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Ligand Specificity of State Transitions in the Cholinergic Receptor: Behavior of Agonists and Antagonists

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SUMMARY

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Several cholinergic ligands, including a series of methylene-linked bisquaternary ammonium compounds, have been examined for their ability to induce a state transition in the membrane-associated cholinergic receptor where there is a slow increase in affinity for the associating ligand. All of the agonists tested, as well as certain antagonists, notably the metaphilic antagonists, convert the receptor to a higher affinity state. Moreover, the rate of receptor conversion appears to be independent of the ligands' capacity to activate the receptor. By applying the two-state cyclic scheme used to explain desensitization to these affinity increases, the apparent binding constants of each ligand for the R and R′ receptor states were determined. Full agonists have a 300-fold higher affinity for the R′ state while the classical antagonists (i.e., d-tubocurarine and gallamine) have the same affinity for both receptor states. Apparent negative cooperativity is observed for the antagonists. Partial agonists, which are represented by the methylene-linked bisquaternary series have ratios of affinities for the two states which lie between these extremes. The metaphilic antagonists, which appear to be unique *in situ* in showing enhanced antagonism for receptors previously conditioned by agonist, will in themselves effect a conversion in receptor state. The structural specificity for the ligands' capacity to effect a conversion in receptor state lends further support to the contention that the conversion from the low to high affinity state *in vitro* and receptor desensitization involve the same molecular transition.

INTRODUCTION

It has been demonstrated in a number of laboratories that cholinergic agonists, upon binding to the isolated membrane-associated receptor, cause a relatively slow conversion in the receptor to a state with enhanced affinity for the agonist. Conversion to the high affinity state has been monitored by the increased competition between agonist and α-toxin binding (1–5), electron spin resonance using a spin-labeled analogue of decamethonium (2), fast filtration techniques (6) and by changes in intrinsic fluorescence of the receptor (7). The temporal response examined in the membrane fragment resembles that of receptor desensitization *in situ* and it has been suggested that the same state transition is being measured *in vitro* and *in situ.* In fact, a detailed kinetic examination of the conversion reveals that the two-state cyclic scheme proposed initially by Katz and

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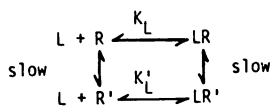
Thesleff (8) is consonant with the data obtained from dissociation and rate constant measurements *in vitro* (3). The scheme is described as follows:



where the R state predominates in the absence of ligand and conversion to the R′ state is a consequence of its higher affinity for the ligand, L. Ligand binding and dissociation are rapid while the isomerizations represented by the vertical steps are slow. Pharmacologic or electrophysiological measurements of desensitization indicate that the process is characteristic of agonist occupation of the receptor. Antagonism of receptor activation usually remains constant following successive test exposures to agonist (9). An exception to this was found for a group of antagonists which exhibit enhanced blockade of receptors previously exposed to agonists. The agents, termed metaphilic antagonists, have been proposed to combine preferentially with the desensitized receptor (9–11). If this is the case, the metaphilic antagonist should be capable of influencing the ratio of R and R′ at equilibrium and measurements of the respective dissociation constants for the two putative states may enable one to compare metaphilic and classical antagonists directly.

Receptor activation and antagonism in relation to ligand structure have been extensively studied in nicotinic receptor systems (12, 13). Since molecular details on the structure of the isolated receptor have begun to emerge, an examination of ligand specificity for the receptor states detectable *in vitro* should prove helpful in delineating the mechanistic basis for the slow transitions in receptor state. Such studies may also yield useful information on the relationship between receptor activation and desensitization.

MATERIALS AND METHODS

Materials. Acetylcholine chloride, carbamylcholine chloride, d-tubocurarine chloride, phenyltrimethylammonium iodide, nicotine, hexamethonium bromide, and decamethonium bromide were purchased from Sigma Chemical Company, St. Louis, Missouri. Pentamethonium bromide and gallamine triethiodide were obtained from K & K Laboratories. Dimethyl-d-tubocurarine iodide, pancuronium bromide, and alloferin dichloride were gifts of Eli Lilly, Indianapolis, Organon, Inc., New Jersey, and Hoffman-La Roche, Basel, Switzerland, respectively. Trimethonium iodide was kindly supplied by Dr. Paul Woodson, University of California, San Diego. Dodecamethonium bromide, tetradecamethonium bromide, and decaethonium bromide were generous gifts of Dr. R. B. Barlow, University of Bristol. Diphenyldecamethonium, decamethylene-1,10-bis [dimethylbenzyl-ammonium bromide] and dinaphthyldecamethonium, decamethylene-1,10-bis[dimethyl (1-naphthylmethylene) ammonium bromide] were kindly supplied by Dr. H. P. Rang, St. George's Hospital Medical School, London. Suberyldicholine diiodide and AH-8165, 1,1′ azobis[3-methyl-2-phenyl-1H-imidazo] (1,2-a) pyridinium, were gifts of Dr. A. Ungar, University of Edinburgh and Dr. R. T. Brittain, Allen and Hanburgs, Ltd., England, respectively (14).

Diaminopyridinium decane, bis(3-aminopyridinium)-1,10-decane, had been previously synthesized by the method of Mooser *et al.* (15). The spin-labeled analogues of decamethonium and hexamethonium, decamethonium mononitroxide, 4-[dimethyl-(10-trimethylammonio) decylammonio]2,2,6,6 - tetramethyl-1-piperidinyloxyl diiodide, decamethonium dinitroxide, 4,4′-[1,10-decanediylbis(dimethylimino)]bis[2,2,6,6-tetramethyl-1-piperidinyloxy] dibromide, and hexamethonium dinitroxide, 4,4′-[1,6-hexanediylbis(dimethylimino)]bis[2,2,6,6-tetramethyl-1-piperidinyloxyl] dibromide, were prepared previously (16) and kindly supplied by Dr. C. F. Chignell. Heptamethonium bromide, octamethonium bromide, and nonamethonium bromide were synthesized and recrystallized according to the method of Barlow and Ing (13). Results of the elemental analyses (Galbraith Laboratories, Knoxville, Tennessee) were heptamethonium, found: C 38.89, H 8.97, N 6.90%; $C_{13}H_{32}N_2Br_2 \cdot 1.8H_2O$ requires: C 38.3, H 8.7, N 6.9%;

octamethonium, found: C 39.63, H 9.25, N 6.53%; $C_{14}H_{34}N_2Br_2 \cdot 1.7H_2O$ requires: C 39.9, H 8.9, N 6.7%; and nonamethonium, found: C 40.83, H 9.10, N 6.29%; $C_{15}H_{36}N_2Br_2 \cdot 1.7H_2O$ requires: C 41.4, H 9.1, N 6.4%. All of the above compounds were dried over phosphorus pentoxide *in vacuo* prior to use. All other chemicals were of reagent grade or of the highest purity available.

Receptor-rich membrane fragments were isolated from *Torpedo californica* electric organs by the density gradient centrifugation procedures outlined previously (3, 17). Monoiodotyrosyl cobra α-toxin was prepared and separated from the non-iodinated and diodotyrosyl α-toxin as described earlier (2).

Kinetics of α-toxin binding. Initial rate measurements of the kinetics of α-toxin binding were conducted as described previously (2). Typically, the receptor-rich membranes (15–25 nM in toxin sites) were present in a 2- to 5-fold stoichiometric excess of α-toxin. Samples were removed for binding measurements at 20 and 40 seconds after initiation of the reaction. The rate constants were calculated from the bimolecular rate equation using the concentrations of total toxin and bound toxin at the time points and toxin bound at equilibrium (3). All reactions were carried out at 22° in 0.01 M sodium phosphate, pH 7.4, containing 0.1 M NaCl.

Analysis of the effect of ligands on the binding of α-toxin. The dependence of initial rate of α-toxin binding on ligand concentration was analyzed empirically by the method of Hill (18) to determine the protection constants, K_p. The effect of ligand-receptor association on the initial rate of toxin binding for a competitive interaction can be described by:

$$\frac{k_T^L}{k_T} = \frac{\text{free receptor}}{\text{total receptor}} = \frac{1}{1 + \left(\frac{L}{K_p}\right)^{n_H}} \quad (2)$$

where k_T^L is the rate of toxin binding measured in the presence of ligand, k_T the rate in the absence of ligand, L the free ligand concentration, and n_H the Hill coefficient. K_p is the ligand protection constant, defined as the concentration of free ligand which inhibits the rate of toxin binding by 50%. Equation 2 can be rearranged to a linear Hill plot formulation for describing the rate of toxin binding as a function of free ligand concentration:

$$\log\left(\frac{k_T^L}{k_T - k_T^L}\right) = n_H \log K_p - n_H \log L \quad (3)$$

$K_p^{10'}$ and $K_p^{30'}$, the protection constants for 10 seconds and 30 minutes of ligand exposure to the membranes prior to toxin addition, and the corresponding Hill coefficients were determined for each ligand from linear regressions of the plots of Equation 3 derived from the experimentally measured curves for $0.1 < k_T^L/k_T < 0.9$. Correlation coefficients from these regressions were rarely less than .98.

To determine the statistical significance of the increase in apparent affinity with prior exposure for each ligand, linear regressions of the data plotted according to Equation 3 for the two exposure durations were used to determine the Y-values and their standard errors at an X-value halfway between the X-intercepts (19). For each ligand a t value was calculated for the difference in these Y-values and a two-tailed t-test employed to evaluate significance (20).

Application of the cyclic scheme for the estimation of K'_L. The applicability of the two-state cyclic scheme to the agonist-induced receptor transition has been demonstrated previously (3). Hence the saturation function, $\bar{Y}$, for the receptor in equilibrium with ligand can be shown to be (21):

$$\bar{Y} = \frac{\text{bound receptor}}{\text{total receptor}} = \frac{L}{L + K_L^{eq}} \text{ where } K_L^{eq} = \frac{1 + M}{\frac{1}{K_L} + \frac{M}{K'_L}} \quad (4)$$

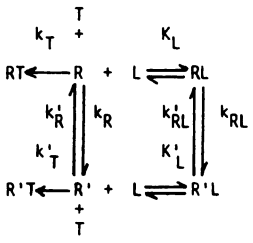
Thus ligand binding at equilibrium should follow a Langmuir binding isotherm with an apparent dissociation constant of K_L^{eq}, determined by K_L, K'_L, and M. These predictions have been verified for carbamylcholine (3). Since the rates of the receptor

conversion induced by diphenyldecamethonium, decamethonium mononitroxide and decamethonium are comparable to those determined for carbamylcholine, the intrinsic rates of conversion for all ligands will be assumed to be relatively slow. Therefore, $K_p^{10'}$, the protection constant determined after 10 seconds prior exposure, is used to approximate K_L (3). K_L^{eq} is estimated from the measured $K_p^{30'}$ since receptor conversion at all ligand concentrations will be complete in less than 30 minutes. M is the ratio of R'/R in absence of ligand and has previously been determined to be 0.1 (3). Thus the experimentally determined values of $K_p^{10'}$ and $K_p^{30'}$ can be utilized to estimate K'_L, the apparent binding constant of the R' receptor state for each ligand:

$$K'_L = K_p^{30'} \cdot M/(1 + M - K_p^{30'}/K_p^{10'}). \quad (5)$$

RESULTS

The influence of duration of prior agonist exposure on α-toxin binding kinetics. We have shown previously that the capacity of agonists to inhibit the initial rate of α-toxin binding provides a measure of the affinity of the respective agonist for the receptor (3). Since initial rates may be measured within 20 second intervals, it becomes possible to monitor a slow conversion between states reflected in low and high affinities for agonists. Using the cyclic scheme and adding the binding of α-toxin (T) which appears competitive with ligand, we have



k_T and k'_T are found to be equivalent and within short time frames dissociation of the α-toxin complex is negligible. For carbamylcholine k_{RL}, k'_{RL}, k_R and k'_R have been estimated from the kinetics of RL conversion to $R'L$ and the subsequent reversion following dilution of the ligand. Likewise K_L is the measured ligand dissociation constant prior to conversion to R' and K'_L can be related to an equilibrium dissociation constant and M by equation 4. Using a Runga-Kutta fourth order approximation and the microscopic reversibility constraint, it was possible to assign the corresponding kinetic and equilibrium constants to the above scheme (3).

A second estimation of K_L and K'_L may be obtained by exposure of the receptor to 10^{-5} M carbamylcholine for 10 minutes followed by a 500-fold dilution of the carbamylcholine-receptor complex and subsequent measurement of the capacity of carbamylcholine to inhibit the initial rate of α-toxin binding. This procedure enables one to drive a major portion of the receptor into the R' state and, before reversion is complete, measure the affinity of the receptor for carbamylcholine. The measurements of carbamylcholine competition with the rate of toxin binding obtained by this procedure along with the competition found for 10-second and 30-minute exposure of carbamylcholine to the receptor are shown in Fig. 1. It is to be expected that only the exposure and dilution condition will yield a curve with a horizontal inflection owing to the non-equilibrium condition and a constant R to R' ratio over the concentration range for carbamylcholine. The two portions of the curve should correspond to K'_L and K_L, respectively. K'_L obtained directly from the exposure and dilution experiments is equal to the computer-derived value for the cyclic scheme. Moreover, K_L calculated from the lower step of the biphasic curve is in accord with the value calculated from the short-term exposure curve. We also find that the fractional population in the low affinity state ~25% is close to that predicted in the experiment. Exposure to 10^{-5} M carbamylcholine will leave 5% of the receptor in R; however, the 30-second interval between dilution of carbamylcholine and toxin addition and the 20-second interval for the toxin binding reaction allows for another 20% conversion of R' to R. The short-term exposure condition should yield a single dissociation constant K_L if $R'/R \leq 0.1$. The long-term (30 minutes) exposure represents an equilibrium condition where the ratio R'/R will vary with each concen-

tration of carbamylcholine and the observed dissociation constant K_{eq} can be related to K'_L, K_L and M (cf. eq. 4).

Dissociation constants of other agonists for the two states. The dissociation constants, K_L and K'_L, have also been estimated for the agonists, nicotine, suberyldicholine, phenyltrimethylammonium and acetylcholine. Despite rather large differences in agonist affinities seen here, the ratio K_L/K'_L is remarkably constant for all of these full agonists (Table 1). For these ligands all of the Hill coefficients appear to be not significantly different from 1.0; thus the protection and dissociation constants can be used interchangeably. To obviate the effects of ester hydrolysis by residual quantities of acetylcholinesterase, with acetylcholine exposure the membranes were incubated with 10 μM echothiophate prior

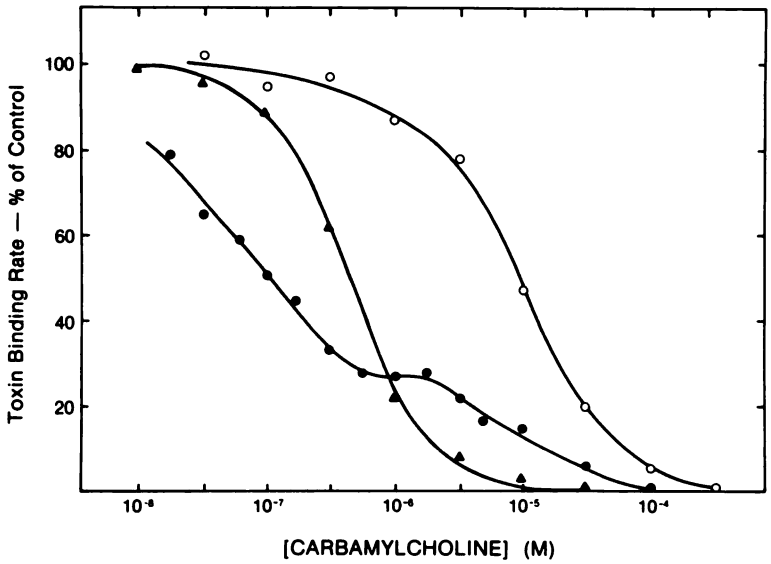


FIG. 1. *The influence of various conditions of carbamylcholine exposure on the initial rate of α-toxin binding to the membrane-associated receptor*

(O——O), Receptor-containing membranes (15 nM in α-toxin sites) were exposed to the specified concentration of carbamylcholine for 10 sec prior to initiation of the α-toxin binding reaction. (▲——▲), Membranes (15 nM in α-toxin sites) were exposed to the specified concentration of carbamylcholine for 30 min prior to initiation of the α-toxin binding reaction. (●——●), Membranes (5 μM in α-toxin sites) were exposed to 10 μM carbamylcholine for 10 min, diluted 500-fold, and the initial rate of α-toxin binding in the specified concentration of carbamylcholine measured 20 sec thereafter.

TABLE 1

Protection (K_p hr) and dissociation (K_L hr) constants for agonists

The protection constants $K_p^{10'}$ and $K_p^{30'}$ were calculated from the concentration dependence of inhibition of α-toxin binding as described in the text.

Agonist	$K_p^{10'}$	$K_p^{30'}$	$K_p^{10'}/K_p^{30'}$	K'_L[a]	K_L/K'_L[b]
	M	*M*		*M*	
Suberyldicholine	1.3×10^{-7}	4.3×10^{-9}	30.2	4.0×10^{-10}	325
Acetylcholine	6.1×10^{-7}	2.4×10^{-8}	25.4	2.3×10^{-9}	269
Carbamylcholine[c]	$(1.5 \pm 0.2) \times 10^{-7}$	$(4.8 \pm 0.3) \times 10^{-7}$	31.3	4.5×10^{-8}	341
Phenyltrimethylammonium	3.5×10^{-5}	1.6×10^{-6}	21.9	1.5×10^{-7}	267
Nicotine	6.4×10^{-5}	2.5×10^{-6}	25.6	2.4×10^{-7}	231

[a] K'_L, the dissociation constant for the R' state, is calculated from Eq. 5.

[b] K_L, the dissociation constant for the R state, = $K_p^{10'}$.

[c] Mean ± SEM (n = 5); the corresponding Hill coefficients were 1.11 ± 0.03 for both $K_p^{10'}$ and $K_p^{30'}$.

to addition of acetylcholine. The dissociation constants found from the agonists' competition with α-toxin binding following 10 sec of agonist exposure compare closely with the constants obtained for activation of the *Torpedo* receptor (22).

Dissociation constants of antagonists for the two receptor states. Binding isotherms as measured by competition with the initial rate of α-toxin binding are shown in Fig. 2 for the antagonists: d-tubocurarine, AH-8165, dimethyl-d-tubocurarine and gallamine. None of these competitive nicotinic antagonists induces a detectable change in the R' to R ratio as would be manifested in enhanced ligand protection with longer prior exposure (Table 2). Hill coefficients for the antagonists all appear to be less than 1.0. Since $M = 0.1$, only when K'_L and K_L differ substantially could we detect differences in apparent affinities of the R and R' states from the above curves. An approach which can discriminate between K_L and K'_L more sensitively involves exposure of the receptor to a concentration of antagonist which saturates the receptor to maximize potential conversion of R to R' by the antagonist. The antagonist is diluted 500-fold to dissociate ligand from receptor. Hence, the ratio of R' to R will be increased by the ratio K_L/K'_L. After dilution 1.0 μM carbamylcholine is added, a concentration that would yield very different ratios of bound to free receptor for the R and R' states (cf. Fig. 1), and the initial rate of α-toxin binding determined immediately thereafter. The sensitivity for detection of changes in the R' to R equilibrium is further increased since carbamylcholine binding at 1.0 μM is extremely sensitive to changes in receptor state. For two representative antagonists, d-tubocurarine and gallamine, no

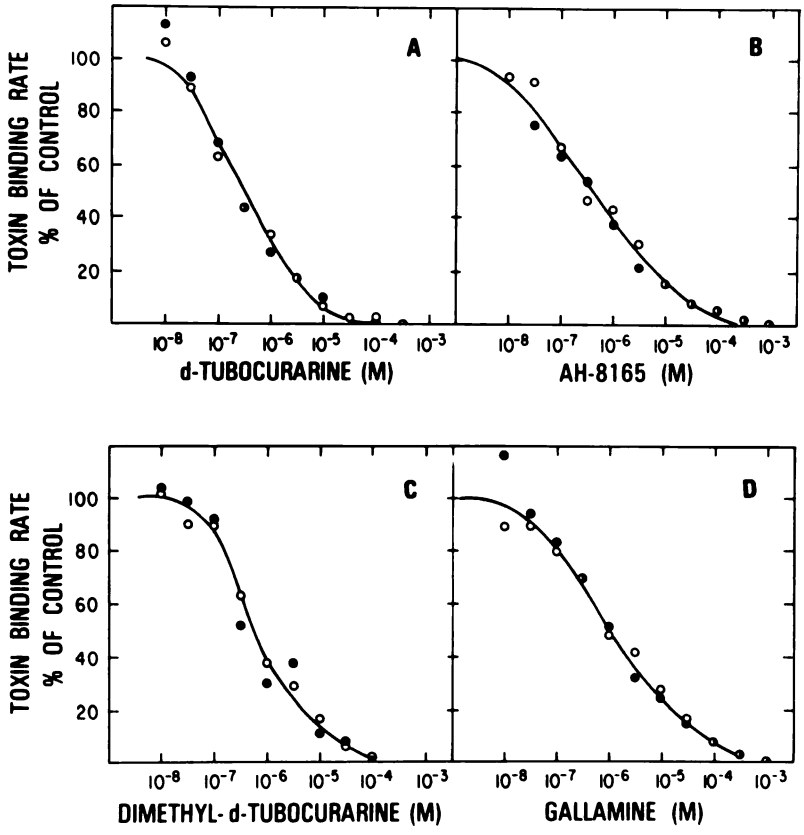


FIG. 2. *The influence of antagonist concentration on the initial rate of α-toxin binding* (O——O), 10 sec exposure prior to initiation of the toxin binding reaction; (●——●), 30 min exposure prior to initiation of the α-toxin binding reaction.

conversion from the R to R' state can be detected by this approach (Table 3). The rate of α-toxin binding in the presence of 1.0 μM carbamylcholine is the same irrespective of whether the membranes had been incubated with antagonists or buffer prior to dilution (compare columns B_1 and B_2). In contrast, if the initial incubations are conducted with carbamylcholine or decamethonium, as expected substantial increases in the ratio of R' to R are evident.

Dissociation constants of the methylene-linked bisquaternary ammonium series. The concentration dependences for protection against the initial rate of α-toxin binding for representative polymethylene bis-

TABLE 2

Protection constants (K_p) and Hill coefficients (n_H) for various antagonists[a]

Antagonist	$K_p^{10''}$	n_H	$K_p^{30'}$	n_H	$K_p^{10''}/K_p^{30'}$ [b]
	M		*M*		
Alloferin	—[c]	—	3.7×10^{-8}	0.72	—
Pancuronium	—[c]	—	3.9×10^{-8}	0.32	—
d-Tubocurarine	3.1×10^{-7}	0.75	3.8×10^{-7}	0.78	0.8
AH-8165	3.9×10^{-7}	0.54	3.5×10^{-7}	0.55	1.1
Dimethyl-d-tubocurarine	8.5×10^{-7}	0.74	8.5×10^{-7}	0.76	1.0
Gallamine	9.8×10^{-7}	0.64	1.1×10^{-6}	0.68	0.9

[a] Protection constants obtained from the initial rate of α-toxin binding following 10 sec and 30 min exposure to ligand as described in the text.

[b] $p > 0.05$ when $K_p^{10''}$ and $K_p^{30'}$ are compared for each antagonist.

[c] In the experiment where the 10 sec and 30 min prior exposures to antagonist were compared, the receptor concentration approached K_p so the protection constants could not be determined accurately. However, the curves for the two exposure conditions appeared superimposable.

TABLE 3

The influence of exposure to ligand on the extent of conversion between receptor states[a]

Ligand for initial incubation	Ligand conc.	Fraction of control initial toxin binding rates			
	During initial incubation/After dilution	1 Initial incubation in buffer		2 Initial incubation with ligand	
		A_1 buffer added following dilution	B_1 carbamylcholine (1×10^{-6} M) added following dilution	A_2 buffer added following dilution	B_2 carbamylcholine (1×10^{-6} M) added following dilution
Carbamylcholine	1×10^{-5} M/2×10^{-8} M	1.03 ± 0.04	0.90 ± 0.04	0.75 ± 0.01	0.24 ± 0.03
Decamethonium	1×10^{-5} M/2×10^{-8} M	0.91 ± 0.03	0.76 ± 0.03	0.77 ± 0.02	0.35 ± 0.02
Diphenyldecamethonium	1×10^{-5} M/2×10^{-8} M	1.01 ± 0.03	0.90 ± 0.04	0.86 ± 0.07	0.48 ± 0.03
Dinaphthyldecamethonium	1×10^{-5} M/2×10^{-8} M	0.83 ± 0.04	0.83 ± 0.05	0.77 ± 0.03	0.48 ± 0.01
d-Tubocurarine	5×10^{-6} M/1×10^{-8} M	0.80 ± 0.03	0.68 ± 0.02	0.83 ± 0.04	0.71 ± 0.02
Gallamine	2×10^{-5} M/4×10^{-8} M	0.97 ± 0.05	0.79 ± 0.01	0.99 ± 0.03	0.76 ± 0.03

[a] Membranes (2–3 μM in α-toxin sites) were incubated in buffer (Group 1) or with the specified concentration of ligand above the diagonal (Group 2). Group 1 was diluted 500-fold into the buffer containing the ligand concentration below the diagonal while Group 2 was simply diluted 500-fold to give the same respective ligand concentrations. Twenty sec later, reaction buffer (A) or 1×10^{-6} M carbamylcholine (B) was added and the initial rate of α-toxin binding determined immediately thereafter. All values are compared with the initial rate of toxin binding to receptor which had not been exposed to ligand initially or to 10^{-6} M carbamylcholine during the toxin binding reaction. With receptor which had not been exposed to ligand the rate obtained in the presence of 1 μM carbamylcholine is 0.83 ± 0.03 of control. Data are expressed as means ± standard errors for three determinations.

trimethylammonium compounds are shown in Fig. 3. Although the magnitude of the increase in affinity resulting from exposure is less than that observed for the full agonists, within this group C_{10}, C_{12}, and C_{14} exhibit the largest changes (Table 4). As the bridging methylene chain is shortened the differences in affinity obtained with prior ligand exposure minimize at C_6 after which an increase is observed for C_5 and C_3. The dissociation constants (K_L) decrease in a regular fashion with chain length (Fig. 4) and show the same trend as the concentrations giving equivalent responses in the frog rectus abdominis (12) and chick biventer cervicus (32) muscles.

The N-substituted analogues of decamethonium also show an increase in apparent affinity upon prior incubation with the receptor, although the increases appear to be less than those observed for the parent compound. Of particular note is the mononitroxide analogue of decamethonium where we have alternative means of measuring dissociation constants for the two states of the receptor. We observe good agreement between the increases in affinity measured directly by electron spin resonance and those obtained from ligand competition with the initial rate of toxin binding (2). Like the parent compound, an increase in apparent affinity for the hexamethonium analogues is not detectable. All of the Hill coefficients are found to be 1.0 or less for the methylene-linked bisquaternary ammonium compounds.

Metaphilic antagonists. Two compounds in this series, diphenyldecamethonium and dinaphthyldecamethonium, have been shown in pharmacological experi-

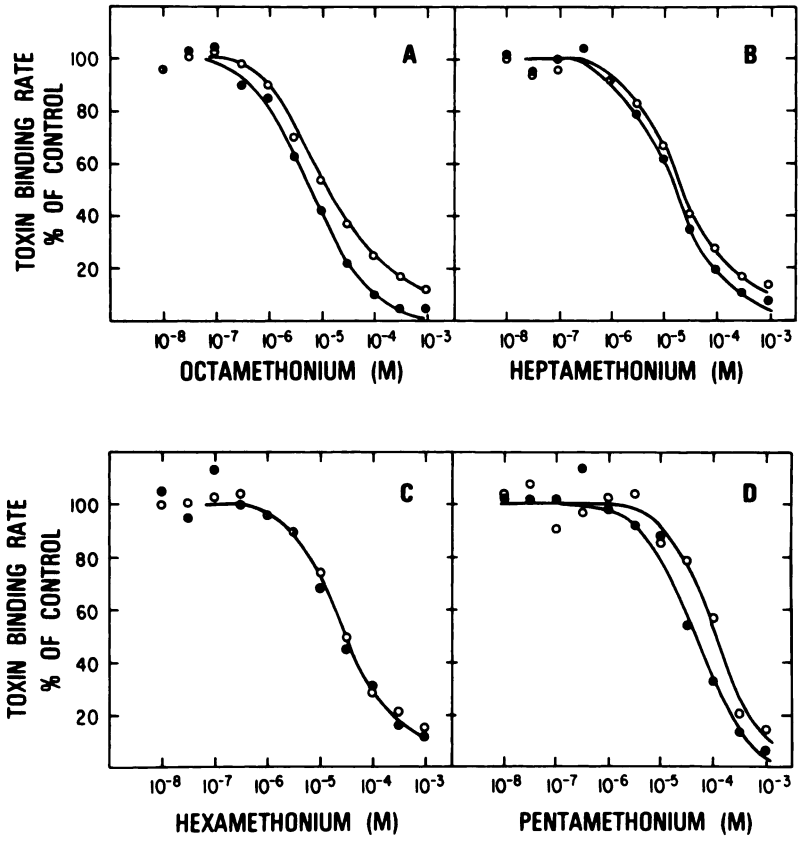


FIG. 3. *The influence of polymethonium concentration on the initial rate of α-toxin binding.* (O——O), 10 sec of exposure prior to the initiation of the toxin binding reaction; (●——●), 30 min exposure prior to initiation of the α-toxin binding reaction.

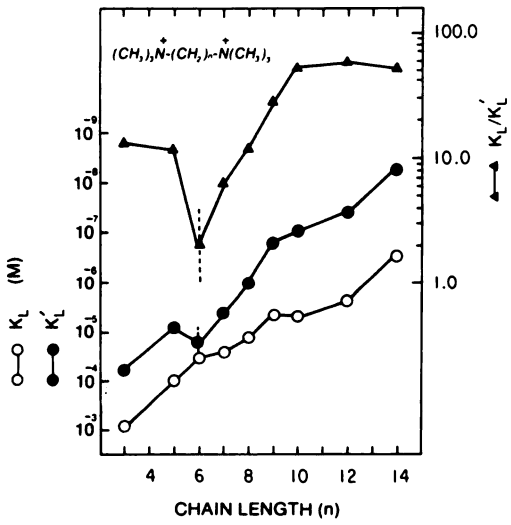


FIG. 4. *Relationship between methylene chain length and polymethonium dissociation constant for the two receptor states R and R'*

Dissociation constants are calculated from data in Fig. 3 as described in METHODS. They are plotted in decreasing order to reflect the increase in affinity. (O——O), K_L (for R or low affinity state); (●——●), K'_L (for R' or the high affinity state); (▲——▲), K_L/K'_L. The vertical dotted lines depict the calculated range in K'_L given the precision in measurement of K_p's. (See METHODS.)

ments to be antagonists, but as antagonists are unique in their capacity to antagonize preferentially receptors which had previously been exposed to agonists (9–11). Through an extensive series of experiments Rang and Ritter were able to show convincingly that these antagonists preferentially combined with the desensitized receptor. Similarly, these agents would be expected to exhibit a higher affinity for the R' state and hence should show enhanced competition with toxin binding upon increasing the duration of exposure to the metaphilic antagonist. The capacity of these ligands to induce the transition is evident in Fig. 5. Moreover, the preference of these ligands for the R' state is more dramatically demonstrated in the exposure and dilution experiments discussed previously (Table 3; compare columns B_1 and B_2). Thus the contrast between the metaphilic and classical antagonists is also clearly evident in the *in vitro* system.

Comparative rates of receptor conversion in state. Since the transition in receptor state can occur with ligands having widely differing capacities to activate the receptor, the relationship between receptor desensitization and activation might be considered in terms of the respective rates of conversion in state for the various ligands. In the absence of data over a complete concentration range, the most meaningful comparison is obtained at concentrations where the ligand concentration is near K_L. It is evident that the rates of conversion to the high affinity state do not vary substantially for the compounds examined (Table 5).

The interaction between antagonist and agonist binding measured by α-toxin binding. All of the classical antagonists appeared to have Hill coefficients less than 1.0, with pancuronium, despite a high affinity, having a particularly low Hill coefficient of 0.32. The low Hill coefficient could reflect two different binding relationships, (a) interacting sites with large negative cooperativity, or (b) multiple classes of independent binding sites with different affinities. To examine the relation between agonist and antagonist association, the apparent binding of carbamylcholine in the presence of pancuronium was studied. The concentration dependence of the inhibition of the initial rate of toxin binding for pancuronium alone and carbamylcholine alone after 10 seconds prior exposure is shown in Fig. 6a. Fig. 6b shows the effect of carbamylcholine in the presence of pancuronium on the initial rate of toxin binding. Also plotted are curves predicted for a competitive interaction of the ligands with one class of sites of pancuronium binding (solid line) and for a non-competitive interaction (dotted line) which might be expected if pancuronium is affecting the apparent rate of toxin binding non-specifically. The data agree most closely with the curve predicted for a competitive interaction at a site represented by a single dissociation constant for pancuronium and substantial negative cooperativity. The results also indicate that the apparent negative cooperativity which may result from the site-to-site interactions is not transferred to the simultaneous binding of carbamylcholine and pancuronium.

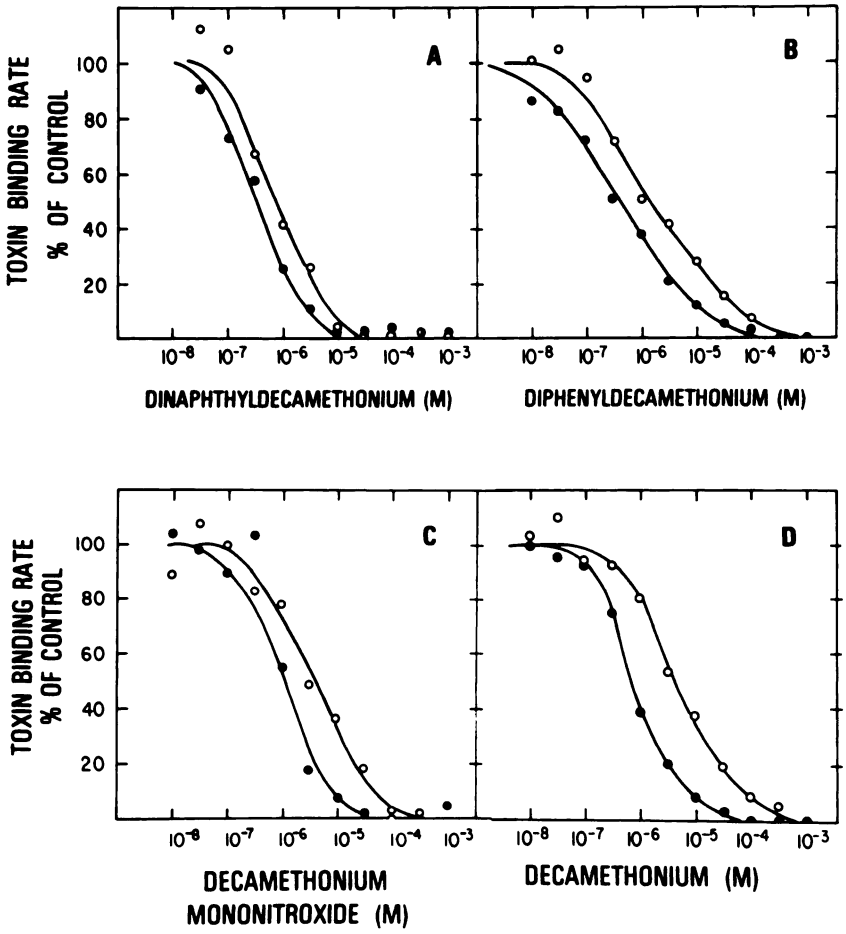


FIG. 5. *Influence of N-substituted decamethonium analogues on the initial rate of α-toxin binding* (O——O), 10 sec of exposure prior to initiation of the α-toxin binding reaction; (●——●), 30 min of exposure prior to initiation of the α-toxin binding reaction.

The calculated dissociation constant for carbamylcholine remains unaffected even with pancuronium occupying over half of the competing sites.

DISCUSSION

Relationship between ligand structure and the state transition. Extending across the pharmacologic spectrum for receptor activation we find that agonists and partial agonists but only certain antagonists can induce a slow conversion in state for the isolated receptor. The differences in affinity between the two states range around 300 for a series of agonists of widely different potencies. If the R' state reflects the desensitized receptor this difference would be anticipated since full agonists elicit maximal desensitization *in vivo* (11). The affinity difference is found to be 50 or less for the partial agonists, characterized by the methylene linked bis-polymethonium series. The ratio of K_L/K'_L is minimal at hexamethonium, an antagonist, and increases with methylene chain length (Fig. 4). The longer chain compounds in this series behave as partial agonists. Interestingly, a decrease in chain length below hexamethonium increases the difference in affinity even though the short chain length compounds behave as antagonists in the chick neuromuscular junction (31). Weber *et al.* (1) have also noted a slight conversion of state induced by hexamethonium.

TABLE 4

Dissociation constants and Hill coefficients for the methylene-linked bisquaternary ammonium ligands

Procedures for determining the protection contants K_p and n and the calculation of K_L are described in METHODS.

Bis-trimethylammonium ligands	Activity	$K_p^{10'}$	n_H	$K_p^{30'}$	n_H	$K_p^{10'}/K_p^{30'}$[d]	K_L/K_L'
trimethonium	Antagonist[a]	8.2×10^{-4}	0.88	3.9×10^{-4}	1.32	2.1*	13.2
pentamethonium	Antagonist[a]	9.7×10^{-5}	0.87	4.9×10^{-5}	0.94	2.0*	11.8
hexamethonium	Antagonist[a]	3.4×10^{-5}	0.86	3.1×10^{-5}	0.84	1.1ns	≤4.0
heptamethonium	Partial agonist[a]	2.5×10^{-5}	0.75	1.7×10^{-5}	0.80	1.5*	6.3
octamethonium	Partial agonist[a]	1.3×10^{-5}	0.77	6.6×10^{-6}	0.78	2.0*	11.8
nonamethonium	Partial agonist[a]	4.5×10^{-6}	1.03	1.3×10^{-6}	1.12	3.5**	28.1
decamethonium	Partial agonist[a]	4.7×10^{-6}	0.79	8.3×10^{-7}	1.00	5.7***	52.2
dodecamethonium	Partial agonist[a]	2.3×10^{-6}	0.96	3.7×10^{-7}	0.82	6.2***	59.0
tetradecamethonium	Partial agonist[a]	2.9×10^{-7}	0.55	5.2×10^{-8}	0.49	5.6***	51.8
N-Substituted decamethonium analogues							
dinaphthyldecamethonium	Metaphilic antagonist[b]	6.2×10^{-7}	0.78	3.0×10^{-7}	1.00	2.1***	12.7
diphenyldecamethonium	Metaphilic antagonist[b]	1.5×10^{-6}	0.55	3.9×10^{-7}	0.62	3.8***	32.6
decamethonium mononitroxide	Partial agonist[c]	4.1×10^{-6}	0.69	9.2×10^{-7}	1.02	4.5***	37.3
decamethonium dinitroxide	—	6.2×10^{-5}	0.47	2.8×10^{-5}	0.44	2.2**	14.4
bis-3-aminopyridinium 1,10 decane	—	4.6×10^{-6}	0.85	2.2×10^{-6}	0.80	2.1*	13.1
decaethonium	Partial agonist[a]	7.8×10^{-6}	0.32	9.2×10^{-6}	0.36	0.8ns	≤4.0
N-Substituted hexamethonium analogues							
hexamethonium dinitroxide	—	3.7×10^{-4}	0.66	2.7×10^{-4}	0.70	1.4ns	≤4.0

[a] Refs. 12, 32, 33, 34.

[b] Refs. 9, 11.

[c] Pharmacologic activity ascertained from agonist stimulated $^{22}Na^+$ flux in BC_3H-1 cells.[2]

[d] For comparison of $K_p^{10'}$ and $K_p^{30'}$: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, $p > 0.05$.

In various pharmacological preparations an increase in methylene chain length from trimethonium to dodecamethonium shifts the equivalence points of the respective dose-response curves to lower concentrations. Maximum potency for stimulation of muscle contracture for chick biventer cervicus and frog rectus abdominus is found between dodecamethonium and tetradecamethonium (12, 32). Usually, in nerve-muscle preparations a maximum in the potency for antagonism of neuromuscular transmission is observed at decamethonium (12, 13, 32). We observe the same trend in the dissociation constants measured *in vitro* where there is essentially a monotonic decrease (i.e., an increase in affinity) with extension of chain length (Fig. 4).

The N-substituted analogues of decamethonium also exhibit the capacity to convert the receptor to a higher affinity state. The effect of all of these compounds on pharmacologic activation parameters have yet to be studied; however, based on extensive data with diphenyldecamethonium and dinaphthyldecamethonium (10, 11) it is likely that bis-aromatic substitutions render these compounds antagonists. Interestingly, however, we find that the Na^+ flux elicited by the mononitroxide analogue of decamethonium is comparable to that of decamethonium[2] and the two compounds are similar in relative affinities for the R and R' receptor states. The nitroxide analogue is a partial agonist and is of particular interest since ligand affinity can be measured by the competition with the initial rate of α-toxin binding and by electron spin

[2] S. Sine, unpublished observations.

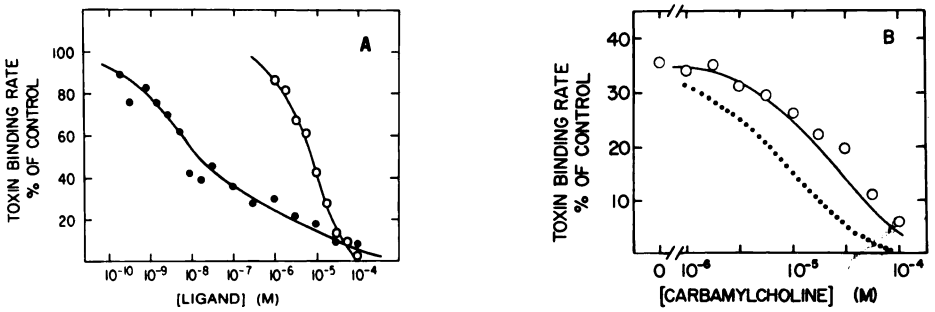


FIG. 6. *The influence of pancuronium and the combination of pancuronium and carbamylcholine on the initial rate of α-toxin binding*

Membrane fragments were 20 nM in toxin sites and α-toxin was 8 nM during the binding reaction. The rate of toxin association was determined from the first 20 and 40 sec of the reaction. A) Inhibition of the rate of α-toxin binding by carbamylcholine and pancuronium alone. (○), Membranes were exposed to carbamylcholine for 10 sec prior to initiating the toxin-binding reaction; (●), membranes were exposed to pancuronium for 30 min prior to initiating the toxin-binding reaction. B) Inhibition of the rate of α-toxin binding by the combination of carbamylcholine and pancuronium. (○——○), Membranes were exposed to 40 nM pancuronium for 30 minutes and then carbamylcholine added for 10 sec prior to initiating the toxin binding reaction. The two curves are theoretical for two possible binding modes: (——), a competitive interaction of carbamylcholine with equivalent sites of pancuronium binding which are negatively cooperative;

$$\frac{k_T{}^L}{k_T} = \frac{1}{1 + \left[\left(\frac{L}{K_p}\right)^{n_H}\right]_{carb} + \left[\left(\frac{L}{K_p}\right)^{n_H}\right]_{pan}}$$

(. . .) a non-competitive interaction between pancuronium and carbamylcholine.

$$\frac{k_T{}^L}{k_T} = \left(\frac{1}{1 + \left[\left(\frac{L}{K_p}\right)^{n_H}\right]_{carb}}\right)\left(\frac{1}{1 + \left[\left(\frac{L}{K_p}\right)^{n_H}\right]_{pan}}\right)$$

The values of $(L/K_p)^{n_H}$ for each ligand were determined empirically. For carbamylcholine each value of $[(L/K_p)^{n_H}]_{carb}$ was calculated from the corresponding concentration in Fig. 6A. For pancuronium $[(L/K_p)^{n_H}]_{pan}$, = 1.86, was calculated from the inhibition of toxin binding in the absence of carbamylcholine.

resonance. We find $K_L^{eq} = 1.00 \times 10^{-6}$ by ESR and 9.2×10^{-7} by α-toxin competition. These data provide an independent means of validation of the toxin competition measurements which require far less receptor for such studies.

The observation that only metaphilic antagonists promote a state transition is entirely compatible with the behavior of these compounds on chick biventer muscle (9–11). Rang and Ritter noted that successive short exposures to carbamylcholine do not enhance the antagonism by d-tubocurarine or gallamine whereas, for the metaphilic antagonists, dinaphthyldecamethonium or diphenyldecamethonium, a diminished response was noted with successive test doses of carbamylcholine. These investigators also established that prior agonist application and removal could convert the receptor to a state where enhanced antagonism was evident. The capacity of agonists to promote the conversion to the state preferred by the metaphilic antagonist paralleled their ability to produce desensitization. Thus, if the metaphilic antagonists have an enhanced affinity for the desensitized receptor, it would be anticipated that K_L and K'_L would differ for these agents.

Other antagonists, such as d-tubocurarine and gallamine, exhibit no detectable difference between K_L and K'_L. This was found to be the case even when conversion was maximized by initial exposure to high concentrations of antagonists and the subsequent examination was carried out with

TABLE 5

Rates of conversion from the low affinity to the high affinity receptor state

Ligand	Concentration	k_{obs}[a]	Fraction of receptor bound $\left(\frac{RL}{RL+R}\right)$[c]
	M	sec^{-1}	
Carbamylcholine	3×10^{-5}	5.2×10^{-3}	0.67
Nicotine	1×10^{-4}	2.1×10^{-2}	0.60
Phenyltrimethylammonium	3×10^{-5}	1.3×10^{-2}	0.46
Decamethonium	1×10^{-6}	7.7×10^{-3}	0.17
Mononitroxyldecamethonium	7×10^{-6}	3.9×10^{-3}[b]	0.63
Diphenyldecamethonium	1×10^{-6}	1.2×10^{-2}	0.40

[a] Rate constant for conversion of RL to $R'L$. According to scheme I this value should approach $k_{RL} + k'_{RL}$.

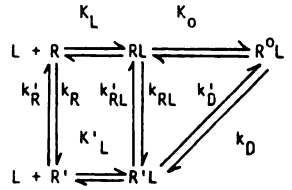
[b] Determined by electron spin resonance (3).

[c] Fraction of receptor bound as RL initially.

carbamylcholine which efficiently discriminates between the R and R' states. This test allows us to detect ratios of K_L/K'_L which are greater than 1.5 while ratios less than 4–5 are not likely to be revealed by the simple equilibrium dose-response curves. Characteristically, the antagonists where we find K_L/K'_L approaching 1.0 are rigid bisquaternary compounds where interquaternary distance is 10–11A. Gallamine is an exception to this but being a trisquaternary amine it may have unique spatial constraints when associated with the receptor. It is possible that flexibility of the ligand is a prerequisite for the R to R' state change since it involves a substantive change in conformation of the ligand-receptor complex.

The dependence on ligand structure seen for the *in vitro* assay lends further support to the contention that the state change observed *in vitro* involves the same molecular transitions as does desensitization *in situ*. In cases where evidence exists for a transition *in situ* either from desensitization or from metaphilic antagonism, we observe a difference in K_L and K'_L, while for classical antagonists $K_L \cong K'_L$. A cyclic scheme has been found to be the most consistent minimal mechanism for desensitization (8) and the transition in state observed *in situ* (3).

The cyclic model in relation to receptor activation. Since we are able to measure the state transition for compounds having different pharmacologic activation parameters, the relationship between the state changes associated with receptor activation and desensitization can be considered. By combining the scheme for receptor activation used by Magleby and Stevens (23) and the two-state cyclic scheme for desensitization (8), an overall scheme can be expanded to include all detectable or known receptor states (cf. 24):



R^o represents the activated state. K_L, K'_L and K_o are equilibrium constants for the fast processes of ligand association and receptor activation (cf. 25). K_L^{app}, the apparent dissociation constant immediately following ligand addition, and K_L^{eq}, the apparent dissociation constant at equilibrium, can be shown for $M \leq 0.1$ to be:

$$K_L^{app} \cong \frac{K_L}{1 + K_o} \tag{7}$$

$$K_L^{eq} = \frac{1 + M}{\frac{1}{K_L^{app}} + \frac{M}{K'_L}} \tag{8}$$

If we assume that desensitization can only follow activation (i.e., $k'_{RL} = k_{RL} = 0$) and that $L > R$, the rate of desensitization will be:

$$k_{obs} = \frac{1}{1 + \frac{L}{K_L^{app}}} k_R + \frac{L}{\left(\frac{1 + K_o}{K_o}\right)(K_L^{app} + L)} k_D + \frac{1}{1 + \frac{L}{K'_L}} k'_R + \frac{L}{L + K'_L} k'_D \tag{9}$$

For conversion we require $k'_D < k_D$ and $K^{eq} < K_L^{app}$. Also for $M \leq 0.1$, $k_R \ll k_D$ and $K'_L \ll K_L^{app}$.

When $L \sim K_L^{app}$, the above equation simplifies to:

$$k_{obs} = \frac{1}{2} k_R + \frac{K_o}{2(1 + K_o)} k_D + \frac{K'_L}{K'_L + L} k'_R + \frac{L}{L + K'_L} k'_D \quad (10)$$

and

$$\frac{K'_L}{K'_L + L} < 1.$$

For a full agonist we might expect $K_o \rightarrow 1$ (cf. 26). Thus, desensitization could proceed efficiently by the R^0L to $R'L$ pathway. For a partial agonist where K_o is reduced by a factor of 5 or 10, a slower rate of desensitization would be anticipated. In the case of the metaphilic antagonist K_o should be less than 0.01 of the value for full agonists and the rate of desensitization should approach $\frac{1}{2}k_R + k'_D$. In this situation isomerization of free R to R' becomes the dominant pathway for conversion to the desensitized state. Therefore, as the pharmacologic efficacy or activation parameter decreases, the rate of receptor conversion should slow dramatically. However, we have found the rate of receptor conversion not to be appreciably diminished for the metaphilic antagonists. With a constancy in rate the scheme eliminating the k_{RL} pathway could only hold if there was a compensatory increase in k_D with a decrease in K_o. Since this is unlikely, there would seem to be no requirement for desensitization to follow activation but rather it can proceed from the initial complex LR.

An upper limit for K_o is not known and so it can not be determined whether k_D and k'_D are comparatively large and the reaction, $R^0L \rightarrow R'L$, is a significant pathway for agonists.

In this respect it should be noted that the monoquaternary compounds that are full agonists yield dissociation constant ratios for the two states R and R' around 300, while the partial agonists have values between 5 and 50. The absence of a continuum in ratios suggests some relation between the activation and desensitization states but the rates of conversion indicate that the two processes involve parallel rather than sequential pathways.

Negative cooperativity among the antagonists. The structurally-rigid antagonists all exhibit Hill coefficients in the toxin competition assay of less than unity with pancuronium's value of 0.32 representing an extreme.[3] Such values may reflect multiple classes of independent binding sites or negative cooperativity where the interactions cause the binding affinity to decrease as a function of ligand saturation (27, 28). There appear to be either two or four ligand binding subunits per receptor so that site-to-site interactions can be accommodated in the models (29–31). Multiple classes of binding sites are often formally indistinguishable from negative cooperativity; however, it would be difficult to fit the low Hill coefficient found for pancuronium to a simple two-site model.

The apparent site interactions observed in the case of binding multiple antagonist molecules are not evident with simultaneous binding of agonist and antagonist since partial saturation of the pancuronium does

[3] With Hill coefficients less than unity, the estimated K_L does not reflect an equilibrium dissociation constant but only a measure of 50% occupation. Nonunitary Hill coefficients will also complicate the measurements of K_L and K'_L. If we assume the same Hill coefficients for the two receptor states, the saturation function at equilibrium would be

$$\bar{Y} = \frac{L^{n_H}}{L^{n_H} + (K_L^{eq})^{n_H}} \quad \text{where}$$

$$(K_L^{eq})^{n_H} = \frac{1 + M}{\frac{1}{(K_L)^{n_H}} + \frac{M}{(K'_L)^{n_H}}}$$

From the protection constants

$$K'_L = \left(\frac{M(K_p^{30'})^{n_H}}{1 + M - \left(\frac{K_p^{30'}}{K_p^{10'}}\right)^{n_H}} \right)^{\frac{1}{n_H}}$$

Because of the error inherent in calculations of n_H, the reported values in Tables 2 and 3 are derived from Equation 5 without consideration of the Hill coefficient. Of the compounds where K_L and K'_L differ, the estimated values of only tetradecamethonium, diphenyldecamethonium and decamethonium dinitroxide are changed significantly when the Hill coefficients are considered.

not induce negative cooperativity in the subsequent binding of carbamylcholine (Fig. 6). Moreover, we find that the binding of carbamylcholine and pancuronium appears competitive. Further examination of antagonist interactions is necessary where both antagonism of toxin binding and the pharmacologic response are analyzed. Existing data indicate that low Hill coefficients are not observed when pharmacologic antagonism is measured (9). This may indicate that pharmacologic antagonism can be achieved without full occupation of all subunit sites by antagonist.

NOTE ADDED IN REVISION

After this manuscript was prepared, an article by U. Quast, M. Schimerlik, T. Lee, V. Witzemann, S. Blanchard and M. A. Raftery appeared (Biochemistry **17**, 2405–2414 (1978)) which used a similar approach to examine ligand binding to the membrane associated receptor from *Torpedo californica*. A different buffer system was used in the two studies, and, although the findings of the two studies are in general ageement, some quantitative differences exist which should be noted:

(1) Quast *et al.* found that the ratio of dissociation constants of the low and high affinity states for acetylcholine was considerably smaller than that found for carbamylcholine (cf Table 1 in the above reference). In contrast, we observed that the ratio (K_L/K'_L) for acetylcholine is comparable to carbamylcholine and other full agonists (Table 1).

(2) d-Tubocurarine in the study by Quast *et al.* appeared to promote a conversion in receptor state, while we found that d-tubocurarine and gallamine did not shift the R to R' equilibrium (Fig. 2 and Table 3). Quast *et al.* employed 2.5 μM carbamylcholine to shift the R to R' equilibrium to the R' state. The system was diluted 40-fold and d-tubocurarine (2.2 μM) inhibition of α-toxin binding in the presence of 60 nM carbamylcholine was measured before and after reversion in receptor state occurred (Fig. 6d). In contrast, we employed 5.0 μM d-tubocurarine to effect a shift in the R to R' ratio which should occur if there is an appreciable difference in affinity for the two states. The system was diluted 500-fold to a d-tubocurarine concentration of 10 nM. Immediately thereafter toxin binding in the presence of 1.0 μM carbamylcholine was employed to ascertain if d-tubocurarine exposure altered the R to R' ratio. No effect of the initial exposure of d-tubocurarine was observed (Table 3).

(3) Quast *et al.* did not find their kinetic data in agreement with the two-state cyclic model for receptor desensitization (8), whereas our earlier kinetic analysis (3) and the additional data in this study are in close accord with the kinetic constraints dictated by the cyclic scheme. The differences largely emerge from a comparison of Fig. 3 of Quast *et al.* with Fig. 1 of this article. We found that K'_L and K'^*_L differed by an order of magnitude while Quast *et al.* show these values differ by a factor of 2. Since equation (8) reduces to $K'^*_L = K_L/M$ when $K'_L \ll K_L$, Quast *et al.* found the value of M to be 0.48 which is inconsistent with the initial predominance of the low affinity state, $M \leq 0.1$. The study of Quast *et al.* employs a buffer system contaning 4.0 mM Ca^{++} which results in more rapid conversions in receptor state ($\tau_{1/2max}$ = 4.9 sec) (4). The rapid rates require an extrapolation to measure the kinetic constant for conversion to the high affinity state and the equilibrium constant for the low affinity state. The buffer system containing Ca^{++} provides a more physiologic medium for the external membrane surface. Neither of the buffers in the two studies, however, would insure physiologic ion concentrations for the internal membrane surface of the vesicle or membrane sheet.

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REFERENCES

1. Weber, M., David-Pfeuty, T., & Changeux, J.-P. (1975) Regulation of binding properties of the nicotinic receptor protein by cholinergic ligands in membrane fragments from *Torpedo marmorata*. *Proc. Natl. Acad. Sci. (USA)*, **72**, 3443–3447.
2. Weiland, G., Georgia, B., Wee, V., Chignell, C. F., & Taylor, P. (1976) Ligand interactions with cholinergic-receptor-enriched membranes from *Torpedo:* influence of agonist exposure on receptor properties. *Mol. Pharmacol.*, **12**, 1091–1105.
3. Weiland, G., Georgia, B., Lappi, S., Chignell, C. F., & Taylor, P. (1977) Kinetics of agonist-mediated transitions in state of the cholinergic receptor. *J. Biol. Chem.*, **252**, 7648–7656.
4. Lee, T., Witzemann, V., Schimerlik, M. & Raftery, M. A. (1977) Cholinergic ligand induced affinity changes in *Torpedo californica* acetylcholine receptor. *Arch. Biochem. Biophys.*, **183**, 57–63.
5. Colquhoun, D. & Rang, H. P. (1976) Effects of inhibitors on the binding of iodinated α-bungarotoxin to acetylcholine receptors in rat muscle.

Mol. Pharmacol., **12**, 519–535.

6. Boyd, N. D. & Cohen, J. B. (1978) Kinetics of binding of cholinergic ligands to cholinergic postsynaptic membranes. *Fed. Proc.*, **37**, 650.
7. Bonner, R., Barrantes, F. J. & Jovin, T. M. (1976) Kinetics of agonist-induced intrinsic fluorescence changes in membrane-bound acetylcholine receptor. *Nature (Lond.)*, **263**, 429–431.
8. Katz, B. & Thesleff, S. (1957) A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol. (Lond.)*, **138**, 63–80.
9. Rang, H. P. & Ritter, J. M. (1969) A new kind of drug antagonism: evidence that agonists cause a molecular change in acetylcholine receptors. *Mol. Pharmacol.*, **5**, 394–411.
10. Rang, H. P. & Ritter, J. M. (1970) On the mechanism of desensitization at cholinergic receptors. *Mol. Pharmacol.*, **6**, 357–382.
11. Rang, H. P. & Ritter, J. M. (1970) The relationship between desensitization and the metaphilic effect at cholinergic receptors. *Mol. Pharmacol.*, **6**, 383–390.
12. Paton, W. D. M. & Zaimis, E. J. (1949) The pharmacological actions of polymethylene bistrimethylammonium salts. *Br. J. Pharmacol.*, **4**, 381–400.
13. Barlow, R. B., & Ing, H. R. (1948) Curare-like action of polymethylene bisquaternary ammonium salts. *Br. J. Pharmacol.*, **3**, 298–304.
14. Brittain, R. T. & Tyers, M. B. (1973) The pharmacology of AH 8165: a rapid-acting, short-lasting, competitive neuromuscular blocking drug. *Br. J. Anesth.*, **45**, 837–843.
15. Mooser, G., Schulman, H. & Sigman, D. S. (1972) Fluorescent probes of acetylcholinesterase. *Biochemistry*, **11**, 1595–1602.
16. Sinha, B. K. & Chignell, C. F. (1975) Synthesis and biological activity of spin-labelled analogs of biotin, hexamethonium, decamethonium, dichlorisoproterenol, and propranolol. *J. Med. Chem.*, **18**, 669–673.
17. Flanagan, S. D., Barondes, S. H. & Taylor, P. (1976) Affinity partitioning of membranes; cholinergic receptor-containing membranes from *Torpedo californica*. *J. Biol. Chem.*, **251**, 858–865.
18. Hill, A. V. (1913) The combinations of haemoglobin with oxygen and with carbon monoxide. *Biochem. J.*, **7**, 471–480.
19. Sokal, R. R. & Rohlf, F. J. (1973) *Biometry*. Freeman, San Francisco, 404–493.
20. Brown, B. W. & Hollander, M. (1977) *Statistics: a Biomedical Introduction*. Wiley, New York.
21. Janin, J. (1973) The study of allosteric proteins. *Prog. Biophys. Mol. Biol.*, **27**, 77–120.
22. Popot, J.-L., Sugiyama, H. & Changeux, J.-P. (1976) Studies on the electrogenic action of acetylcholine with *Torpedo marmorata* electric organ. II. The permeability response of the receptor-rich membrane fragments to cholinergic agonists *in vitro*. *J. Mol. Biol.*, **106**, 469–483.
23. Magleby, K. L. & Stevens, C. F. (1972) The effect of voltage on the time course of end-plate currents. *J. Physiol. (Lond.)*, **223**, 151–171.
24. Magazanik, L. G., & Vyskocil, F. (1976) Desensitization at the neuromuscular junction, in *Motor Innervation of Muscle*. (S. Thesleff, ed.) Academic Press, New York, 151–176.
25. Sheridan, R. E. & Lester, H. (1975) Relaxation measurements on the acetylcholine receptor. *Proc. Natl. Acad. Sci. (USA)*, **72**, 3496–3500.
26. Dionne, V. E., Steinbach, J. H. & Stevens, C. F. (1978) An analysis of the dose-response relationship at voltage-clamped frog neuromuscular junctions. *J. Physiol. (Lond.)*, **281**, 421–444.
27. Hammes, G. G. & Wu, C.-W. (1974) Kinetics of allosteric enzymes. *Annu. Rev. Biophys. Bioeng.*, **3**, 1–33.
28. Levitzki, A. & Koshland, D. E., Jr. (1976) The role of negative cooperativity and half-of-the-sites reactivity in enzyme regulation. *Curr. Top. Cell. Regul.*, **10**, 1–40.
29. Raftery, M. A., Vandlen, R. L., Reed, K. L. & Lee, T. (1975) Characterization of *Torpedo californica* acetylcholine receptor: its subunit composition and ligand-binding properties. *Cold Spring Harbor Symp. Quant. Biol.*, **40**, 193–202.
30. Karlin, A., Weill, C. L., McNamee, M. G. & Valderrama, R. (1975) Facets of the structures of acetylcholine receptors from *Electrophorus* and *Torpedo*. *Cold Spring Harbor Symp. Quant. Biol.*, **40**, 203–210.
31. Damle, V. N. & Karlin, A. (1978) Affinity labelling of one of two α-neurotoxin binding sites in acetylcholine receptor from *Torpedo californica*. *Biochemistry*, **17**, 2039–2052.
32. Rang, H. P. & Ritter, J. M. (1971) The effect of disulfide bond reduction on the properties of cholinergic receptors in chick muscle. *Mol. Pharmacol.*, **7**, 620–631.
33. Barlow, R. B. & Zoller, A. (1964) Some effects of long chain polymethylene bisonium salts on junctional transmission in the peripheral nervous system. *Br. J. Pharmacol.*, **23**, 131–150.
34. Katz, B. & Miledi, R. (1973) The characteristics of 'end-plate noise' produced by different depolarizing drugs. *J. Physiol. (Lond.)*., **230**, 707–717.