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Vulnerability of Desensitized or Curare-Treated Acetylcholine Receptors to Irreversible Blockade by Cobra Toxin

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SUMMARY

Inactivation of frog myoneural acetylcholine receptors by cobra toxin (24–120 nM) is studied in the presence of reversible antagonists (*d*-tubocurarine, dihydro-β-erythroidine) and agonists (carbachol, nicotine). During agonist treatments most receptors are densensitized rather than activated. Neither agonists nor antagonists reverse the blockade produced by the toxin.

At 26 μM *d*-tubocurarine, receptors are partially protected from the toxin. At 5 μM *d*-tubocurarine, a concentration which blocks 97 % of the receptors, or lower concentrations, curare-blocked and free receptors are equally vulnerable to the toxin.

Receptors are protected against the toxin at 7–140 μM carbachol, concentrations which desensitize 60–99.6 % of the receptors. During combined treatment with agonist and toxin, end plate potential amplitudes decline in 300–600 sec to an irreversible "plateau" rather than exponentially to zero. The plateau is well described, as a very slow decline in the number of available receptors, by the following scheme. (A) The receptor population undergoes three simultaneous processes with first-order rate constants in the range 10^{-4} to 10^{-2} sec^{-1}: reversible desensitization by agonists, recovery from desensitization, and irreversible inactivation by toxin. (B) Desensitized receptors are essentially invulnerable to the toxin.

The agonist data are consistent with the model for desensitization proposed by Katz and Thesleff [*J. Physiol.* (*London*) **138**, 63 (1957)].

INTRODUCTION

Evidence has accumulated recently that a polypeptide toxin from the venom of cobras and other elapid snakes blocks neuromuscular transmission by binding irreversibly to postsynaptic acetylcholine receptors (1). Important support for this view is the fact that receptors are protected from irreversible blockade by toxin during simultaneous exposure to either cholinergic agonists or antagonists. First demonstrated with recordings of tension in muscles (3–6), this finding has been extended with intracellular recording from muscle fibers (1, 2, 7, 8), with a preparation of acetylcholine-sensitive microsacs from *Electrophorus* electric organs (9), and with binding of radioactive toxin to partially purified receptor macromolecules from electric organs (10, 11) and muscles (8, 12, 13).

An important complication in interpreting these experiments is that cholinergic agonists

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and antagonists undoubtedly bind to more than 1 molecule in the postsynaptic membrane. It is therefore possible that the toxin acts only indirectly on the receptor and that the site where reversible agonists and antagonists protect against the toxin is distinct from the one to which the reversible drugs bind to produce their physiological effects.

One way to overcome these objections is to show that protection fails to result from drugs which probably have similar "nonspecific" binding but do not bind to the receptor. Thus atropine fails to protect against the toxin (10, 13), and choline does so only at concentrations (above 1 mM) high enough to show effects on the receptors (1). In the present study the protective effects of various drugs are compared, with respect to extent and time course, with their depolarizing or blocking effects on the end plate receptors.

The results suggest that curare blockade has only a rather weak protecting effect, and that the protection produced by depolarizing drugs is related to their desensitizing rather than directly to their depolarizing action.

Brief accounts of the work (2, 7, 14), a detailed treatment of the mathematics (1), and a description of protection against the toxin with iontophoretically applied agonists (1) have been published.

METHODS

The frog sartorius myoneural junction preparation was employed; most of the procedures have been described (1, 15).

An improved flow system enabled solution changes in the experimental chamber which were more than 95% complete in 100 sec.

Dihydro-β-erythroidine was a gift of Merck Sharp & Dohme.

Multiple Penetrations

For "incubation and recovery" experiments (Fig. 2) the results for several fibers in a single muscle were averaged. The EPP[2] was monitored in about five identified superficial fibers over the complete experiment (often as long as 8 hr). This was accomplished by sketching a portion of the muscle as it appeared under the stereomicroscope and impaling the same fibers for measurements at various stages in the experiment.

Measurement of Small EPPs

Two techniques enabled the measurement of EPP amplitudes during blockade by rather high antagonist or agonist concentrations. First, computerized signal averaging (15) was sometimes used. Second, the Ca^{++} concentration was augmented during treatment with *d*-tubocurarine, usually from 0.6 mM initially to 2.0 mM. This caused an increase in quantal content, consequently a larger EPP. The change in Ca^{++} concentration did not markedly influence the effects of *d*-tubocurarine (16) or of the toxin.[3]

Special Corrections during Agonist Treatment

Wave forms of EPPs shortened considerably during the first few minutes of exposure to agonists, because the increased conductance of the end plate caused the time constant of the muscle fiber to decrease. This necessitated a modification in the automatic procedure, in which the wave form of the individual EPP was fitted to a "template" consisting of the average EPP response (15). In order to keep the wave form of the template similar to that of the individual response, a new averaging sequence (from which the template was constructed) was started each time the wave form changed appreciably.

Amplitudes of EPPs were corrected to give a linear measure of the peak end plate conductance, from which the fraction of available ACh receptors was calculated (15). In a modification of the equivalent circuit given by Martin (17), the effect of the agonist was allowed for by adding a steady synaptic conductance in parallel with the transient conductance associated with the EPP. The steady conductance was measured by the steady depolarization, assuming a "standard" resting potential of −85 mV and a reversal potential of −15 mV. The observed EPP amplitude

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

[3] H. A. Lester, unpublished results.

was multiplied by the correction factor

$$\frac{(70)^2}{(V_{DC} + 15)(V_{DC} + 15 + \Delta V)}$$

Here V_{DC} is the resting potential during agonist action; ΔV is the peak amplitude of the observed EPP.

Measurement of Toxin Potency

This was provided by the inactivation rate constant α_{31} (called simply α in ref. 15), which is the inverse of the time constant for decline of EPP amplitude. A control run had to be taken to establish α_{31} at each end plate because of variations among fibers.

Measurement of Reversible Blockade

During both blockade by antagonists and desensitization by agonists, it was possible to measure the parameter μ. This is the ratio of EPP amplitude during steady-state reversible blockade to amplitude in the control period.

Possible Effects of Drugs on Quantal Content

There seem to be no presynaptic effects of *d*-tubocurarine (18). However, initial experiments with carbachol suggested that a significant decrease in quantal content (m), often by a factor of 10, accompanied desensitization, as found by Ciani and Edwards (19). Therefore the quantitative kinetic studies employed another agonist, nicotine, which apparently had a much smaller effect on m, and care was taken to locate the recording electrode at the center of the end plate (18). Nevertheless, because of the large diminutions in EPP amplitude (μ = 0.035–0.13), changing cable characteristics, and large corrections for depolarization, one cannot rule out a decrease in m by a factor of 2 during nicotine desensitization.

RESULTS

Survey Experiments

Actions of tubocurarine and carbachol on EPP.

Figure 1 presents results of an experiment in which carbachol and *d*-tubocurarine were applied to an end plate at concentrations selected for approximately equal diminution of the EPP ($\mu \cong 0.1$).

As described by previous authors (20), *d*-tubocurarine rapidly caused a decline to a new steady state of EPP amplitude. Recovery from *d*-tubocurarine blockade usually required a longer time than onset and was seldom complete. Very similar results were obtained in a few experiments with dihydro-β-erythroidine.

Desensitization by agonists followed the

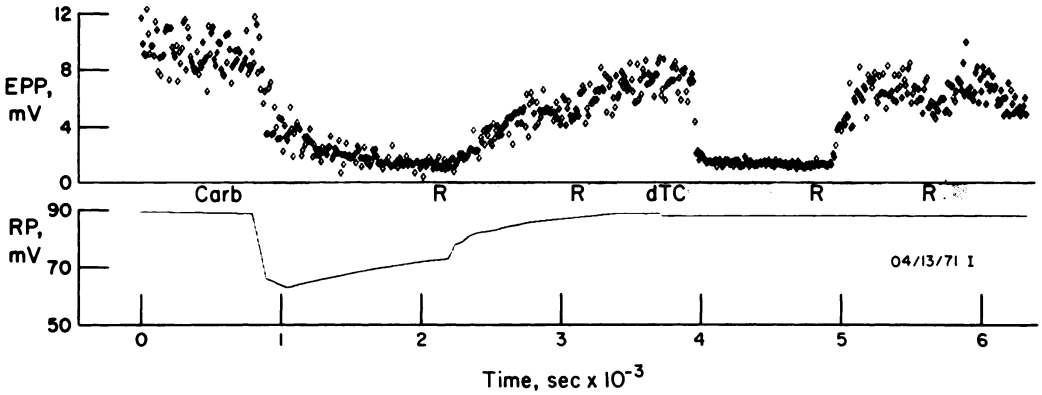

FIG. 1. *Time course of desensitization by an agonist (carbachol, Carb, 28 μM) or blockade by an antagonist (d-tubocurarine, dTC, 1.5 μM) at frog myoneural junction*

Upper: amplitudes of individual EPPs, elicited at 0.1/sec and measured as described in METHODS. Lower: resting potential (RP) of the muscle fiber. Shading represents times when the chamber was flushed with the indicated solutions. The control Ringer's solution (R) (0.6 mM Ca^{++}) was also present at the beginning of the experiment.

description given by previous investigators (21, 22). When Ringer's solution containing carbachol entered the chamber, the membrane became depolarized. After about 200 sec the potential moved toward the normal resting level. End plate potential amplitude (corrected as described in METHODS) also decreased, eventually attaining a steady state. The onset and recovery from desensitization occurred with roughly the same time course (23) and were much longer than for *d*-tubocurarine action. Very similar results were obtained with nicotine as the agonist.

Protection against action of cobra toxin by d-tubocurarine.

Two types of experiments were used to study the interaction between the effects of *d*-tubocurarine and cobra toxin.

Incubation and recovery experiments. In these experiments the irreversible blockade produced by the toxin (110 nM) applied alone was compared with the blockade produced when the toxin was applied in the presence of tubocurarine. The irreversible blockade was assessed 3 hr after both drugs had been washed out of the bath. Measurements made during blockade with 26 μM *d*-tubocurarine showed that μ

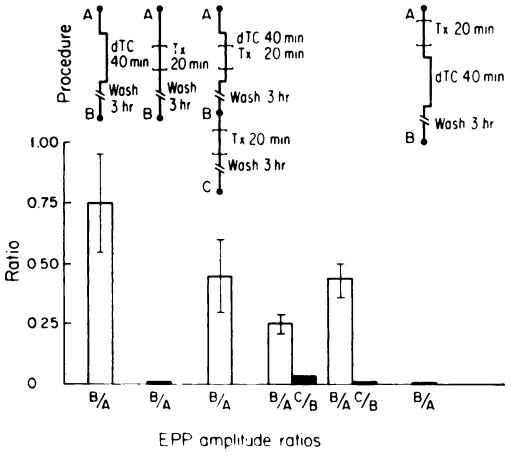

FIG. 2. *Incubation and recovery experiments with cobra toxin (Tx) and d-tubocurarine (dTC)*

Each ratio (or set of two ratios), ± the standard error of the mean, represents EPP measurements on five fibers in a different muscle, as described under METHODS.

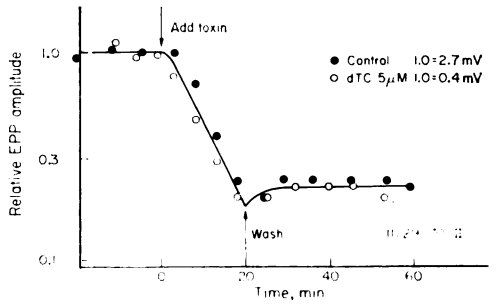

FIG. 3. *Kinetics of blockade of receptors by cobra toxin (48 nM) in a partially curarized preparation*

Amplitudes of 32 or 64 averaged EPPs, elicited at 0.5/sec, are shown. O, control run, with 0.6 mM Ca^{++}. Only toxin was added to the Ringer's solution and was present only between the arrows. *d*-Tubocurarine (dTC) was then added (not shown); μ was not measured here but was about 0.05 in other preparations at this tubocurarine concentration. The calcium concentration was then raised (not shown) to 2 mM to give a measurable EPP. After 1 hr toxin was again added (●) between the arrows; tubocurarine and a high Ca^{++} concentration remained present throughout.

was 4–10 × 10^{-3}, which is somewhat smaller than would be expected from the measurements of Jenkinson (16).

As Fig. 2 shows, muscles treated with tubocurarine alone, followed by washing for 3 hr in Ringer's solution, displayed little permanent loss of EPP amplitude (first procedure). The same was true for MEPP amplitude. A 20-min treatment with toxin, followed by a 3-hr wash, resulted in an EPP amplitude only about 1% of the control value (second procedure). Toxin applied during treatment with 26 μM tubocurarine, however, caused a smaller decline in the EPP (third procedure). Roughly the same ratios of decline were seen with MEPPs. The EPP amplitude remained depressed when tubocurarine was applied after the toxin (fourth procedure), and MEPPs remained too small to measure.

These experiments show that *d*-tubocurarine protects the ACh receptors against blockade by the toxin, but cannot reverse the blockade once it has occurred.

Somewhat surprisingly, these experiments failed to show protection when performed with lower tubocurarine concentrations (5 μM or less; $\mu > 0.03$). This

finding was confirmed with the kinetic experiments.

Kinetic experiments. When the toxin was applied to end plates in the presence of 5 μM tubocurarine, the result was an exponential decrease in EPP amplitude (Fig. 3). Furthermore, the semilogarithmic plot of this decline could be superimposed on the control plot for the toxin acting on the same end plate, but without tubocurarine. Therefore the presence of *d*-tubocurarine failed to alter the inactivation rate constant, α_{si}.

This lack of effect was seen whether the test in the presence of tubocurarine preceded that in its absence, or vice versa. The result was true for tubocurarine concentrations of 5 μM or below ($\mu > 0.03$). Higher concentrations (26 μM), which showed protection in the "incubation and recovery" experiments (Fig. 2), could not be tested by this method because the EPP amplitude was too small to permit accurate measurement. Similar results in the kinetic experiments were obtained with dihydro-β-erythroidine.

Protection by carbachol.

Incubation and recovery experiments. These were conducted as in Fig. 2, with a toxin concentration of 110 nM, but with carbachol replacing *d*-tubocurarine as the protecting agent. In contrast to tubocurarine, carbachol afforded protection over a wide range of concentrations.

For instance, at 7 μM, the lowest concentration tested, carbachol caused only slight desensitization ($\mu = 0.4$). The B/A ratio for protection with carbachol (see the third procedure in Fig. 2) was 0.15 $\pm$ 0.03 (five fibers); in the same fibers the C/B ratio, representing the unprotected case, was 0.01.

At 140 μM carbachol, the highest concentration tested, desensitization was nearly complete, with $\mu < 0.004$. B/A ratios greater than 0.3 for protection were observed in all 15 fibers tested; the C/B ratio, during the test without protection, was again close to 0.01. However, there was some indication that 140 μM carbachol alone caused permanent decreases in quantal content. To eliminate this presynaptic effect, measurements were made of MEPP amplitudes in eight fibers under the same conditions. The B/A ratio for protection was 0.90 $\pm$ 0.15 with the MEPPs. Thus practically none of the receptors were inactivated by the toxin during protection by 140 μM carbachol.

The fourth procedure in Fig. 2 was also performed with carbachol concentrations in the range 7–140 μM. As in the case of tubocurarine, carbachol completely failed to reverse the inactivation caused by the toxin.

Kinetic experiments. These experiments were carried out in the same way as the kinetic experiments with tubocurarine (Fig. 3), but the results were quite different.

Figure 4 shows a typical result. In the control run, without carbachol, the toxin produced the usual irreversible exponential decline. In the presence of carbachol the

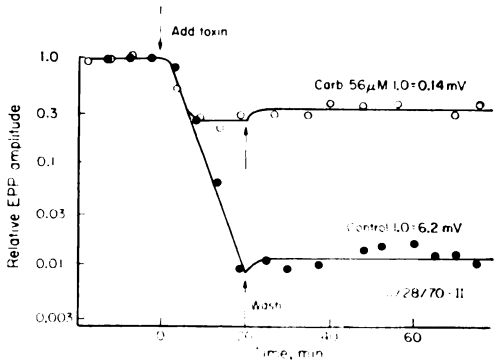

FIG. 4. *Kinetics of blockade of receptors by cobra toxin (48 nM) in a partially desensitized preparation*

Amplitudes of 32 or 64 averaged EPPs, elicited at 0.5/sec, are shown. O, control run, with 0.6 mM Ca^{++}. Only toxin was added to the Ringer's solution and was present only between the arrows. Carbachol (Carb) was then added (not shown); μ was not measured here but was about 0.05 in other preparations at this carbachol concentration. The calcium concentration was then raised to 2 mM (not shown) to give a measurable EPP. After 1 hr toxin was again added (●) between the arrows; carbachol and a high Ca^{++} concentration remained present throughout. Notice that the vertical scale differs from that in Fig. 3. The control run for this end plate had a different value of α_{si} than the control run for the end plate in Fig. 3, although the toxin concentrations were identical (15).

toxin caused an initial decline in EPP amplitude. After about 10 min the EPP amplitude reached a "plateau" with no further decline. The response remained at the plateau level after extensive washing with Ringer's solution containing carbachol.

The plateau was always observed with this carbachol and toxin concentration; it was also consistently observable with several other conditions of combined agonist and toxin application, as described below. It occurred whether the test in the presence of carbachol preceded or followed the control test in its absence. During the plateau, the EPP wave form matched that of the untreated end plate.

Further observations on plateau. Several causes may be eliminated for the plateau. It does not represent an equilibrium level of binding between toxin and receptors, because the action of the toxin was essentially irreversible during carbachol application, just as in the control run (Fig. 4). The plateau cannot have arisen because of a slow reaction between carbachol and toxin: it made no difference whether the carbachol-toxin solution was mixed 1 min or 1 hr before it was applied to the preparation.

While it is true that agonists cause complex changes in EPP amplitude because they affect membrane conductance, resting potential, and cable properties, (24), these factors could not have caused the plateau, since it was found that the membrane conductance had returned to normal in those fibers that were desensitized by high concentrations of carbachol. The plateau could not be ascribed to removal of toxin molecules from the bathing solution, for replenishing the bath with fresh toxin-containing solution caused no effect during the plateau (Fig. 5).

Possible causes of plateau. Other mechanisms cannot easily be eliminated. For instance, desensitization could create (or unmask) a population of receptors that can produce a response to ACh but cannot be inactivated by the toxin. This scheme was not readily testable with further experiments of the present type.

The plateau could, however, be explained if it were assumed (a) that desensitization protects receptors against the toxin (at least partially), and (b) that desensitization occurs at a rate similar to the rate of action of the toxin.

Thus, if a population of receptors, in an equilibrium state of desensitization; is exposed to toxin (Fig. 4), the toxin starts to inactivate available receptors and the EPP amplitude begins to decline. Part of this decline is counterbalanced by a conversion of receptors from the desensitized

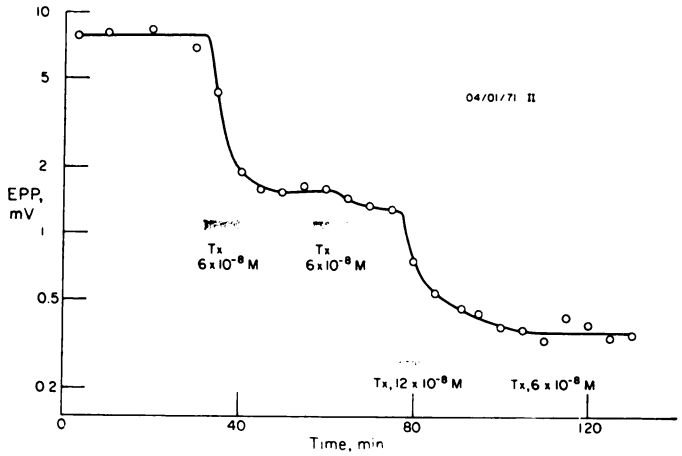

FIG. 5. *Plateau*

Amplitudes of 32 or 64 averaged EPPs, elicited at 0.5/sec, are shown. Shading represents solution flow, as in Fig. 1. Carbachol (56 μM) was added to the preparation 1 hr before the experiment and was present throughout; resting potential during the experiment was 80 mV. Tx, toxin.

to the available fraction. The available pool declines until the rate of this conversion nearly equals the rate of irreversible inactivation of receptors by toxin. Thereafter the size of the available fraction declines much more slowly; this is the plateau. Eventually all the receptors spend enough time in the available state to be blocked by the toxin; therefore the EPP eventually reaches zero. However, the desensitized state provides a "trap" for receptors and protects them against the toxin for long periods of time.

The two phases of the action of the toxin could thus represent (a) rapid depletion of the available fraction of the receptors and (b) very slow depletion of the desensitized fraction.

In qualitative agreement with this scheme is the finding that the amplitude of the plateau decreases with increasing toxin concentration (Fig. 5).

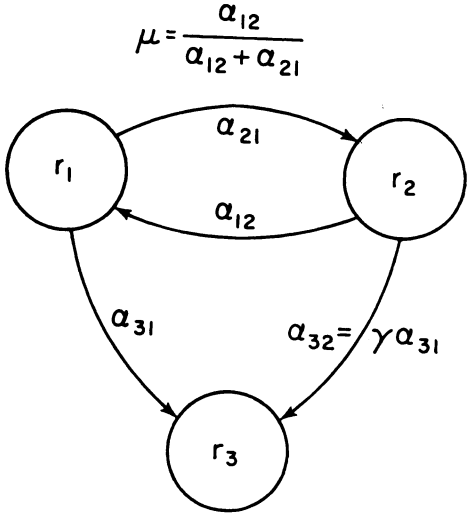

FIG. 6. *Kinetic scheme*
Transitions occur among available (state 1), desensitized (state 2), and toxin-blocked (state 3) receptors. See the text.

Quantitative Kinetic Studies with Desensitization

The aim of these experiments was to predict the parameters of the plateau by utilizing the measured parameters of desensitization and of inactivation by the toxin, on the assumption that desensitized receptors are less vulnerable to the toxin than are normal receptors.

Theory.

Figure 6 presents the working model. This scheme is not meant to represent molecular events, but merely phenomenological states which can be measured during an experiment. It was thought inadvisable to utilize directly the more complex molecular model favored by the experiments of Katz and Thesleff (23) and of Rang and Ritter (25), because some constants in the latter model are not determinable in these experiments. However, the present scheme is perfectly compatible with these more explicit models (see APPENDIX).

Three different states of the ACh receptor are assumed to exist. Only state 1 produces conductance increases when presented with ACh or other agonists. State 2 is the inactive, desensitized receptor, which can revert to state 1. State 3 is the inactive, irreversibly blocked toxin-receptor complex.

Let r_i be the number of receptors in state i; α_{ji} is the first-order rate constant for transition from state i to j.

For any end plate with constant quantal content, r_1 is assumed to be proportional to the amplitude of the peak conductance during an EPP. It is assumed that the steady depolarization by bath-applied agonists involves a negligible fraction of r_1. Steady-state desensitization reduces r_1 to fraction μ of its original size.

The kinetic scheme (Fig. 6) leads to the differential equations

$$\dot{r}_1 = -(\alpha_{21} + \alpha_{31})r_1 + \alpha_{12}r_2, \quad (1a)$$

$$\dot{r}_2 = \alpha_{21}r_1 - (\alpha_{12} + \alpha_{32})r_2, \quad (1b)$$

$$\dot{r}_3 = \alpha_{31}r_1 + \alpha_{32}r_2. \quad (1c)$$

Parameters of desensitization. α_{12} seems to depend on agonist concentration (23, 25) and may be measured as follows. Let the initial conditions be $r_1 = s$, with $r_2 = r_3 = 0$ and no toxin present ($\alpha_{31} = \alpha_{32} = 0$). If agonist is added at $t = 0$, Eqs. 1 lead to

$$r_1 - \mu s = s(1 - \mu) \exp[-(\alpha_{12} + \alpha_{21})t], \quad (2)$$

$$\alpha_{12} = \mu(\alpha_{12} + \alpha_{21}). \quad (3)$$

Equations 2 and 3 allow the determination of α_{21} and α_{12} from the observable rate of desensitization, $\alpha_{12} + \alpha_{21}$, and from the observable ratio μ.

Parameters of toxin action. Inactivation of receptors by cobra toxin follows pseudo-first-order kinetics:

$$\alpha_{31} = k_T[T], \quad (4)$$

where k_T is approximately equal to 1.5×10^5 M^{-1} sec^{-1} (15), and $[T]$ is the toxin concentration near the receptors.

Protection parameter γ. We define γ to measure the vulnerability of desensitized receptors to the toxin; thus

$$\alpha_{32} = \gamma\alpha_{31}. \quad (5)$$

Complete protection by densensitization would correspond to $\gamma = 0$. If the toxin can inactivate desensitized receptors as rapidly as available ones, $\gamma = 1$.

Parameters of plateau. If the preparation is in a steady state of desensitization and toxin is added at $t = 0$, Eq. 1 predicts that r_1 will decline according to a double-exponential equation.

$$r_1 = \beta_+ \exp(-\lambda_+ t) + \beta_- \exp(-\lambda_- t) \quad (6)$$

where

$$\lambda_\pm = \frac{A \pm Q}{2} \quad (7)$$

with

$$A = \alpha_{12} + \alpha_{21} + \alpha_{31} + \alpha_{32} \quad (8a)$$

and

$$Q = [A^2 - 4(\alpha_{12}\alpha_{31} + \alpha_{21}\alpha_{32} + \alpha_{31}\alpha_{32})]^{1/2}. \quad (8b)$$

The boundary conditions

$$\beta_+ + \beta_- = 1 \quad (9a)$$

and

$$\lambda_+\beta_+ + \lambda_-\beta_- = \alpha_{21} + \alpha_{31} - \alpha_{12}\frac{1-\mu}{\mu} \quad (9b)$$

give

$$\beta_\pm = \tfrac{1}{2}\left[\frac{1 \pm (\alpha_{31} - \alpha_{21} - \alpha_{12} - \alpha_{32})}{Q}\right]. \quad (10)$$

The "fast" component of exponential decline has an amplitude of β_+ and a time constant of λ_+; the "slow" component, whose parameters are β_- and λ_-, corresponds to the plateau. Equations 7, 8, and 10 give $\lambda_\pm$ and $\beta_\pm$ as functions of the experimentally measurable rate constants α_{12}, α_{21}, α_{31}, and the protection parameter γ.

The problem of predicting the plateau quantitatively in terms of the kinetic scheme (Fig. 6) thus involves first measuring α_{12} and α_{21} for desensitization alone, and α_{31} for the toxin alone, and then, on the basis of these values, finding the value of γ which predicts the measured $\lambda_\pm$ and $\beta_\pm$ during combined desensitization and toxin action.

Measured values.

For a limited range of agonist and toxin concentrations, it was possible to test the kinetic scheme by measuring all the parameters on a single end plate. Averaging of successive EPP amplitudes, as in Figs. 3–5, tended to distort the time course of drug actions. Therefore individual EPPs, elicited at 0.1/sec, were measured automatically (see METHODS). In order to eliminate errors caused by recording noise, EPP amplitudes were maintained at greater than 0.2 mV by varying the Ca^{++} concentration between 0.6 and 2 mM.

Figure 7 presents results of a typical experiment. The toxin concentration was 60 nM, and the agonist was nicotine at 25 μM. The first decay, initiated by changing the solution from Ringer's to nicotine, is used for determining α_{12} and α_{21} for desensitization by means of Eqs. 2 and 3.

The second decay and the plateau are caused by adding toxin in the presence of nicotine. This section of the experiment furnished the parameters of the plateau: the rate constants $\lambda_\pm$ and the coefficients $\beta_\pm$. Actually the rate constant λ_- is too small to measure, so that the plateau merely provides an upper limit on this parameter.

The third decay, caused by toxin alone, furnishes the rate constant α_{31}.

This analysis was performed on six end plates, and the results are gathered in Table 1. In the calculations λ_- (rate constant of the

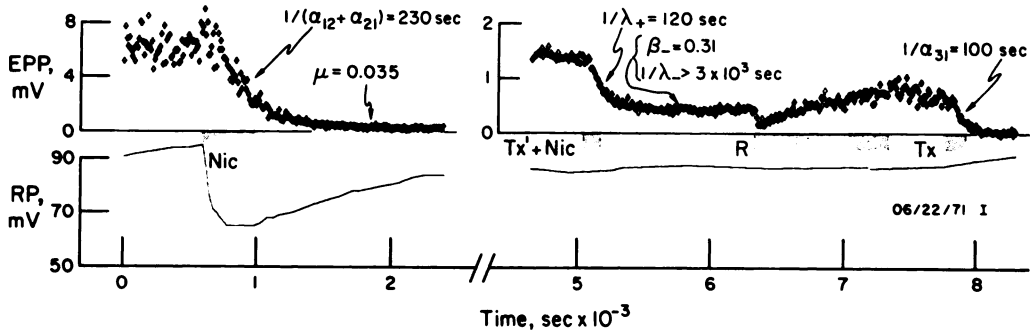

FIG. 7. *Testing kinetic scheme*

Amplitudes of EPPs and resting potentials (RP) were measured and drawn directly by the computer from on-line data (see METHODS). The initial solution was Ringer's with 0.6 mM Ca^{++}, called R. Nicotine (Nic, 25 μM) was then added. During the break in the illustrated data, EPPs continued to be elicited for measurements of quantal content (see METHODS). Then Ca^{++} was increased to 1.5 mM (not shown), with nicotine still present, and EPP amplitude increased to a new steady state. Tx' + Nic, same as the immediately preceding solution plus toxin, 60 nM. The solution was next changed to control Ringer's (R); EPP amplitude fell rapidly at first, because quantal content decreases with reduced Ca^{++}. Recovery from desensitization eventually dominated and EPP increased. Reduced Ca^{++} was still necessary to maintain EPPs at subthreshold levels in deep fibers, where toxin penetrates slowly. Tx, 60 nM in control Ringer's solution. All three measured decays fit exponentials with the indicated rate constants. The abscissa runs from 0 to 8000 sec.

TABLE 1

Summary of kinetic analysis

The agonist was nicotine, 25 μM. The experiment labeled 6/22 I is presented in Fig. 7; data labeled 6/22 II were recorded simultaneously at another end plate in the same muscle. Notice that α_{12} and α_{21} were not observed directly but were calculated from the observed rate ($\alpha_{12} + \alpha_{21}$) and the observed ratio μ according to Eqs. 2 and 3. See the text and Fig. 7 for further particulars.

Experiment	Toxin conc.	α_{21}	α_{12}	Observed μ	Observed α_{21}	Observed λ_+	Calculated λ_+	Observed λ_-	Calculated λ_- $\gamma = 0$	Calculated λ_- $\gamma = 0.1$	Observed β_-	Calculated β_- ($\gamma = 0$)
	nM	10^{-3} sec^{-1}			10^{-3} sec^{-1}	10^{-3} sec^{-1}		10^{-3} sec^{-1}	10^{-3} sec^{-1}			
6/22 I	60	4.2	0.15	0.035	10	8.3	14	<0.3	0.11	1.1	0.31	0.30
6/22 II	60	3.2	0.14	0.045	3.3	4.0	6.6	<0.3	0.07	0.4	0.36	0.50
6/23 I	60	7.2	1.1	0.13	7.7	8.3	15	~1.0	0.55	1.3	0.17	0.52
6/23 II	60	3.1	0.21	0.062	20	20	23	<0.3	0.18	2.2	0.13	0.14
6/24 I	24	3.0	0.38	0.12	4.0	6.4	7.1	<0.3	0.22	0.61	0.26	0.45
6/24 II	24	3.1	0.19	0.056	4.0	8.3	7.2	<0.3	0.10	0.50	0.30	0.45

slow decay) is most sensitive to γ and, in each case, the best fit was with $\gamma < 0.1$. The calculated amplitude of the slow decline, β_-, was most sensitive to α_{21} and α_{31} but was always fitted best with $\gamma = 0$, giving a mean ratio β_- (obs)/β_- (calc) = 0.67. The calculated rate constant of the fast decline, λ_+, was very nearly the sum $\alpha_{31} + \alpha_{21}$ for all values of γ, and the mean ratio λ_+ (obs)/λ_+ (calc) was 1.25.

Sources of error. The kinetic analysis as performed in these experiments is subject to several errors.

1. The rate constant for desensitization, $\alpha_{12} + \alpha_{21}$, increases with increasing Ca^{++} (22, 24, 26–28). It is difficult to estimate the magnitude of this effect, because there are no experiments reported with the present conditions: viz. bath-applied nicotine in Ringer's solution with nearly normal ionic concentrations.

2. The rate constant for desensitization

may be affected by depolarization. This has been seen with iontophoretically applied ACh (28), but it is not know whether it also occurs with the slower desensitization caused by bath-applied drugs.

3. Desensitization could have been accompanied by a decrease (up to 50%) in quantal content (see METHODS). If so, the rate constant $\alpha_{21} + \alpha_{12}$ would have been overestimated.

4. The steady depolarization during nicotine treatment could have involved a significant fraction of receptors in state 1, which would decrease the number of receptors available to produce an EPP; thus the assumption of linearity between r_1 and peak end plate conductance would no longer hold. If the apparent dissociation constant for nicotine on the eel electroplax (25 μM) is correct for frog end plates, this could have led to an underestimate of μ by as much as 50%.

In qualitative terms corrections for errors 1 and 2 would take the ratios β_- (obs)/β_- (calc) and λ_+ (obs)/λ_+ (calc) further from unity, and corrections for errors 3 and 4 would bring the ratios toward unity. However, none of these corrections seriously affects the conclusion, based primarily on the small observed λ_-, that protection was nearly complete ($\gamma < 0.1$).

DISCUSSION

Interaction between Tubocurarine and Cobra Toxin

The puzzling feature of the experimental results was the failure of tubocurarine at concentrations less than 5 μM to afford any protection, measured either by the kinetic method or by "incubation and recovery" experiments.

The dissociation constant for tubocurarine at the frog end plate is 0.4 μM (16), and the rate constants for association and dissociation are sufficiently large that the reaction is diffusion-limited under most circumstances (20, 29, 30). Thus equilibrium between curare-blocked and nonblocked receptors will be maintained as the toxin takes its effect. If μ is a measure of the fraction of the receptors not blocked by curare molecules, and γ measures the relative association rate constant of the toxin for curare-blocked compared with nonblocked receptors (γ would be zero if curare-blocked receptors were completely inaccessible to the toxin), the rate constant for inactivation by the toxin should decrease from α_{31} in the absence of tubocurarine to $\alpha'_{31} = \alpha_{31}[\mu + \gamma(1 - \mu)]$ in the presence of tubocurarine. The results showed no change in the rate constant in the presence of tubocurarine (i.e., $\alpha'_{31} = \alpha_{31}$) although μ was as low as 0.03, implying that γ is close to unity, and that at this concentration tubocurarine does not protect the receptors at all.

On the other hand, incubation and recovery experiments with 26 μM tubocurarine (although not with 5 μM tubocurarine) showed almost complete protection, corresponding to $\gamma \approx 0.15$.

The simple interpretation of protection by tubocurarine in terms of competition for the toxin-binding site thus appears unable to account fully for the results.

Other Experiments on Protection by Antagonists

The available binding studies on protection of ACh receptors by antagonists form an incomplete picture. For muscles, the data agree with the present findings, in that protection has not been shown for tubocurarine concentrations less than 5 μM (8, 12, 13). Miledi and Potter (8) recently reported that the binding of radioactive α-bungarotoxin to frog sartorius muscles decreased to only 50% of the control level at very high tubocurarine concentrations, suggesting a receptor population in which half the sites are completely unprotectable by tubocurarine.

On the other hand, membrane fragments and partially purified receptors from eel electroplax cells can be completely protected by *d*-tubocurarine and other antagonists against both α-bungarotoxin and cobra toxin from *Naja nigricollis* (9, 11). The protection is well described in terms of competition for the toxin-binding site.[4]

[4] At present only the experiments of Weber *et al.* (34) on binding of toxin to eel electroplaque microsacs, a preparation in which agonist-induced ionic fluxes show no desensitization (35), imply that *activated* as well as *desensitized* receptors are substantially invulnerable to the toxin.

Protection during Desensitization

The quantitative kinetic experiments described above show that the combined action of toxin and agonists can be explained by the kinetic scheme (Fig. 6) and by the assumption of nearly complete protection. Unfortunately, the complete kinetic analysis is possible for only a narrow range of agonist and drug concentrations.

To extend these findings to other conditions, the incubation and recovery experiments with carbachol desensitization, from $\mu < 0.004$ to $\mu = 0.4$, were re-examined on the basis of values of α_{12}, α_{21}, and α_{31} obtained from other experiments, and calculation of the value of the protection parameter (γ) required to give the experimentally measured degree of protection (1). This analysis also gave estimates of $\gamma = 0.15$ or less.

Thus the results suggest that desensitization results in the receptors becoming virtually inaccessible to cobra toxin.

Fundamental Events

An unsatisfactory feature of the present analysis is that it deals with phenomenological states which may have no relation to actual molecular events. The results show, however, that desensitization and inactivation by the toxin cannot occur simultaneously at the same receptor. These processes may therefore involve the same area of the receptor.

The kinetics of desensitization and recovery depends upon the agonist and its concentration, the tonicity of the bathing solution, divalent cations, and membrane potential (22, 24–28, 31). A constant and striking feature of the process is its long time course, in contrast to the action of tubocurarine and other competitive inhibitors (Fig. 1). This and other facts led to models (23, 25) whose dominant rate constants represent slow conversions rather than agonist binding to the receptors. According to one suggestion, these transitions interfere with the part of the receptor which controls ionic permeation (22, 27, 28). The molecular mechanism remains undetermined, nor is it certain that the dominant mechanism is the same for bath-applied and iontophoretically applied agonists.

Katz and Thesleff (23) proposed a model for desensitization at the frog end plate in terms of transitions between a normal and a desensitized conformation of the receptor, and this was supported by studies on "metaphilic" antagonists (25). The present scheme, which defines functional states rather than molecular transitions, is fully compatible with the Katz-Thesleff model (see APPENDIX). It is of interest that the alkylating antagonists studied by Rang and Ritter (25, 32) had the property that they bound selectively to the desensitized receptors (i.e., $\gamma > 1$); cobra toxin has the reverse property, binding to normal but not to desensitized receptors.

The kinetic experiments are consistent with the conjecture by Katz and Thesleff (23) that the transition from desensitized to available receptors occurs very slowly in the presence of agonists. This transition has the rate constant α_{12} in the scheme of Fig. 6. For $\gamma = 0$ the calculated rate constant λ_- of the slow decay—the plateau—is within a factor of 2 of α_{12} (see Table 1). Thus the plateau appears flat only because α_{12} is very small during agonist action.

Other Experiments on Protection by Agonists

Miledi *et al.* (10), using membrane fragments and solubilized receptor protein from *Torpedo*, found greater and more persistent protection by carbachol (200 μM) than by *d*-tubocurarine (100 μM) against the binding of [^{131}I]α-bungarotoxin. *Torpedo* receptors show desensitization (33). These data therefore agree qualitatively with my findings that desensitization protects better than curare blockade.

Other experiments (8, 12, 13) with the same toxin in vertebrate striated muscle have demonstrated that carbachol protects the receptor against binding of the toxin. The kinetics of binding (8) suggests a plateau of the sort observed and explained with the present methods; however, complicating factors such as diffusion preclude a

quantitative analysis of the available binding experiments.

APPENDIX

We examine here the relation between the scheme used to evaluate protection by desensitization (Fig. 6) and the model proposed by Katz and Thesleff (23). Others (1, 23, 25) give details of the evidence for the Katz and Thesleff model and variants which conserve the principle of microscopic reversibility.

The model is

$$\begin{array}{ccc} S + R & \underset{K}{\rightleftharpoons} & SR \\ k_2 \uparrow & & \downarrow k_1 \\ S + R' & \underset{K'}{\rightleftharpoons} & SR' \end{array} \qquad \text{(A-1)}$$

The agonist is S. The free receptor is R; the state SR produces conductance increases. The two states R' and SR' produce no change in conductance. Our "available" state 1 corresponds to the sum R and SR (although the kinetic analysis assumes $SR \ll R$). The "desensitized" state 2 is R' and SR'.

The affinity constants K and K' govern equilibria which occur, for our purposes, instantaneously. The two rate constants k_1 and k_2 are measured in fractions of inverse seconds.

The following relations hold:

$$\alpha_{21} = \frac{k_1 K[S]}{1 + K[S]} \qquad \text{(A-2a)}$$

$$\alpha_{12} = \frac{k_2}{1 + K'[S]} \qquad \text{(A-2b)}$$

These relations show that Eq. 2 in the text is a restatement of Katz and Thesleff's expression for $1/\tau$ (their model 4). Also $\mu = 1 - I$.

In order to evaluate γ, we make the further assumption that R' is as vulnerable to the toxin as R, but SR and SR' are completely resistant. In other words, the receptor is protected from the toxin only when agonist is bound (ref. 36, Fig. 9). This leads to

$$\gamma = \frac{\alpha_{32}}{\alpha_{31}} = \frac{[R']/([R'] + [SR'])}{[R]/([R] + [SR])} = \frac{1 + K[S]}{1 + K'[S]}. \qquad \text{(A-3)}$$

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