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

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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 (Kd = 2.1 × 10^-18 M at 23°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, at higher concentrations, to accelerate receptor-toxin complex dissociation. This accelerating effect is not saturable. It is proposed that nicotinic ligands block association and induce dissociation of toxin by interaction with the same site on the receptor derived from the optic lobe.

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 neuromuscular junction (1). Specific binding sites for αBuTX have been observed on nerve cells as well, especially in autonomic gan gia (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 neuromanometric evidence in support of a neurotransmitter receptor role of these toxin binding sites, yet a physiological analysis of certain cholinergergic 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 muscle and the central nervous system of the chick.

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1 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 tetramethylammonium chloride (Eastman). Quinclidinyl benzilate was a gift from Dr. H. Mautner, Tufts University, and dihydro-β-erythroxydine 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 and suspended in 10 mM sodium phosphate, pH 7.4, 1.0% 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 × 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 [125I] as described previously (14). A chromatographically homogeneous fraction, tentatively identified as monoiodo-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 20 to 3 × 10^6 Ci/mmol, depending on the age of the preparation. No decline in binding activity was observed upon storage for up to two half-lives of [125I]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 [125I]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 [125I]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 [125I]-αBuTX was added to the mixture to give final concentrations of about 10^-7 M receptor and 2 × 10^-7 M toxin at the desired time point (2% to 5 min for determination of Kd, 3 h to overnight for determination of Kd), 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 Kd (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 5 \times 10^{-8} \text{ M} \alpha\text{-BuTX} overnight at 23°C, in a

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^{-8} \text{ M} unlabeled \alpha\text{-BuTX}.

RESULTS
The existence of specific \alpha\text{-BuTX} binding sites in the central
nervous system was established by a saturation study, as
described in the text. Portions (0.2 ml) of the extract were incubated
with different concentrations of \textsuperscript{125}I-\alpha\text{-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

heat of drug were added, and bound toxin determined
after an additional 5 min. The drug concentration at which
receptor-toxin complex half-life equals 5 min is defined as \textit{K}_c. In
some cases, receptor-toxin complex was determined as a function of
both drug concentration and incubation time. All drug binding experi-
ments were carried out at 37°C, unless stated otherwise.

concentrations the drug were added, and bound toxin determined

Fig. 1. Binding of \textsuperscript{125}I-\alpha\text{-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 \textsuperscript{125}I-\alpha\text{-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 \textsuperscript{125}I-\alpha\text{-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^{-8} \text{ M} unlabeled \alpha\text{-BuTX} (A). ---, corrected binding
curve.

Fig. 3. Association rate constants at 23°C. Optic lobe (A) and
muscle (C) extracts, containing 1.3 \times 10^{-7} \text{ M} and 0.8 \times 10^{-7} \text{ M} toxin
binding sites (Rc), respectively, were incubated with 5.6 \times 10^{-8} \text{ M}
and 3.1 \times 10^{-8} \text{ M} \textsuperscript{125}I-\alpha\text{-BuTX} (Tc), respectively, at 23°C in a total volume
of 1 ml of standard buffer. Reactions were started by adding radio-
active 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\text{-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^{-7} \text{ M} in toxin binding sites) (A) and muscle (2.7 \times 10^{-9}
M) (C) were incubated overnight at 23°C with 10^{-8} \text{ M} \textsuperscript{125}I-\alpha\text{-BuTX} (2.6 \times 10^{-8}
M and 5.7 \times 10^{-9} \text{ M}, respectively). At time zero (t0) native toxin in 0.1
ml of water was added to give a final concentration of 2.5 \times 10^{-9} \text{ M}
in a total volume of 1 ml. At the times indicated, 0.1-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 the slight perturbation of the
equilibrium upon increasing the volume at t0, and (b) potential
receptor denaturation during prolonged periods of incubation. Alterna-
atively, 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 \textsuperscript{125}I-\alpha\text{-BuTX}. At this point (defined as t0) the first disc was
removed and counted; subsequently discs were analyzed at the indi-
cated intervals (A). Muscles extract (4.4 \times 10^{-9} \text{ M} in toxin binding
sites), after overnight incubation at 23°C with 10^{-8} \text{ M} \textsuperscript{125}I-\alpha\text{-BuTX} (Tc), respectively, at 23°C 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) (O). Nonspecific binding in all experiments was
determined by maintaining high levels of native toxin in extracts,
prior to and during exposure to \textsuperscript{125}I-\alpha\text{-BuTX}. All values were corrected
for nonspecific binding.
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Fig. 5. Inhibition of binding of \(\text{^{125}I-\alpha\text{BuTX}}\) by \(d\)-tubocurarine at 22°C. Experimental conditions were those described in legend to Fig. 2, in the absence (C) and presence of \(d\)-tubocurarine at \(3.16 \times 10^{-5} \text{ M} \) (\(\bullet\)), \(10^{-5} \text{ M} \) (\(\triangle\)), \(3.16 \times 10^{-6} \text{ M} \) (\(\triangleleft\)), and \(10^{-6} \text{ M} \) (\(\triangle left\)). Inset, a replot of the apparent dissociation constants \(K_{D\text{(app)}}\) as a function of inhibitor concentration \((I)\). The inhibition constant \((K_i)\) is calculated from the equation

\[
K_{D\text{(app)}} = K_D \left(1 + [I]^n \right)^{-1}
\]

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

For muscle (Fig. 3). Three methods were used to determine dissociation rate constants. Preformed radioactive \(\text{^{125}I-\alpha\text{BuTX}}\) 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 dioce 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 (16) on the other are indistinguishable. For brain receptors, an off rate constant of \(9.6 \times 10^{-4} \text{ s}^{-1}\) was determined (23°C, standard buffer). The dissociation constant calculated from the rate constants is \(1.3 \times 10^{-10} \text{ M}\), 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-\(\alpha\text{BuTX} complex must be of the order of weeks or greater.

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

<table>
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<th>Ligand</th>
<th>Muscle, (K_i) M</th>
<th>(K_i) M</th>
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<tr>
<td>Nicotine</td>
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<td>(d)-Tubocurarine</td>
<td>(2.11 \times 10^{-7})</td>
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<td>Gallamine</td>
<td>(3.76 \times 10^{-6})</td>
<td>(3.82 \times 10^{-6})</td>
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<td>Lobeline</td>
<td>(6.80 \times 10^{-7})</td>
<td>(1.20 \times 10^{-5})</td>
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<td>Tetramethylammonium</td>
<td>(7.08 \times 10^{-5})</td>
<td>(1.90 \times 10^{-5})</td>
</tr>
<tr>
<td>Decamethonium</td>
<td>(2.35 \times 10^{-8})</td>
<td>(2.66 \times 10^{-5})</td>
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<td>Acetylcholine (in presence of (3 \times 10^{-4} \text{ M} ) eserine)</td>
<td>(1.26 \times 10^{-6})</td>
<td>(3.75 \times 10^{-5})</td>
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<tr>
<td>Carbamylcholine</td>
<td>(9.44 \times 10^{-6})</td>
<td>(6.35 \times 10^{-5})</td>
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<td>Quinuclidinyl benzilate</td>
<td>(1.00 \times 10^{-3})</td>
<td>(8.41 \times 10^{-5})</td>
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<td>Dihydro-(\beta)-erythroidine</td>
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<td>Decamethonium</td>
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<td>Acetylcholine (in presence of (3 \times 10^{-4} \text{ M} ) eserine)</td>
<td>(4.2 \times 10^{-7})</td>
</tr>
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<td>Carbamylcholine</td>
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<tr>
<td>Quinuclidinyl benzilate</td>
<td>(2.0 \times 10^{-7})</td>
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\(K_i\) determined at 37°C as described under "Experimental Procedures."
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 $\alpha$BuTX (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 $R \cdot L$ (=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).

The drug concentration required to reduce the initial toxin binding rate to 50% of the control value (protection constant, $K_P$) 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 $\alpha$BuTX 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 $\alpha$BuTX, they also accelerate its dissociation from the brain receptors. The effect of nicotine on the stability of the brain receptor-$\alpha$BuTX 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 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 longer.
measurable at very high ligand concentrations. However, it appears that nicotine above 0.2 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 aBuTX and Naja naja siamensis a-toxin were without effect on complex half-life at concentrations up to 2.5 X 10^{-5} M and 10^{-5} M, respectively.

**DISCUSSION**

Until now, a comparison of the drug binding properties of aBuTX 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 aBuTX 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 aBuTX binds reversibly, 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, i.e. on neuronal receptors, than on skeletal muscle (25). Comparing affinities of the two types of receptor for the nicotinic drugs 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 Naja naja nigricollis 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_D 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 derivative 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 X 10^{-5} 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, Naja naja siamensis 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 X 10^{-5} M), there exists an accessory, low affinity binding site for benzoquinonium (K_D = 2 X 10^{-5} 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 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
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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 benzoquinonium-eel receptor-saminisien a-neurotoxin). 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 (K0 and K∞) 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 (12). However, one may assume that toxin receptors in the central and autonomic nervous system of the chick are similar. Drug affinities in sympathetic ganglion neurons have been reported (2) 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 chich 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, nicotinic 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 lobe, where applied αBuTX 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 binding is without leading to receptor inactivation. The data presented above provide such a mechanism. Due to the postulated "microreversibility" 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 αBuTX (12, 27).

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