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Blockade of Acetylcholine Receptors by Cobra Toxin: Electrophysiological Studies

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SUMMARY

Microelectrode techniques were used to investigate the manner in which a pure polypeptide toxin from cobra venom blocks transmission at the frog myoneural junction. At concentrations of 16–114 nM, the toxin causes an irreversible exponential decline in the amplitude of the end plate potential as a result of a decrease in the sensitivity of the postsynaptic receptors for acetylcholine. Other processes, such as spontaneous and impulse-evoked acetylcholine release, acetylcholinesterase activity, and passive electrical properties of the muscle fiber membrane, remain unaffected. The rate constant for inactivation of receptors increases linearly with toxin concentration. The constant of proportionality probably describes the binding of toxin to the receptor and equals 1.5×10^5 M^{-1} sec^{-1}. Certain complications in these experiments arise because diffusion barriers limit the access of toxin molecules to the receptors.

INTRODUCTION

Venoms of hydropheid (sea snakes) and elapid (cobras, mambas, sambas, corals, kraits) snakes contain several protein toxins which have diverse effects on cell membranes (1–3). The most potent toxins have long been known to interfere with myoneural transmission. Within the past few years microelectrode techniques have been used to study the effects of α-bungarotoxin [from the Formosan banded krait (4)] and of cobra toxin (5–7) and have led to the conclusion that these toxins act by blocking postsynaptic sensitivity to acetylcholine.

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The present experiments were designed to provide more information about the site and kinetics of action of cobra toxin (8) at the neuromuscular junction.

There is substantial evidence that these toxins combine irreversibly with postsynaptic acetylcholine receptors. (a) Rat diaphragms treated with [^{131}I]toxin retain radioactivity only at the end plates (9–11). Furthermore, in denervated muscles, in which acetylcholine sensitivity spreads to involve the whole fiber (12), the labeling spreads similarly (13). (b) The acetylcholine receptor macromolecule has been partially purified from several muscles and electric organs with elapid toxins serving as bound radioactive markers (14–17), or as the selective element in affinity chromatography (18). (c) Desensitization by agonists and blockade by competitive antagonists, two processes thought to occur at the receptors themselves, inhibit the action of the toxin

at the myoneural junction (19, 20). Nicotinic drugs inhibit the binding of the toxin to solubilized receptor preparations (14, 15, 18, 21). (d) These toxins have no detectable enzymatic activity in solution (reviewed in ref. 2).

This paper presents further support for the postulated site and mode of action. Brief reports have been published elsewhere (22–24).

METHODS

Solutions. The standard Ringer's solution contained NaCl, 112 mM; KCl, 2.5 mM; $MgCl_2$, 4 mM; $CaCl_2$, 2 mM; and Tris, 5 mM (pH 7.3). Calcium concentration was often decreased to 0.4–0.7 mM in order to block neuromuscular transmission (25).

The experiments were performed with toxin T_3 from the venom of *Naja naja siamensis* (*kaouthia*) (Thailand cobra), kindly supplied as the crystalline powder by D. Eaker (8). The toxin (mol wt 7820) was stored at 4° at a concentration of 1 mg/ml in 50 mM acetate buffer, pH 5.0. Concentrations were checked by measuring optical density at 280 nm. The toxin solutions were diluted in the appropriate Ringer's solution just before use. *d*-Tubocurarine chloride was obtained from K & K Laboratories, Inc.; all other organic chemicals were purchased from Sigma.

Experimental chamber. The chamber held 3–5 ml of solution. Studies with a marker dye showed that a solution change was 50% complete after 10 ml, 95% after 25 ml, and more than 99% after 50 ml had been flushed through the chamber. Flow rates of 10–15 ml/min were employed.

Recording. Glass microelectrodes were used (26), with resistances ranging from 5 to 15 MΩ. Low-resistance pipettes, which had lower recording noise, were preferred for measurements on miniature end plate potentials. The recording amplifier has been described elsewhere (27). The stimulus frequency was 0.5/sec.

Micro-iontophoresis. Glass microelectrodes were also used for focal iontophoretic delivery of ACh[2] and carbachol (28). Some experiments required the same iontophoretic dose over a period of 1–2 hr; only preparations giving constant responses during an initial 15–30-min period were used. A controlled-current circuit ensured that the micropipette transferred the same quantity of charge despite any change in its electrical properties. Braking currents (28) of 3–10 namp were used.

Data collection and analysis. The results were analyzed by an on-line computer (Control Data model 160-A) adapted for neurophysiological experiments (19). The computer digitized sweeps at a sampling interval of 100 μsec, transferred them to magnetic tape, and displayed running averages.

Individual records were measured off-line by one of two methods designed to overcome recording noise as far as possible. In the first procedure each sweep was displayed on a screen, accompanied by horizontal "pointer" lines, which the operator set to the baseline and peak of the EPP. The computer then recorded the EPP amplitude. In the second, totally automatic procedure the computer first constructed a "template" from the average record by subtracting the baseline from the five samples which preceded the peak and the 80 which followed it. The record for the same time segment of each individual record, minus the baseline, was then analyzed. The computer calculated by the method of least squares the scaling factor which allowed the best fit between the individual response and the "template." This scaling factor was proportional to the individual response amplitude. The two methods of measurement gave equivalent results for EPPs and ACh potentials, but the "template" procedure could not be used for MEPP measurements.

Responses in excess of 1 mV were corrected for nonlinear summation (29).

RESULTS

Effects of cobra toxin on amplitude of end plate potentials. When cobra toxin was introduced into the solution bathing the muscle there was often a delay, quite variable in length, before the EPP ampli-

[2] The abbreviations used are: ACh, acetylcholine; EPP, end plate potential; MEPP, miniature end plate potential.

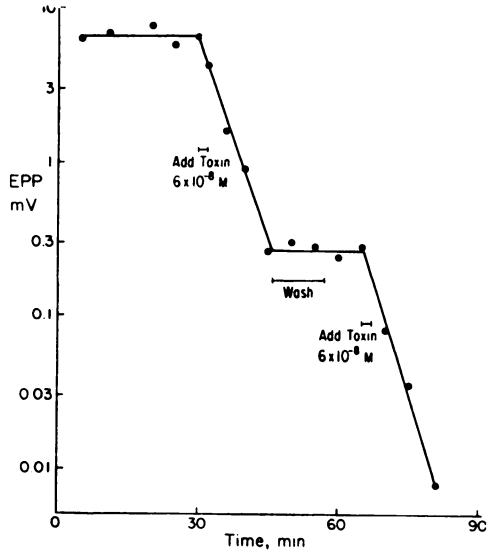

FIG. 1. *Myoneural transmission during treatment with cobra toxin*

Each point represents the average of 64 EPPs, except for the last two, which represent 128. Horizontal lines indicate the times when the chamber was flushed with new solutions.

tude started to decrease. The EPP amplitude then declined at a steady exponential rate (Fig. 1). The decline halted, but failed to reverse, when the chamber was flushed with toxin-free solution. When the preparation was again subjected to the same toxin concentration, the decline resumed with the same exponential rate constant. This rate constant, α, is a convenient measure of the activity of the toxin.

The experiments failed to reveal any recovery from the action of the toxin with a time constant less than about 24 hr. Within this limit, then, the toxin acts irreversibly.

Quantal content of end plate potentials. In most of these experiments the toxin concentration was 16 nM with a Ca^{++} concentration of 0.4 mM. The quantal content, m, ranged from 1.5 to 15 but usually was near 3. This value lies in the range in which three independent estimators of m may reliably be employed (30): m_0, from the number of failures; m_1, the ratio of the mean EPP to the mean MEPP amplitudes; and m_2, from the coefficient of variation of the EPP amplitude distribution. Calculation of m_2 used a grouped linear regression analysis similar to that described by Colomo and Rahamimoff (31).

Figure 2 shows results of this analysis for for two end plates. After a delay the toxin caused a decline in both EPP (curve *A*) and MEPP (*C*) amplitudes. The decreasing curves remain nearly parallel on semilogarithmic coordinates, and quantal content (*B*) is roughly constant, showing that the main effect of the toxin is to reduce quantal size with only a marginal reduction in quantal content.

The end plates shown in Fig. 2 are typical, in that the curve for MEPP amplitude (*C*) decreased slightly less steeply than that for EPP amplitude (*A*), and the toxin usually caused a corresponding decline in m (most clearly seen in the right-hand section of Fig. 2). Averaged results for 15 end plates in which the EPP had dropped to about one-fourth its original amplitude showed that the calculated value of m had decreased to 0.67 of the control value. These results suggest that the toxin acts presynaptically to diminish the release of ACh, as well as postsynaptically. Such an effect has been proposed for several drugs thought to act mainly on the postsynaptic membrane (32, 33). It is possible, however, that a nonuniform postsynaptic action of the toxin on different regions of the end plate may have caused m to be underestimated in these experiments.

If the toxin acts unevenly along an end plate, then the coefficient of variation of the MEPP amplitude distribution should tend to increase as the average MEPP amplitude decreases. Recording noise made it somewhat difficult to measure this effect, but analysis of results on 14 end plates in which the EPP amplitude had been reduced by a factor of 2 or more showed a significant increase, from 0.23 to 0.36, in the coefficient of variation of the MEPPs. This broadening of the MEPP distribution would tend to lower all three estimates of m. The number of failures, which determines m_0, is overestimated because responses consisting of one small MEPP are measured as failures. The average MEPP amplitude is overestimated, as small MEPPs go undetected,

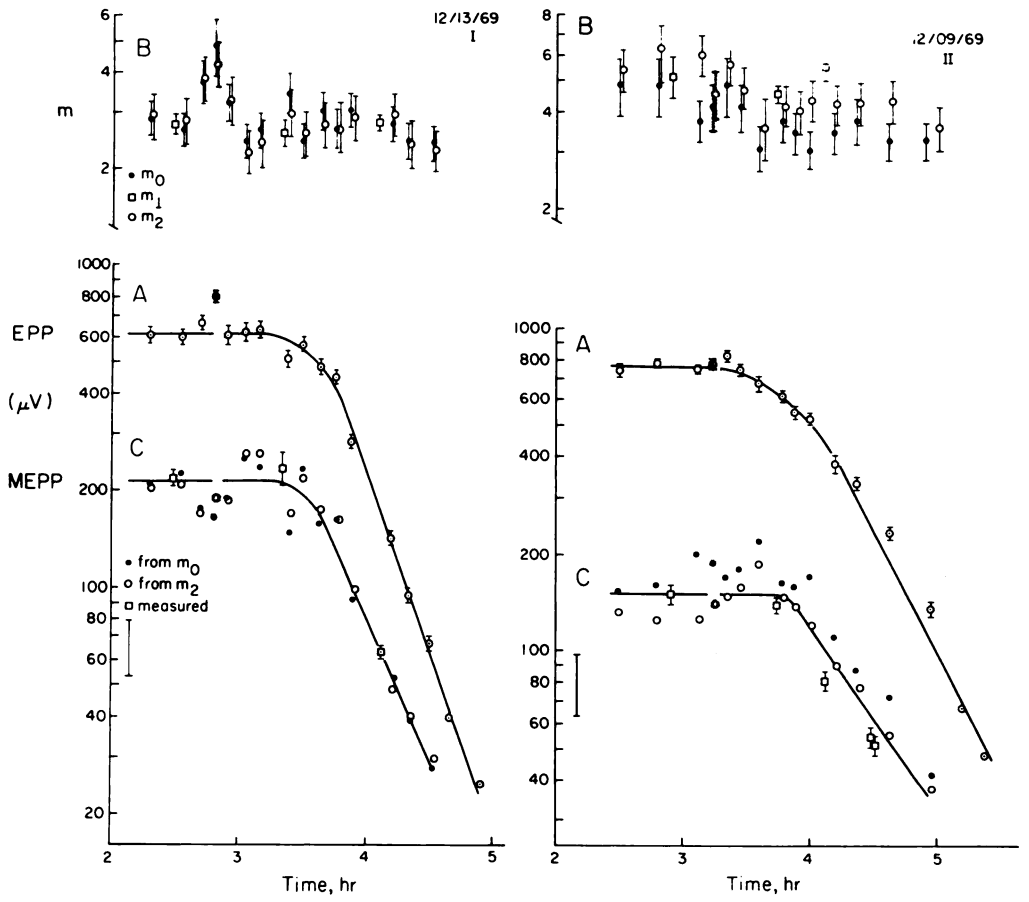

FIG. 2. *Postsynaptic action of cobra toxin on myoneural transmission*

Two end plates from different preparations are shown. At stippling, the chamber was flushed with a solution containing toxin. ⊙, ●, and ○, measurements on a series of 128 EPPs; □, measurements on spontaneously occurring MEPPs. *A*, EPP amplitude (mean ± SE). Points without error flags at later times were too small, relative to the recording noise, for accurate measurements on individual responses. *B*, quantal content (mean ± SE): ●, m_0, computed from the number of failures; ○, m_2, computed from the coefficient of variation of the EPP amplitude distribution; □, m_1, computed by dividing EPP by MEPP amplitudes. *C*, MEPP amplitude: □, measured directly; ●, computed as EPP/m_0; ○, computed as EPP/m_2. The vertical line at the lower left gives approximate error limits for computed MEPPs.

thus decreasing m_1. Finally, the broadened MEPP distribution leads to a broader EPP amplitude distribution, decreasing m_2. It is therefore likely that the apparent decrease in quantal content caused by the toxin was an artifact resulting from its nonuniform action.

Experiments at higher toxin concentration (114 nM) also showed that the main action was postsynaptic, since it was found (Fig. 3) that the coefficient of variation of the EPP (estimating m_2) remained unchanged as the EPP amplitude decreased.

Micro-iontophoretic application of acetylcholine. The diminution of quantal size caused by cobra toxin could arise either presynaptically, from decreased ACh content per quantum, or postsynaptically, from decreased ACh sensitivity of the muscle fiber membrane. The possibilities were tested by iontophoretic ACh application (Figs. 4 and 5; Table 1). The toxin reduced

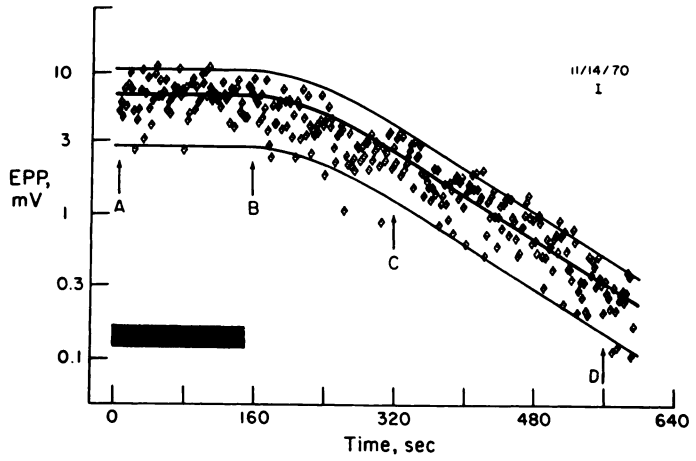

FIG. 3. *Postsynaptic action of cobra toxin at a higher concentration (114 nM)*
The amplitude of individual EPPs during treatment with 114 nM toxin is shown. The shaded area represents the time required to flush the experimental chamber with the toxin solution. The Ca^{++} concentration was 0.6 mM. The average (middle, heavy line) and two standard deviations (upper and lower, lighter lines) have been marked for the first 80 points before the toxin begins to act (segment *A* to *B*). An exponential decline was fitted to the 120 EPPs between *C* and *D*, and the fractional limits around *AB* are continued to *CD*. In both segments (*AB* and *CD*) 5% of the EPPs lie outside the limits.

the ACh potential at least as rapidly as it reduced the EPP, from which it is concluded that the block is entirely postsynaptic.

The possibility that the apparent reduction in quantal content produced by cobra toxin was an artifact resulting from its uneven action at different parts of the end plate was tested by comparing the time courses of decline of ACh potentials and EPPs in the presence of cobra toxin. Since there is no appreciable diffusion barrier between the external solution and the receptors that can be reached by microiontophoretically applied ACh (28, 34, 35), the kinetics of inactivation of these receptors by the toxin should show little variation, and should be faster than the kinetics for the decline of the EPP.

These predictions were borne out by experimentation on five end plates (Fig. 5 and Table 1). The mean value of α for ACh potentials was about 40% greater than that for EPPs; the coefficient of variation of α was 11% for ACh potentials but 31% for the latter. Furthermore, the delay before the action of the toxin on ACh potentials, measured by $t_{1/2}$, was not significantly less than the delay for the action of *d*-tubocurarine, a much smaller molecule whose action reflects the speed of the solution change near the receptors (34).

The average α value for EPPs in Table 1 exceeds by 40% the average for the end plates subjected to the same toxin concentration for the data in Fig. 6. This is because the end plates in Table 1 were all selected for sensitivity to micro-iontophoretic application of ACh; therefore at least a fraction of their ACh receptors were freely accessible to the external solution. This bias is not present in the results of Fig. 6.

Denervated fibers. Thirty-three days after severance of the sciatic nerve, ACh sensitivity was detected for several millimeters around the end plate region (12). The newly induced receptors were irreversibly inactivated by cobra toxin. Although detailed quantitative studies were not made, it appeared that the toxin inactivated these receptors at about the same rate as at normal end plates. Others have reported similar findings with denervated rat (37) and frog (14) fibers.

Time course of EPP. In most cases the toxin failed to alter the time course of the EPP. Although the absolute amplitudes often differed by a factor of 100 before and

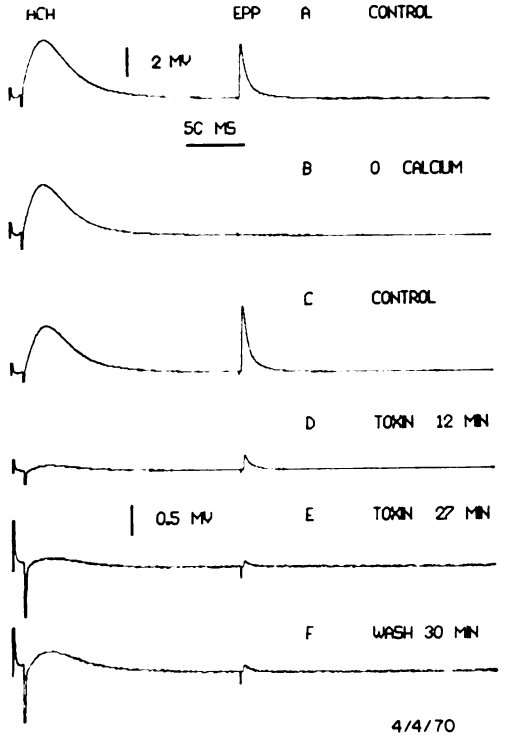

FIG. 4. *Effect of cobra toxin on response to microiontophoretically applied ACh*

The control solution contained 0.6 mM Ca^{++}; toxin, 50 nM, was added as noted. The iontophoretic current was 15 namp for 10 msec. Brief capacitative transients (retouched) mark the beginning and end of the ACh pulse. Each trace is the average of 16 sweeps. Vertical calibration: *A–D*, 2 mV; *E* and *F*, 0.5 mV.

after treatment, the averaged wave forms could be superimposed after magnification to the same peak amplitude (22).

Inhibitors of acetylcholinesterase prolong both the rise and fall of the EPP (26). Therefore the toxin does not appear to affect the acetylcholinesterase. The rising phase of the EPP was constant, so that the kinetics of ACh release was not grossly altered (35, 38). The constancy of the falling phase also indicates that the passive resistance and capacitance of the muscle fiber remained unaffected.

Also, the latency between the stimulus artifact and the start of the EPP remained unaffected; therefore propagation of the presynaptic impulse and synaptic delay (35, 39) did not change significantly. Chang and Lee (6) provided valuable evidence on this point. They recorded the impulse at the nerve terminal and found that it remained unaltered during treatment with cobra toxin; the end plate current recorded by the same electrode, however, declined.

Absence of effects on other processes. The resting potential showed no significant changes for any of the toxin treatments used. Furthermore, in 13 end plates, when the EPP had dropped to less than half its control value, the average frequency of MEPPs was unchanged. Chang and Lee (6) observed no major changes in the muscle fiber action potential after toxin treatment.

Variation in α with concentration. The toxin acts with first-order kinetics, irreversibly blocking a given fraction of the receptors per unit time (Figs. 1–3 and 5). The rate constant, α, for this process was measured as a function of toxin concentration (Fig. 6). The lowest concentration (16 nM) produced results like those in Fig. 2; the highest (114 nM) is represented by Fig. 3. Among end plates treated with equal toxin concentrations, α showed appreciable scatter. Nevertheless, α clearly increases with concentration, and the data fit a straight line.

DISCUSSION

The simplest model for the action of the toxin is represented by

$$T + R \xrightarrow{k_T} TR$$

A toxic molecule, T, and a receptor, R, form the irreversible complex TR, which is insensitive to ACh. This scheme leads to the differential equation

$$\dot{r} = -k_T[T]r$$

where r is the number of available receptors R and $[T]$ is the toxin concentration near the receptors (see below). For $[T]$ constant, we have

$$\alpha = k_T[T]$$

and r declines exponentially with the rate constant α.

The experimental data describe the decline of MEPP, EPP, and ACh potential amplitudes during treatment with the toxin.

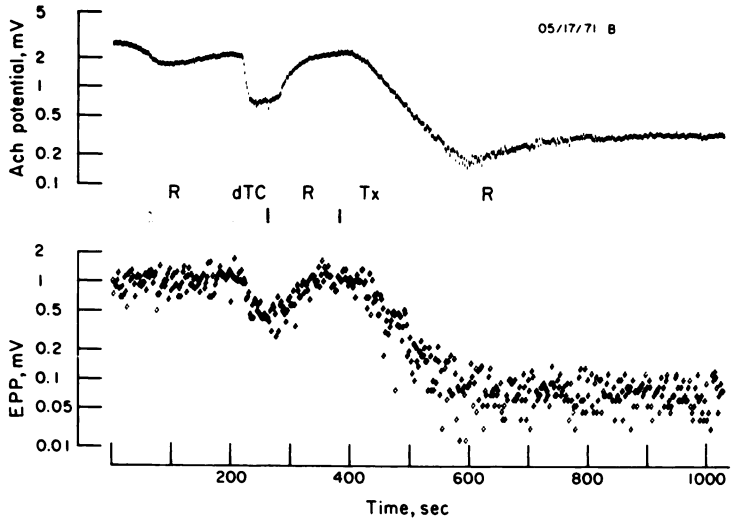

FIG. 5. *Rapid action of cobra toxin in the absence of diffusion barriers*

The amplitudes of ACh potentials (top) and EPPs were recorded at the same end plate. Individual records were similar to those used for the experiment in Fig. 4. Both responses were reduced reversibly by *d*-tubocurarine (dTC, 0.4 μM) and irreversibly by cobra toxin (Tx, 114 nM). Shading indicates the times when solutions were flushed through the chamber. R, control Ringer's solution (0.6 mM Ca^{++}). The first flushing with Ringer's solution gives an indication of how mechanical artifacts alone affect the response.

TABLE 1

Comparison of d-tubocurarine and cobra toxin effects on ACh potentials and EPPs

Data are like those shown in Fig. 5. As usual, α is the final rate of exponential decline during treatment with cobra toxin. For the toxin, $t_{1/2}$ is the time from the beginning of the solution change until the rate of exponential decline reaches $\alpha/2$. For *d*-tubocurarine, $t_{1/2}$ is the time required for the ACh potential to change halfway from its initial to its final value. Because of quantal fluctuations in EPP amplitude (see Fig. 5), $t_{1/2}$ for *d*-tubocurarine on EPPs could not be measured.

	EPPs		ACh potentials		
	$t_{1/2}$ (toxin)	α	$t_{1/2}$ (*d*-tubocurarine)	$t_{1/2}$ (toxin)	α
End plate	*sec*	10^{-3} *sec*$^{-1}$	*sec*		10^{-3} *sec*$^{-1}$
05/13/71a	22	9.5		22	18
05/13/71b	65	12	42	60	20.0
05/17/71a	96	14	50	64	16
05/17/71b	30	17	20	24	15
05/18/71	36	6.2	26	34	18
Averages	50	12	35	41	17

These amplitudes were corrected (29) to give a linear measure of the end plate conductance.

In order to relate the experimental observations to the model, two factors have to be considered. The first is the relationship between the fraction of receptors inactivated and the observed conductance. The simplest assumption here is that the response is directly proportional to the fraction of the receptors that have not been inactivated. The finding that the relationship between ACh concentration and the end plate conductance is markedly nonlinear (28) implies that this assumption is open to question, but there is no firm basis for modifying it at present.

The second factor concerns the effect of diffusion, which may mean that the toxin concentration $[T]$ at the receptors is not the same as the concentration $[T]_o$ in the bath. That this is an important factor is suggested (a) by the delay before any effect was seen, even in surface fibers, and (b) by the fact that end plates on interior fibers often showed no effect even when those on surface

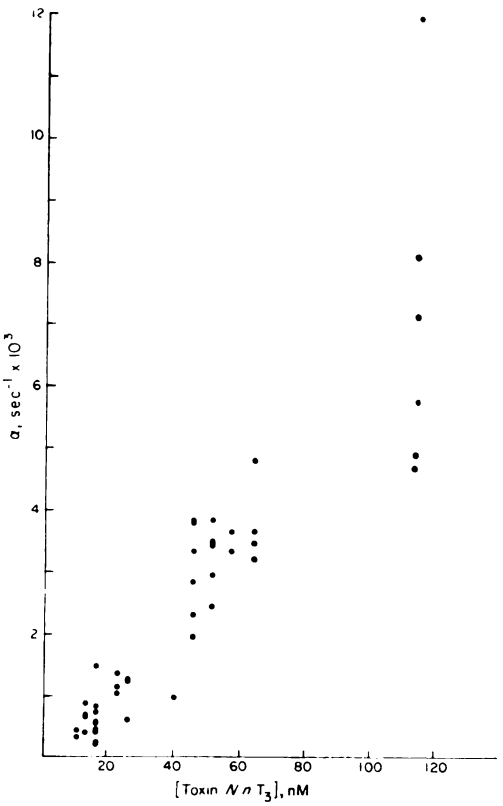

FIG. 6. *Inactivation rate constant, α, as a function of toxin concentration*

Forty-seven different end plates were measured over a 1-year period. Experiments like that of Fig. 1 were used to determine α. End plates were chosen at random in surface fibers and were usually monitored in pairs; one such pair, 400 μ apart, is represented by the highest and lowest values for α at 114 nM. *N.n.* T_3 on the abscissa refers to the *Naja naja* toxin preparation used. The ordinate runs from 0 to 12 $\times$ 10^{-3} sec^{-1}.

fibers were 99% blocked. An analysis based on the known diffusion properties of the toxin and of the frog sartorius muscle shows that diffusion processes account for the observed variability in α among fibers (19). That increasing the toxin concentration generally shortened the delay before action (compare Figs. 2 and 3) suggests that a saturable population of binding sites initially prevented toxin molecules from reaching the receptors (19).

The important point, however, is that diffusion was probably not a serious rate-limiting step during the period of exponential decline, because (a) the rate constant measured by the decline of iontophoretic ACh potentials (for which diffusional delays are presumably minimal) was only slightly greater than for EPPs (Table 1 and Fig. 6), and (b) the toxin rapidly ceased acting when the external concentration was reduced to zero (Fig. 1). Within a factor of about 2, therefore, the measured rate constant, α, provides an approximate estimate of the reaction rate constant $k_T[T]$.

The value obtained from the slope of the line in Fig. 6 is $k_T = 5.5 \times 10^4$ M^{-1} sec^{-1}. Allowing for the effects of diffusion might increase this estimate to about 10^5 M^{-1} sec^{-1}, which agrees well with the value of 1.5×10^5 M^{-1} sec^{-1} obtained from the data on ACh potentials in Table 1.

The linear relation between α and toxin concentration (Fig. 6) rules out the necessity for 2 or more toxin molecules to act cooperatively.

If the toxin-receptor binding were reversible,

$$T + R \underset{k_{-T}}{\overset{k_T}{\rightleftharpoons}} TR,$$

the present experiments would serve to set an upper limit of 10^{-5} sec^{-1} on k_{-T}, since recovery from the action of the toxin on the EPP was never seen. In contrast to the EPP, the ACh potential often increased slightly during prolonged washing (Figs. 4 and 5); however, further experimentation with iontophoretic pipettes showed that this effect was caused by receptors which had been protected from the toxin by desensitization (19, 36). [Protection by desensitization is described in the following paper (20).] In no case was there evidence that receptors ever recovered their ACh sensitivity after being blocked by the toxin.

The dissociation constant for the toxin-receptor complex is given by k_{-T}/k_T and must therefore be less than 0.1 nM. The complex shows at least 10 times higher affinity than the reversible binding of tetrodotoxin to frog sodium channels (39) and at least 4000 times higher affinity than that of *d*-tubocurarine binding to the receptor (40). Because the actual rate constants are several

orders of magnitude smaller in the case of cobra toxin, its action is essentially irreversible in the present experiments.

Note added in proof: Lee (*Ann. Rev. Pharm.* **12**, 1972) distinguishes two classes of elapid and hydrophid curare-like toxins. Sea snake toxins and "Type I" cobra toxins contain 61–62 amino acids and four disulfide bonds; the "Type II" cobra toxin (71 amino acids) used in the present study and α-bungarotoxin (74 amino acids) have five disulfide bonds. Whereas receptor binding with the first group is slowly reversible, with k_{-T} between 10^{-5} and 10^{-3} sec^{-1}, the second group shows little or no reversibility. This difference makes it advisable to carefully describe the toxin employed.

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