d]	0.94
		30	2.5×10^5	0.76
	1	0.5	2.1×10^5[d]	0.80
		30.0	6.7×10^4	0.89
	1.5	0.5	1.3×10^5[d]	0.90
		30.0	3.0×10^4	0.90
	3	0.5	1.5×10^5[d]	0.65
		30.0	2.4×10^4	0.65
	10	0.5	1.9×10^5[d]	0.58
		30.0	3.0×10^4	0.45
	15	0.5	1.2×10^5[d]	0.30
		30.0	1.4×10^4	0.33
Bisquaternary mononitroxide spin label (I) ($C_{10}A$)	8.5	0.5	7.7×10^4[d]	0.79
		30.0	2.4×10^4	0.79
	10	0.5	4.1×10^4[d]	0.55
		30.0	1.6×10^4	0.52
	13	0.5	5.3×10^4[d]	
		30.0	1.5×10^4	
	15	0.5	8.3×10^4[d]	0.35
		30.0	2.2×10^4	0.45
	30	0.5	8.3×10^4[d]	0.21
		30.0	8.0×10^3	0.40
Solubilized receptor				
None			7.4×10^5 (2)	1.0
Carbamylcholine	80	0.5	1.6×10^5	0.82
		30.0	1.8×10^5	0.83

[a] The 0.5- and 30-min exposure conditions were used in paired reactions in the same experiment.

[b] Ratio of bound toxin at equilibrium in the presence and absence of inhibitor.

[c] Mean ± S.E.M.

[d] Multiphasic kinetics was consistently observed following 0.5 min of exposure to carbamylcholine and the bisquaternary spin label (Fig. 4). Rate constants were estimated from the linear portion of the seimilogarithmic plots of the second-order equation. This neglects an initial fast phase, which consituted 10–35% of the total reaction. Thus the apparent rate constants represent estimated values for toxin binding at 30 sec of exposure. The equation in footnote 4 should provide a more complete description of the reaction kinetics following 30 sec of exposure of ligand.

TABLE 2

Influence of prior carbamylcholine exposure on kinetics of cobra α-toxin binding to isolated membrane fragments

Isolahed receptor-containing membranes were conditioned for 29.5 min at the concentration of carbamylcholine specified in the left-hand column. Then, 0.5 min prior to initiating the toxin binding reaction, they were diluted in volume to achieve the concentration given in the second column. During the toxin binding reaction the concentration of receptor sites was between 6 and 10 nM. Each group of experiments was conducted on the same day as a comparative pair or triad.

Carbamylcholine concentration		k_T	Equilibrium ratio of toxin bound in presence and absence of carbamylcholine
Initial 29.5-min exposure	0.5 min before and during toxin binding		
μM	μM	$M^{-1}\ sec^{-1}$	
3	0.003	3.2×10^5	1.0
	0.003	3.18×10^5	1.0
1	0.01	6.7×10^5	1.0
	0.01	4.7×10^5	1.0
3	0.3	4.4×10^4	0.84
0.3	0.3	3.3×10^5	0.80
15	1.5	2.1×10^4	0.88
1.5	1.5	3.0×10^4	0.90
15	15	1.4×10^4	0.33
3	1	5.1×10^4	0.77
1	1	6.7×10^4	0.89
3	3	2.4×10^4	0.65

less than the number of toxin binding sites would thus seem appropriate, since a sufficient number of sites are available to accommodate unbound ligand.

DISCUSSION

The apparent competition between certain ligands and toxin for receptor sites, which is dependent on the duration of ligand exposure, presents an additional complication to interpretation of the relationship between agonist and antagonist binding to the receptor. For example, procedures such as equilibrium dialysis or high-speed centrifugation, which require a long time to establish equilibrium or to achieve separation of phases, do not enable one to distinguish whether association is a consequence of rapid binding of ligand or a slow, ligand-induced change in receptor state. Competition with toxin binding also suffers from the substantial time required for monitoring toxin association, and, as is evident following 30 sec of ligand exposure, a changing population in receptor state occurs during the assay interval. Second, the competitive assay makes it necessary to ascertain whether the change in receptor state affects the binding of either toxin or competing ligand, or both. Although the number of suitable ligands is limited, a spectroscopic measure of ligand binding, being direct, obviates many of these difficulties. Since the correlation time for ESR is shorter than the mean residence time of the ligand on the receptor, the signal from the unbound species is distinct from the broadened resonances arising from a single or multiple bound species, and ratios of free to bound ligand can be calculated irrespective of a change in state for the bound species.

After we had initiated this work, a study appeared which also demonstrated that competition between toxin and various nicotinic agonists for *Torpedo marmorata* membrane fragments was dependent on the duration of exposure to the agonist (9). In that study receptors were present in a 6-fold stoichiometric excess over toxin and only initial rates of toxin association were monitored. In the studies described here, we monitored the complete time course to equilibrium and employed toxin in excess. Thus a conversion between receptor states could be demonstrated for the entire receptor population. Studies by Hess and his colleagues have shown competitive behavior between α-bungarotoxin and *d*-tubocurarine binding, but not between the toxin and carbamylcholine; however, a dependence on ligand exposure duration was not reported (3, 19).

Over a limited concentration range the binding of *d*-tubocurarine appears competitive with toxin, for the initial rate was affected without appreciably decreasing the binding at equilibrium. This observation, along with the finding that the duration of *d*-tubocurarine exposure does not influence toxin binding kinetics, suggests the following scheme to be applicable:

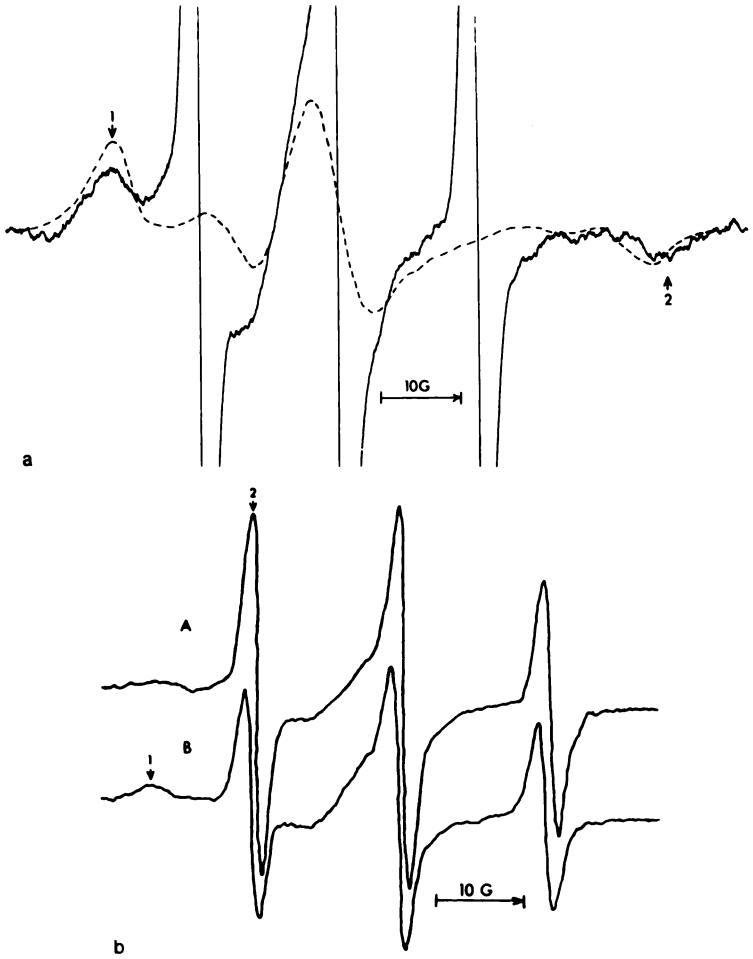


FIG. 5. *Electron spin resonance spectrum of bisquaternary mononitroxide in association with receptor-enriched membranes (a) and time dependence of spectral changes (b)*

a. Membranes, 8.0 μM in α-toxin binding sites, were mixed with 8.36 μM spin label, 4-[dimethyl-10-(trimethylammonino)decylammonino]-2,2,6,6-tetramethyl-1-piperidinyloxyl diiodide, and the spectrum was measured after 30 min at 25° in 0.1 N NaCl–0.01 M sodium phosphate, pH 7.4. Arrows *1* and *2* depict the low- and high-field lines, respectively, of the bound species ($2T_{\parallel}$ = 70 G), while the resonances of the unbound ligand extend off scale. Addition of toxin in a 1.15-fold molar excess gives rise to a sharp, three-line spectrum characteristic of isotropic tumbling. The ESR spectrum (- - -) of the same label (10 μM) in ethylene glycol at −30° is shown for comparison. The instrumental gain setting for the receptor-bound label was 2×10^4, while that for the ethylene glycol solution was 5×10^3.

(b). The mononitroxide decamethonium analogue (8.87 μM) was added to the receptor membrane (10.3 μM in α-toxin sites) in 10 mM sodium phosphate buffer, pH 7.4, and 0.1 M NaCl. Spectrum *A* was recorded 30 sec after addition of the label, and spectrum *B* was recorded 30 min after label addition. Arrow *1* indicates the position of the low-field line of receptor-bound label, while arrow *2* shows the low-field line of the free label.

$$\begin{array}{l} T + R \xrightarrow{k_T} RT \\ \quad + \\ \quad L \\ \quad \downarrow\uparrow K_L \\ \quad RL \end{array} \qquad (1)$$

where T, L, and R are toxin, *d*-tubocurarine, and receptor, respectively.

In the case of carbamylcholine or the spin-labeled bisquaternary ligand, the time-dependent change in apparent com-

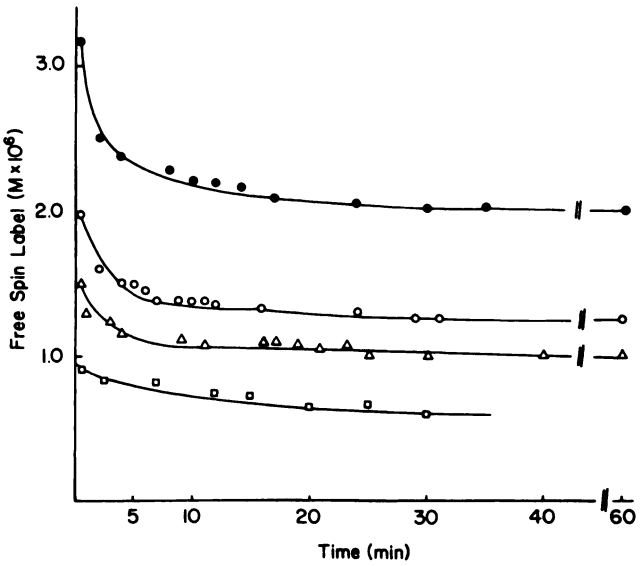


FIG. 6. *Kinetics of decrease in free spin label concentration following addition to receptor for separate membrane preparations*

Conditions were the same as those described for Fig. 5. The concentrations of free and bound ligand were determined from signal intensities of the low-field resonance of the free peak (arrow 2, Fig. 5b) and the total quantity of ligand added to the system. Each symbol represents a separate membrane preparation. ●——●, 10.6 μM α-toxin sites, 8.87 μM spin label; ○——○, 12.45 μM α-toxin sites, 9.19 μM spin label; △——△, 8.27 μM α-toxin sites, 5.84 μM spin label; □——□, 14.5 μM α-toxin sites, 7.25 μM spin label. The calculated dissociation constants at 0.5 and 30 min are given in Table 3. The time dependence of ligand binding was measured in six separate membrane preparations, and the apparent half-time for conversion ranged between 2.8 and 10 min.

petition must be added to the scheme, which then can be represented as follows:

$$\begin{array}{ccc} RT & \rightleftharpoons & RT \\ k_T\uparrow & & \uparrow k'_T \\ T + R & \rightleftharpoons & R' + T \\ + & & + \\ L & & L \\ K_L\downarrow\uparrow & & \downarrow\uparrow K'_L \\ RL & \rightleftharpoons & R'L \end{array} \qquad (2)$$

where R and R' denote the two states of the receptor. This formulation is consistent with the findings of Weber *et al.* (9) and our dilution experiments, since the toxin binding rate is unaffected by prior ligand exposure if the carbamylcholine is diluted to 3 nM (Table 2). Here ligand binding is assumed to be more rapid than conversion between receptor states, and the exposure-dependent, apparent competition between L and T is simply a consequence of the smaller dissociation constant of LR' relative to LR. Also, the toxin association rate constant, k_T, is equivalent for the R and R' states. The slow dissociation of toxin, whether measured in the presence of carbamylcholine or excess unlabeled toxin, allows one to consider toxin binding to be irreversible for the duration of these experiments (Fig. 1). Scheme 2 is analogous to that proposed by a number of workers to explain receptor desensitization (20–22).

Although this formulation is completely compatible with initial rate data and with the observation that prior ligand exposure has no effect on toxin binding kinetics following sufficient dilution of ligand (Table 2), two observations suggest that the binding of toxin and carbamylcholine may not be competitive. First, toxin binding following 30 sec and 30 min of carbamylcholine exposure approaches the same equilibrium value, but one which is depressed from the value observed in the absence of ligand (Fig. 4

and Table 1). This occurs despite very different rates of association. Second, the slow increase in affinity for the bisquaternary inhibitor observed directly by ESR is not always sufficient to account for the degree of inhibition of toxin binding which is exposure-dependent (Table 3).

Since dissociation of the toxin-receptor complex is not enhanced by carbamylcholine (Fig. 1b), the first observation suggests that carbamylcholine and α-toxin binding are not mutually exclusive. A reaction pathway involving a ternary complex, with toxin binding to the ligand-receptor complex (LR), could account for the depression in equilibrium binding following 30 sec of exposure to carbamylcholine. Thus this alternative does not require toxin and carbamylcholine binding to be competitive and only necessitates that LR and LR' have different vulnerabilities to toxin.

Concerning the second point, if toxin were to combine only with free receptor as depicted in Scheme 2, the ratio of rates of toxin binding measured after carbamylcholine exposure for 30 sec and 30 min would be represented as

$$\frac{\text{rate}_{30\text{ sec}}}{\text{rate}_{30\text{ min}}} = \frac{k_T}{k'_T} \frac{\left(\frac{R}{R + LR}\right)}{\left(\frac{R'}{R' + LR'}\right)} \cdot \frac{(R_0)\,(T)}{(R_0)\,(T)} = \frac{\left(1 + \frac{L}{K_L}\right)}{\left(1 + \frac{L}{K'_L}\right)} \frac{k_T}{k'_T} \tag{3}$$

The experiments with dilution to low carbamylcholine concentrations suggest that $k_T = k'_T$. Thus the ratio of dissociation constants for the spin label, K_L/K'_L, can be estimated from the rates of toxin binding following 30 sec and 30 min of exposure to the ligand. It is likely that the ratio of toxin binding rates will underestimate the difference between K_L and K'_L owing to the substantial time required to monitor toxin binding. This contention is also supported by the deviations from bimolecularity in toxin-receptor binding that we observed following 30 sec of ligand exposure. The comparison between ratio of rate constants estimated form the toxin binding kinetics and those calculated from direct ESR measurements shows that the ESR-determined increase in affinity is less than that estimated from toxin binding measurements. This disparity, while difficult to quantitate precisely, would also be supportive of a more complex mechanism than is depicted by a competitive interaction between the spin label and toxin for R and R' as represented by Scheme 2.

The slow conversion of receptor state *in vitro*, which has been demonstrated here by two independent means, has a temporal dependence similar to receptor desensitization *in situ* rather than to an initial depolarization. The recent voltage-current relaxation measurements using electroplax cells provide strong evidence that the agonist binding step associated with the depolarization event is intrinsically rapid and would occur in milliseconds (23). While desensitization can be monitored electrophysiologically for nicotinic agonists, these experiments do not yield direct information on whether the degree of receptor occupation is altered in the desensitization process. However, Rang and his colleagues have provided convincing evidence that certain antagonists exhibit an increased affinity for the receptor which is dependent on the duration and concentration of prior agonist exposure (24). The effect was not evident for *d*-tubocurarine, and the particular antagonists exhibiting this behavior were termed metaphilic antagonists. The parameters of agonist exposure which induced the metaphilic behavior closely paralleled those effecting receptor desensitization; thus both phenomena may reflect the same change in receptor state (21, 25).

Lester (22) has found in the frog neuromuscular junction that receptors desensitized by carbamylcholine become invulnerable to cobra α-toxin. These electrophysiological findings, however, do not resolve whether the decrease in nearly irreversible antagonism by α-toxin is due to enhanced binding of carbamylcholine

TABLE 3

Dissociation constants for complex between mononitroxide analogue of decamethonium (I) and receptor-containing membranes measured by electron spin resonance and inhibition of α-toxin binding following 0.5 and 30 min of exposure to ligand

ESR					Toxin binding	
Concentration of toxin binding sites	Concentration of spin label	ESR-derived K_L at 0.5 min	ESR-derived K'_L at 30 min	Calculated ratio of rates, $k_{T,0.5}/k_{T,30.0}$ [a]	Ligand concentration[b]	Measured $k_{T,0.5}/k_{T,30.0}$
μM	μM	μM	μM		μM	
10.6	8.90	2.99	1.11	2.34	10	
8.3	5.84	1.36	0.73	1.74	8.5	3.21
12.5	9.19	1.43	0.72	1.99	14	3.53
14.6	7.25	1.18	0.72	1.57	10	2.59

[a] Calculated from the equilibrium constants determined by ESR, K_L and K'_L, following 0.5 and 30.0 min of exposure to the spin label, respectively, where

$$\frac{k_{T,0.5}}{k_{T,30.0}} = \frac{(1 + L/K_L)}{(1 + L/K'_L)}$$

[b] The ligand concentration, L, is that employed for the measurement of toxin binding kinetics.

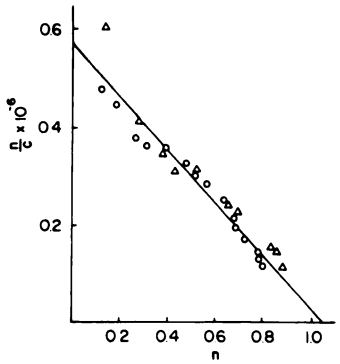


FIG. 7. *Scatchard plot of mononitroxide decamethonium analogue binding to receptor-enriched membranes*

The procedures for titration and estimation of the free and bound ligand concentrations are detailed in the text. Between 8 and 10 min were allowed to elapse between each incremental addition of spin label. The number of binding sites, n, is the determined number of toxin binding sites on the membrane preparation. Binding site titrations by ESR were conducted on three separate membrane preparations, and the ratio of spin label to toxin binding sites ranged between 0.95 and 1.28. The different symbols represent separate titrations on the same preparation of membranes.

by the desensitized receptor or to a change in its affinity for toxin. Rang and Ritter have examined the magnitude of desensitization and its rates of onset and recovery in relation to various models for ligand-induced transitions in receptor state (25). Their extensive analyses with the frog and chick neuromuscular junction indicated that the cyclic mechanism for desensitization (Eq. 2) most closely described their findings. A comparison of desensitization and activation parameters produced by carbamylcholine in the presence and absence of the antagonist, *d*-tubocurarine, suggested that carbamylcholine-elicited desensitization is not associated with an increase in binding affinity for this agonist. However, other agonists may differ in this respect, since the alkyltrimethylammonium ligand appeared to have a preferential affinity for the desensitized receptor (25), as do the metaphilic antagonists (21, 24).

Since the slow, exposure-dependent inhibition of toxin binding by carbamylcholine is large with respect to the total inhibition, we initially screened nitroxide analogues of carbamylcholine synthesized in one of our laboratories (11) for exposure-dependent inhibition of toxin binding. However, the affinity of these derivatives is too low to make them practical for ESR spectroscopy. The mononitroxide analogue of decamethonium had a sufficiently high affinity to enable us to monitor binding at reasonably low receptor concentrations and exhibited exposure-dependent inhibition of toxin binding.

However, its exposure-dependent inhibition of toxin binding relative to total inhibition of toxin binding is not as large as that seen with carbamylcholine. It is possible that an entire spectrum of compounds with different capacities for inhibiting toxin or antagonist binding by simple competition, as opposed to inducing a change in receptor state, could be uncovered (9). ESR offers an attractive means of directly monitoring the association for certain ligands, and it would be of importance to obtain electrophysiological data for these spin labels.

Although we have been able to monitor directly a slow, ligand-induced change in receptor state, the molecular basis of this phenomenon is unknown. Since it appears to be lost upon solubilization of the receptor, a ligand-induced change in assembly of receptor protomers within the membrane is an intriguing possibility. Changes in protein conformation per se are usually more rapid processes than the change in receptor state that we have observed here.

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