Ligand Responses of α-Bungarotoxin Binding Sites from Skeletal Muscle and Optic Lobe of the Chick*

Ging-Kuo Wang, Stephen Molinaro, and Jakob Schmidt

From the Department of Biochemistry, School of Basic Health Sciences, State University of New York, Stony Brook, New York 11794

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Binding properties of detergent-solubilized receptors for α-bungarotoxin from skeletal muscle of the 13th day chick embryo and from optic lobe of the hatching chick were compared. It was found that both types of receptor are nicotinic, although they differ in their affinities for individual ligands and in the rank order of ligands. In contrast to the muscle receptor, the neuronal receptor binds the toxin in a reversible fashion ($K_D = 2.1 \times 10^{-19} \text{M at } 23^\circ C$). Small ligands inhibit brain receptor-toxin association, as analyzed by kinetic and equilibrium procedures. Toxin and ligands compete for a single type of noninteracting site, and the ratio of toxin binding sites to ligand-binding sites is unity. The inhibitory potency of ligands parallels their ability to accelerate receptor-toxin complex dissociation. This accelerating effect is not saturable. It is proposed that nicotinic ligands block higher concentrations, to accelerate receptor dissociation.

The present study was initiated to find an explanation for this apparent discrepancy, by analyzing in detail the binding properties of toxin receptors extracted from the skeletal musculature and the central nervous system of the chick.

The molecular characterization of nicotinic acetylcholine receptors from electric tissue and skeletal muscle owes a great deal to α-bungarotoxin and similar snake venom polypeptides which bind with extreme selectivity and affinity to these receptors and thereby block synaptic transmission across the neuroeffector junction (1). Specific binding sites for αBuTX' have been observed on nerve cells as well, especially in autonomic ganglia (2, 3) and retina (4, 5), and more recently on motoneuron terminals (6), sensory ganglia (7), and a large variety of structures in the brain and spinal cord (8), and it is of great neurobiological interest to determine if these binding sites are somehow associated with acetylcholine receptors, because the toxin might provide a powerful tool for their identification, characterization, and purification. There is considerable biochemical and neuronomatological evidence in support of a neurotransmitter receptor role of these toxin binding sites, yet a physiological analysis of certain cholinergeic synapses in spinal cord and sympathetic ganglia has revealed that the nicotinic receptors involved are not blocked by αBuTX and its congeners (9-12).

The present study was initiated to find an explanation for this apparent discrepancy, by analyzing in detail the binding properties of toxin receptors extracted from the skeletal musculature and the central nervous system of the chick.

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The abbreviation used is: αBuTX, α-bungarotoxin.

EXPERIMENTAL PROCEDURES

Chemicals—Drugs were obtained from Sigma Chemical Co., except atropine sulfate and gallamine triethiodide (from ICN), and tetraethylammonium chloride (Eastman). Quinuclidinyl benzilate was a gift from Dr. H. Maurter, Tuttis University, and dl-dihydro-beta-erythroidine was supplied by Merck, Sharp & Dohme.

Preparation of Brain and Muscle Extracts—Fertilized eggs were bought from Spafas (Norwich, Conn.) and incubated at a relative humidity of 98%. Leg muscles from 13th day chick embryos and optic lobes from 20th day chick embryos were used for the majority of tissue preparations; these are the times at which receptor concentrations reach a maximum (Fig. 1; see also Ref. 13). Freshly dissected tissue was homogenized in 20 volumes of 10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100, 0.4 mM phenylmethylsulfonyl fluoride, 13 mM dimethylformamide, 0.02% sodium azide (extraction buffer), and homogenized in a Thomas tissue grinder (brain) or in a Polytron homogenizer (muscle). After additional stirring for 1 h at 4°C the homogenate was centrifuged at 35,000 x g for 30 min, and the supernatant fluid was stored at -70°C until use. Unless samples were repeatedly thawed and frozen, no significant loss of binding activity was observed for periods up to 1 month.

Toxin Binding Assay—αBuTX was purified from Bungarus multicinctus venom (Miami Serpentarium) and labeled with $^{125}$I as described previously (4). A chromatographically homogeneous fraction, tentatively identified as mononido-αBuTX, proved to be 100% biologically active as determined by its ability to bind to acetylcholine receptor from Torpedo californica; its specific radioactivity varied from 3 × 10$^6$ to 3 × 10$^7$ Ci/mol, depending on the age of the preparation. No decline in binding activity was observed upon storage for up to two half-lives of $^{125}$I. Binding of $^{125}$I-αBuTX to Triton X-100 extracts was measured by means of a DEAE-cellulose disc technique (15) which involves incubation of aliquots of detergent extract in 10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100 (standard buffer) with appropriate amounts of $^{125}$I-αBuTX followed by adsorption of the incubation mixture onto DEAE-cellulose discs (Whatman DE81) and removal of unbound toxin by washing with standard buffer. During the wash, 15% of the brain receptor-bound toxin is released from the disc; all binding data presented in the present report have been corrected for this loss. Nonspecific binding to both extract and disc was determined by incubation in the presence of excess nonradioactive toxin. As a rule, nonspecific binding amounted to less than 5% of total toxin used. Occasionally, large volumes of reaction mixture were filtered through two apposed DEAE-cellulose paper discs (DEAE-filter assay, Ref. 16). Binding of $^{3}$H-αBuTX to membrane preparations was measured as described previously (17).

Drug Binding Analysis—On rate and equilibrium experiments followed a similar protocol. Ligands were dissolved in standard buffer just prior to use. Tissue extracts were incubated with serial dilutions of a drug for 30 min, then $^{3}$H-αBuTX was added to the mixture to give final concentrations of about 10$^{-9}$ M receptor and 2 × 10$^{-8}$ M toxin; at the desired time point (2% to 5 min for determination of $K_D$; 3 h to overnight for determination of $K_T$), an aliquot was transferred to a DEAE-cellulose disc to terminate the reaction. To analyze drug effects on toxin binding rate, incubation conditions were chosen such as to allow receptor saturation to go to approximately 45% of completion; this ensures approximation of initial rate conditions. The drug concentration required to reduce initial binding rate to 50% is defined as $K_T$ (protection constant). To determine off rate effects, the order of addition of ingredients was reversed; after permitting the reaction of receptor and toxin to go to completion (usually overnight), various
Correct for nonspecific binding, control samples were incubated in the absence of radiolabeled toxin, in order to correct for (a) the slight perturbation of the equilibrium upon increasing the volume at $t_0$, and (b) potential receptor denaturation during prolonged periods of incubation. All drug binding experiments were carried out at 37°C, unless stated otherwise.

RESULTS

The existence of specific $\alpha$-BuTX binding sites in the central nervous system was established by a saturation study, as shown in Fig. 2. The dissociation constant for the brain receptor, measured at 23°C, is $2.1 \pm 0.8 \times 10^{-12}$ M ($n = 9$), whereas for muscle it is not accurately measurable, but certainly below $10^{-12}$ M. Experiments were then performed to study the kinetics of association between $\alpha$-BuTX and these high affinity binding sites. The receptor-toxin association reaction was analyzed assuming second order kinetics; the experimental values yield a linear plot throughout a time course corresponding to more than 80% of complex formation. The association rate constants (23°C, standard buffer) were calculated to be $7.4 \times 10^{8}$ M$^{-1}$ s$^{-1}$ for brain and $3.0 \times 10^{8}$ M$^{-1}$ s$^{-1}$ for muscle.

FIG. 1. Binding of $^{125}$I-$\alpha$-BuTX to extracts from optic lobe (O.L.) and skeletal muscle (M.) during development. At the indicated times, extracts from optic lobe (O.L.) and thigh muscle (M.) were prepared, and binding sites were quantitated as described in the legend to Fig. 2.

FIG. 2. Saturation of optic lobe extracts with $^{125}$I-$\alpha$-BuTX at 23°C. Optic lobes were dissected from 20th day embryos and extracted as described in the text. Portions (0.2 ml) of the extract were incubated with different concentrations of $^{125}$I-$\alpha$-BuTX overnight at 23°C, in a total volume of 0.25 ml. Two 0.1-ml aliquots at each concentration were pipetted onto DEAE-cellulose discs. One disc was washed and counted (a), while the other was counted without washing; the concentration of free toxin was computed from the difference. To correct for nonspecific binding, control samples were incubated in the presence of $5 \times 10^{-9}$ M unlabeled $\alpha$-BuTX (A). - - - -, corrected binding curve.

CONCENTRATIONS OF [FREE TOXIN] NO

[EXTRACTED WET TISSUE]

FIG. 3. Association rate constants at 23°C. Optic lobe (A) and muscle (C) extracts, containing $1.3 \times 10^{-7}$ M and $0.8 \times 10^{-7}$ M toxin binding sites (R$_0$), respectively, were incubated with $5.6 \times 10^{-9}$ M and $3.1 \times 10^{-9}$ M $^{125}$I-$\alpha$-BuTX (T$_0$), respectively, at 23°C in a total volume of 1 ml of standard buffer. Reactions were started by adding radioactive toxin. At the times indicated, 0.05-ml aliquots were withdrawn and pipetted onto DEAE-cellulose discs which were processed as described in the text. Control experiments were carried out in the presence of excess unlabeled $\alpha$-BuTX to determine the extent of nonspecific binding at any time point; the concentration of specifically bound toxin is designated X. Data were plotted according to the integrated second order rate equation. The half-times of reaction were 145 s for optic lobe extract and 54 s for muscle extract.

FIG. 4. Dissociation rate constants at 23°C. Extracts of optic lobe (O.L.) ($1.0 \times 10^{-5}$ M in toxin binding sites) (A) and muscle ($2.7 \times 10^{-8}$ M) (C) were incubated overnight at 23°C with $^{125}$I-$\alpha$-BuTX ($2.8 \times 10^{-8}$ M and $5.7 \times 10^{-9}$ M, respectively). At time zero ($t_0$) native toxin in 0.1 ml of water was added to give a final concentration of $2.5 \times 10^{-6}$ M in a total volume of 1 ml. At the times indicated, 0.05-ml aliquots of the mixture were pipetted onto DEAE-cellulose discs, and the amount of bound toxin was determined. Values were normalized with respect to a control experiment in which buffer was added instead of native toxin, in order to correct for (a) the slight perturbation of the equilibrium upon increasing the volume at $t_0$, and (b) potential receptor denaturation during prolonged periods of incubation. Alternatively, after overnight incubation, 0.1-ml aliquots of the optic lobe mixture were pipetted onto several DEAE-cellulose discs, which were washed for 15 min in vigorously stirred standard buffer to remove free $^{125}$I-$\alpha$-BuTX. At this point (defined as $t_0$) the first disc was removed and counted; subsequently discs were analyzed at the indicated intervals (A). Muscle extract ($4.4 \times 10^{-9}$ M in toxin binding sites), after overnight incubation at 23°C with $^{125}$I-$\alpha$-BuTX ($7.5 \times 10^{-9}$ M) in a total volume of 1 ml, was diluted 100-fold with 50 mM Tris-HCl, pH 7.4, and bound toxin was determined, as a function of time after the dilution step, by processing 5-ml aliquots using the DEAE-filter assay (see text) (C). Nonspecific binding in all experiments was determined by maintaining high levels of native toxin in extracts, prior to and during exposure to $^{125}$I-$\alpha$-BuTX. All values were corrected for nonspecific binding.
Peripheral versus Central α-Bungarotoxin Receptors

FIG. 5. Inhibition of binding of 125I-oBuTX by d-tubocurarine at 23°C. Experimental conditions were those described in legend to Fig. 2, in the absence (○) and presence of d-tubocurarine at 3.16 × 10^{-5} M (●), 10^{-5} M (□), 3.16 × 10^{-6} M (△), and 10^{-6} M (■). Insert, a plot of the apparent dissociation constants (K_{D(app)}) as a function of inhibitor concentration (I). The inhibition constant (K_i) is calculated from the equation

\[ K_{D(app)} = K_D \left(1 + [I]/K_i\right) \]

assuming a ratio of binding sites for d-tubocurarine to toxin binding sites of n = 1.

![Graphical Representation](https://example.com/graph.png)

FIG. 6. Competitive binding of drugs to the oBuTX receptor in optic lobe at 37°C. Extracts (from 3.4 to 6.4 × 10^{-3} M receptor) were incubated with excess 125I-oBuTX (1.5 to 2.0 × 10^{-6} M) and different drug concentrations at 37°C overnight. The dissociation constant used was 5.72 × 10^{-10} M, calculated from kinetic data obtained at 37°C. Inhibition constants (K_i) are obtained as intercepts of the abscissa. The ratio of ligand to toxin binding sites is obtained as the negative slope of each curve. Tx, concentration of free toxin; R, concentration of receptor-ligand complex; R, concentration of receptor-toxin complex.

for muscle (Fig. 3). Three methods were used to determine dissociation rate constants. Preformed radioactive 125I-oBuTX receptor complexes were incubated with a large excess of unlabeled toxin, and the concentration of radioactive complex remaining was measured as a function of time. Alternatively, complexes were diluted into a large volume of buffer to initiate dissociation of radioactive toxin. The third method is a modified dilution procedure whereby discs holding equal amounts of receptor-toxin complex are washed for different periods of time in a large volume of standard buffer; this latter experiment reveals that during the wash procedure required for the standard binding assay (30 min at room temperature), 15% of the toxin bound to optic lobe extracts is lost and must be corrected for. Results of dissociation experiments are shown in Fig. 4; quite obviously the off rates determined using unlabeled ligand competition on one hand and the "negative concentration jump" technique on the other are indistinguishable. For brain receptors, an off rate constant of 9.6 × 10^{-5} s^{-1} was determined (23°C, standard buffer). The dissociation constant calculated from the rate constants is 1.3 × 10^{-10} M, comparable to the value derived from the equilibrium study. In the case of muscle extracts, no dissociation of the toxin could be detected. When binding to muscle membrane preparations was investigated, the interaction of receptor and toxin appeared to be slowly reversible; a similar observation has been made by Colquhoun and Rang in rat muscle (18). However, comparable apparent off rates were obtained in a control experiment in which a volume of buffer, small enough to produce no measurable dilution effect, was substituted for an equal volume of concentrated toxin. This leads us to believe that the observed reduction of receptor-toxin complexes over long periods of time is a consequence not of binding reversibility, but of proteolysis or other nonspecific effects; consequently, the half-life of the muscle receptor-oBuTX complex must be of the order of weeks or greater.

Drugs effects on toxin binding at equilibrium were investigated with brain receptors only. In Fig. 5, a double reciprocal plot is presented for binding of 125I-oBuTX in the presence of four different concentrations of curare. Intersection of all five curves at the same point on the y axis indicates that toxin and curare compete for a single noninteracting type of binding site. An approximately straight line was obtained when the

### Table 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Muscle, K_i (M)</th>
<th>Optic lobe, K_i (M)</th>
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<tr>
<td>Nicotine</td>
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^a Determined at 37°C as described under "Experimental Procedures."

^b Determined at 37°C as described in legend to Fig. 6.

^c Determined at 23°C as described in legend to Fig. 5.
Peripheral versus Central a-Bungarotoxin Receptors

The drug concentration required to reduce the initial toxin binding rate to 50% of the control value (protection constant, $K_D$) equals the inhibition constant obtained by equilibrium binding studies, as was first demonstrated by Weber and Changeux (19) for the acetylcholine receptor from electric tissue. Good agreement between the two kinds of constant is also observed in chick optic lobe; the correlation coefficient computed for Columns 2 and 3 of Table I is 0.98. Measurement of $K_P$ values permits the in vitro pharmacological analysis of muscle receptors (which because of their irreversible interaction with aBuTX cannot be studied at equilibrium). Affinities of several drugs for the chick muscle receptor, determined by this procedure, are compiled in Column 1 of Table I.

Small ligands not only slow down binding of aBuTX, they also accelerate its dissociation from the brain receptors. The effect of nicotine on the stability of the brain receptor-aBuTX complex can be demonstrated by following complex dissociation at specific drug concentrations (Fig. 7a), or by measuring complex remaining after a given incubation time as a function of drug concentration (Fig. 7b). With increasing concentrations of the drug, the half-life of the toxin-receptor complex was progressively reduced to less than 1 min. This effect is independent of whether the receptor is embedded in the membrane or solubilized; raising the temperature from 4°C to 37°C decreases the half-life of the complex by an order of magnitude, in the absence and at any given concentration of small ligand (Fig. 8). The low temperature experiment was carried out with the expectation that half-lives would become apparent $K_D$ values were plotted as a function of the concentration of curare (Fig. 5, inset), suggesting that there were as many receptor sites for curare as for aBuTX (16). Similar data were obtained with carbamylcholine and nicotine. Accurate determination of the number of binding sites for small ligands requires toxin-independent procedures such as equilibrium dialysis with radioactive compounds. Such studies are currently not feasible with the brain receptor because of the low concentration of the latter and the relatively high apparent $K_D$ values for the ligands in question. Nevertheless, it is possible to quantify the number of ligand-binding sites that are accessible to competition with neurotoxin. In the equation $R_T = R \cdot Tx + R \cdot L + R_P$, $R_T$ (total receptor concentration) as well as $R \cdot Tx$ (=receptor-toxin complex) are measurable, whereas the remaining two species, $R \cdot L$ (=receptor-ligand complex) and $R_P$ (=free receptor), are not. A simplification of this situation can be achieved according to Reich and his colleagues (16), by employing a sufficient excess of toxin to make $R_P$ negligible even in the absence of added competing ligand. The equation then reduces to $R_T = R \cdot Tx + R \cdot L$, thereby permitting the quantitation not only of $R \cdot L$, but also of the ratio of toxin and ligand binding sites. This type of analysis, carried out with several ligands, clearly established that the ratio of toxin binding sites to ligand binding sites is unity (Fig. 6).

![Fig. 7. Effect of nicotine on the dissociation rate of the receptor-toxin complex. Optic lobe extract was incubated with $^{125}$I-aBuTX at 37°C for 3 h. The experiment was begun by adding the desired amount of nicotine. Final receptor concentration was $7 \times 10^{-10}$ M, final toxin concentration was $2 \times 10^{-9}$ M. $a$, nicotine was added to the incubation mixture to give final concentrations of $4.5 \times 10^{-11}$ M (○), $4.5 \times 10^{-10}$ M (△), $4.0 \times 10^{-9}$ M (□), and $4.5 \times 10^{-8}$ M (◇) in a total volume of 1 ml; the incubation was continued at 37°C. At each time point indicated, a 0.1-ml aliquot was pipetted onto a DEAE-cellulose disc and processed as described in the text. Data were corrected for background and normalized with respect to the amount of toxin bound in the absence of nicotine. $b$, nicotine was added to the incubation mixture to give the indicated concentrations in a total volume of 0.08 ml. After an additional incubation for 5 min at 37°C, 0.075-ml aliquots were pipetted onto DEAE-cellulose discs and processed as described in the text. Values were corrected for nonspecific binding. The drug concentration corresponding to the midpoint of the curve is defined as $K_D$. Two independent experiments were performed (○, △).

![Fig. 8. Receptor-toxin complex half-life as a function of nicotine concentration and temperature. Rates of toxin dissociation were determined, at the indicated temperatures, as described in Fig. 7a. Membrane-bound receptors were analyzed by a centrifugation procedure, as follows. Optic lobe homogenate, in extraction buffer free of Triton X-100, was incubated with $^{125}$I-aBuTX for 3 h at 37°C. At the start of the experiment, nicotine was added to the mixture to give a total volume of 0.5 ml. Final concentrations were: receptor, $9.8 \times 10^{-10}$ M; toxin, $2.2 \times 10^{-9}$ M; nicotine, as indicated. At various times, 0.06-ml aliquots were pipetted into 1.5-ml microcentrifuge tubes, containing 1.2 ml of 120 mM NaCl in detergent-free extraction buffer. Tubes were spun in an Eppendorf model 5800 centrifuge for 2 min. The supernatant was removed by aspiration, and the pellet was analyzed in a Searle 1105 gamma counter. Half-lives were estimated from the resulting dissociation curves. Open symbols, Triton X-100 extract; closed symbol, membrane-bound receptor.](http://www.jbc.org/doi/10.1074/jbc.851086501)
measurable at very high ligand concentrations. However, it appears that nicotine above 0.2 \text{M} impairs binding of receptors to DEAE-cellulose. All nicotinic cholinergic drugs tested were found to increase toxin dissociation in essentially the same manner as nicotine. The potency of a drug to induce dissociation is related to its potency to block binding (Fig. 9). Only the snake toxins themselves violate this rule; native \textit{a}{BuTX} and \textit{Naja naja siamensis} \textit{a}-toxin were without effect on complex half-life at concentrations up to $2.5 \times 10^{-5} \text{M}$ and $10^{-3} \text{M}$, respectively.

**DISCUSSION**

Until now, a comparison of the drug binding properties of \textit{a}{BuTX} receptors from the central nervous system and peripheral tissues has had to rely on data obtained in different laboratories on different species with different methods (see e.g. McQuarrie et al. (20)). In the present study, some of these variables have been eliminated. The chick embryo is well suited for such an analysis because it contains, at specific stages of its development, high concentrations of \textit{a}{BuTX} receptors in the optic lobes and skeletal muscle which are readily and reproducibly measured using the same assay procedure.

A conspicuous difference between central and peripheral receptors concerns their toxin affinity. Our observation that \textit{a}{BuTX} binds reversibly, \textit{i.e.} relatively weakly, to neuronal binding sites confirms a number of earlier findings on brain and autonomic nervous system from various species, including chick (3, 14, 21, 22). This weaker interaction is not unexpected since the curarimimetic toxins were “designed,” by evolution, for the neuromuscular junction, and their affinity for neuronal sites presumably is accidental, arising from a general structural similarity of nicotinic receptors.

Drug effects on the toxin binding rate reveal the nicotinic nature of both kinds of receptor. As a rule, acetylcholine receptors from muscle were found to display higher affinities; a case in point is decamethonium which, as an inhibitor of the toxin association rate, is about 1,000 times more effective in muscle extracts than in brain extracts. Nicotine, on the other hand, clearly has a higher affinity for the central receptor.

The strong interaction of nicotine with neuronal toxin receptors has been noted previously (2, 17, 23, 24); it is conceivable that the neuronal toxin receptor in fact represents the physiological target for nicotine which is known to have far more pronounced effects on the central and autonomic nervous system, \textit{i.e.} on neuronal receptors, than on skeletal muscle (25). Comparing affinities of the two types of receptor for the nicotinic drugs \textit{d}-tubocurarine, gallamine, nicotine, acetylcholine, carbamylcholine, decamethonium, and hexamethonium, we detect a moderate similarity ($r = 0.49$). A better correlation emerges after muscarinic and non-cholinergic ligands are included in the analysis (for all compounds listed in Columns 1 and 2 of Table I, $r = 0.81$). We conclude that both receptors are nicotinic, but as nicotinic receptors they differ substantially.

The equilibrium binding studies on optic lobe extracts show that inhibition and protection constants agree very well, suggesting that the ligand produces its effects on toxin association rate and toxin binding equilibrium by interacting with one and the same site. An analysis of the inhibitory effect of several drugs on toxin binding reveals that it is of a strictly competitive nature; from the reciprocal plot of the inhibition data one may further deduce that the toxin binding sites involved in the competition belong to a single noninteracting type. Experiments carried out at large toxin excess reveal, furthermore, that the ratio of sites accessible to both drug and toxin is unity.

The acceleration of receptor-toxin dissociation by added ligand has been observed before in studies of the interaction of the acetylcholine receptor from eel electric tissue, cobra neurotoxin, and nicotinic effectors. Weber and Changeux (19) who used tritiated \textit{Naja naja nigricollis} \textit{a}-neurotoxin were able to speed up complex dissociation with several agonists and antagonists. In all cases which these authors studied, ligand concentrations: several orders of magnitude higher than their $K_p$ values were required to produce this effect, and there was no obvious relationship between the ability of a drug to shorten receptor-toxin complex half-life and its affinity to the toxin site as measured directly by equilibrium dialysis of radioactive derivatives or indirectly as a protection constant (effect on toxin binding rate) or inhibition constant (effect on toxin binding at equilibrium). In the case of decamethonium, the effect appeared to be saturable, and a half-maximal concentration of $5 \times 10^{-3} \text{M}$ was estimated suggesting a low affinity accessory binding site through which the ligand might exert its effect. Reich and his colleagues (16) analyzed this phenomenon in the system eel electric organ acetylcholine receptor, \textit{Naja naja siamensis} \textit{a}-neurotoxin. They observed that all cholinergic ligands tested accelerate toxin dissociation. Benzoquinonium decreased the half-life of the receptor by several orders of magnitude to a limit beyond which further increases in ligand concentration were without effect. From this saturation, the authors were able to calculate a dissociation constant for the formation of the ternary complex consisting of the small ligand and the preformed receptor-toxin complex. It was concluded that beside a high affinity site ($K_D = 5 \times 10^{-5} \text{M}$), there exists an accessory, low affinity binding site for benzoquinonium ($K_D = 2 \times 10^{-3} \text{M}$). The chick brain receptor differs from that of eel electric tissue in that the toxin off rate increases monotonically with the concentration of small ligand. The best example is nicotine which was tested at concentrations up to $0.5 \text{M}$ beyond which analysis is difficult, partly because of the temporal resolution of the assay procedure as currently employed (about 10 s), partly because of the interference of the ligand with the DEAE-cellulose disc technique. This does not rule out saturation at some even higher concentration. However, the dissociation constants for the
two kinds of binding site for nicotine would then differ by over 6 orders of magnitude (compared to less than 3 orders of magnitude for the system benzoxquinonium-eel receptor-sperm flagella). The absence of saturability at near molar concentrations leads us to believe that for all practical purposes, there is no such second site in the neuronal toxin receptor. The fundamental dilemma of the off rate effects is as follows. There is kinetic evidence that the effect is nonspecific, i.e. it is not saturable even at very high ligand concentrations; at the same time, however, there is pharmacological evidence that the effect is specific, i.e. the strength of the effect is related to the nicotinic cholinergic nature of the ligand investigated or more precisely, there is a good correlation between blocking and dissociating potency ($K_{b}$ and $K_{d}$) of a series of compounds tested. How can this dilemma be resolved? "Accessory" cholinergic sites as postulated for the eel receptor are ruled out because the saturability criterion is not met. We then have to assume that the low affinity binding actually occurs to the high affinity binding site. Let us briefly consider the plausibility of this proposition. All we have to assume is that, on a molecular scale, the complex is not as static as a half-life of 2 h at room temperature (and 30 min at 37°C) might imply and that, in fact, a given toxin molecule diffuses off, and returns to, the binding site many times before it escapes irreversibly, i.e. sufficiently to be replaced by any other nearby toxin molecule in the medium. The frequency with which associations of close proximity are established may be assumed to be higher than the frequency with which loose associations of greater distance between binding site and toxin are realized. The important point is that a large number of configurations are formed transiently, each characterized by a specific rate of formation, rate of return to the bound state, and rate of complete dissociation. It is now postulated that during the macroscopic half-life of the complex, "loose" configurations occur with sufficient frequency to allow the small ligand to penetrate beyond the "local toxin concentration" and reach the binding site. Obviously, the binding site that the small ligand "sees" is distorted sterically and electrostatically (both the toxin and cholinergic drugs are cations at physiological pH) by the vicinity of the toxin, and therefore, the affinity is expected to be reduced. Since no specific receptor-toxin configuration presents itself to the approaching ligand, rather an entire range of such configurations, a superposition of binding curves results, characterized by a continuum of dissociation constants that reach high values; consequently, saturation is not observed.

The results presented in this communication may be important for assigning a function to neuronal toxin receptors. Clearly, it would have been preferable to analyze binding properties of receptors in sympathetic ganglia rather than in the brain of the chick, since physiological data are presently available for the former only. However, one may assume that toxin receptors in the central and autonomic nervous system of the chick are similar. Drug affinities in sympathetic ganglion neurones have been reported and agree reasonably well with data on optic lobe presented here; it is of special interest that $d$-tubocurarine was observed by Carbonetto et al. (12) to accelerate toxin dissociation from receptors on chick sympathetic ganglia.

The contradictory findings concerning the identity of neuronal snake toxin receptors as neurotransmitter receptors can be reconciled in various ways. It is conceivable, for instance, that there exist multiple receptors, nicotine acetylcholine receptors that do or do not bind toxin, and toxin receptors that are or are not acetylcholine receptors. In such a scheme, toxin receptors from sympathetic ganglia would not be related to synaptic transmission, whereas toxin receptors in the toad optic tectum, where applied $a$BuTX blocks synaptic transmission (26), could be classified as bona fide nicotinic acetylcholine receptors. Alternatively, one may assume that all toxin binding sites are located on acetylcholine receptors but that in some cases, toxin binds without leading to receptor inactivation. The data presented above provide such a mechanism. Due to the postulated "micromovement" of the toxin-receptor complex, the transmitter is thought to gain access to its binding site before this site is completely vacated by the spontaneous dissociation of the toxin molecule. In other words, the transmitter activates the receptor while the toxin ostensibly remains bound. By the same token, other small ligands continue to interact with a toxin-occupied site, which would explain the fact that $d$-tubocurarine retains its inhibitory potency in the presence of $a$BuTX (12, 27).

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