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Allosteric Interactions between the Membrane-Bound Acetylcholine Receptor and Chemical Mediators. Kinetic Studies†

James E. Bulger,‡ Juian-juian L. Fu,§ Ellen F. Hindy, Richard L. Silberstein,¶ and George P. Hess*

ABSTRACT: The kinetics of the specific irreversible reaction of a snake neurotoxin, α-bungarotoxin, with the acetylcholine receptor of electroplax membrane preparations have been investigated. The effects of activators (decamethonium, carbamylcholine) and inhibitors (α-bungarotoxin, *d*-tubocurarine) of neural transmission on this reaction have been measured and the following new information obtained. (1) The irreversible reaction is preceded by the reversible formation of toxin-receptor complexes. (2) Two types of receptor binding site exist. *d*-Tubocurarine directly competes with the toxin for one type of binding site. Decamethonium and carbamylcholine are noncompetitive inhibitors of the toxin reaction. (3) The data are *inconsistent* with binding sites on separate and distinct molecules or with preexisting nonequivalent binding sites. A simple model is proposed to explain both the kinetic data and equilibrium measurements which indicated that activators and inhibitors of neural transmission compete for only one-half of the receptor sites available to them. The model proposes that for the compounds investigated the binding sites of activators do not overlap with those of inhibitors and that ligand-induced conformational changes of the receptor result in changes in the affinities of the binding sites. The model is simple and is based on mechanisms which have been found to be valid for many well-characterized regulatory enzymes.

After appropriate corrections of equilibrium measurements for unspecific binding of ligands to electroplax membrane preparations, and for the volume occupied by the membranes, we found that compounds (carbamylcholine, decamethonium) which initiate changes in the permeability of nerve and electroplax membranes to inorganic ions, and compounds which inhibit this permeability change (*d*-tubocurarine, α-bungarotoxin) occupy different binding sites on the membrane-bound receptor (Bulger and Hess, 1973). These binding sites interact only partially with each other (Fu et al., 1974, 1977). Different molecules, preexisting nonequivalent binding sites (MacQuarrie and Bernhard, 1971), or an allosteric mechanism which involves ligand-induced conformational changes (Koshland et al., 1966; Koshland, 1970; Conway and Koshland, 1968), are often invoked to account for such observations with well characterized enzymes. The kinetic investigations described in this paper were undertaken in the hope of discovering whether these mechanisms also apply to the more complex acetylcholine receptor-mediated processes.

In this paper, we describe our studies of the kinetics of the specific and irreversible reaction of [^{125}I]iodo-α-bungarotoxin (Chang and Lee, 1963; Lee and Chang, 1966; Lee et al., 1967; Lee, 1972) with the membrane-bound receptor of electroplax. Our investigations indicated the existence of two types of receptor-binding site, and provided information about the interconversion of the sites induced by α-bungarotoxin and about the competition of reversibly binding ligands with the toxin sites.

Several investigators have reported the reaction of α-bungarotoxin with the membrane-bound receptor (Barnard et al., 1971; Kasai and Changeux, 1971; Raftery et al., 1971; Bosmann, 1972; Franklin and Potter, 1972; Weber et al., 1972). Preliminary reports of some of our studies have appeared (Bulger and Hess, 1973; Hess et al., 1975).

† From the section of Biochemistry, Cornell University, Ithaca, New York 14853. *Received October 17, 1975.* This work was supported by Grants GM 704842 and NS08527 from the National Institutes of Health, and Grant BMS572-01908 from the National Science Foundation.

‡ National Institutes of Health Postdoctoral Fellowship NS008527. Present address: Department of Chemistry, Memphis State University, Memphis, Tenn. 38152.

§ National Institutes of Health Postdoctoral Fellowship GM53265. Present address: Miami Valley Laboratory, Research and Development, Procter & Gamble Co., Cincinnati, Ohio 45239.

¶ Some of this work formed an Honors Thesis for A.B. Cornell University, May 1974. Present address: Stanford University Medical School, Palo Alto, Calif. 94303.

Experimental Procedure

Materials

Live electric eels were purchased from Paramount Aquarium, Ardsley, N.Y. Crude *Bungarus multicinctus* venom was obtained in lyophilized form from the Miami Serpentarium, Florida. $Na^{125}I$ was supplied by ICN Laboratories, Cleveland, Ohio. Decamethonium bromide and *d*-tubocurarine were obtained from K & K Laboratories, Plainview, N.Y. Crystallized bovine serum albumin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Microgranular CM52-cellulose cation exchanger, series 14, was obtained from Whatman (1 mequiv/g dry weight), cellulose dialyzer tubing (3787-D40, 1¾ in. flat width) was obtained from A. H. Thomas Co., Philadelphia, Pa. Pipets with disposable tips were obtained from the Centaur Chemical Co., Stanford, Conn. All other chemicals and reagents were supplied by Fisher Scientific Co., Rochester, N.Y., or by Mallinckrodt, St. Louis, Mo.

Apparatus. An Amicon diafiltration apparatus equipped with UM-2 filters was used to concentrate the toxin and for the exchange of buffer solutions. A Technicon Auto-Analyzer fitted with a N-72 manifold, or on occasion a Cary, Model 14, recording spectrophotometer, was used to determine the protein in the pellets of excitable membrane bound with [^{125}I]-iodo-α-bungarotoxin. Radioactivity was counted on a Nuclear Chicago model C120-1 γ counter.

Methods

Preparation of Membrane Fragments. Excitable membrane fragments were prepared from *Electrophorus electricus* electroplax by a modification (Fu et al., 1977) of the method of Changeux (Changeux et al., 1969; Kasai and Changeux, 1971).

Radioactive Toxin Preparation. The α fraction of the crude venom was isolated according to Li (1968). Radioactive (^{125}I) iodination of the α-bungarotoxin was according to the method of Reif (1967). The reaction mixture, containing approximately 13.4 mg of toxin in 2 ml of 0.1 M NH_4OAc, pH 5.3, diluted to 10 ml with 0.05 N KI, was concentrated slightly in an Amicon diafiltration apparatus and the solvent was exchanged for 0.01 M sodium phosphate buffer, pH 6.5. The material was then chromatographed on a 0.9 × 49 cm CM-cellulose column. The column was allowed to equilibrate with 0.01 M sodium phosphate buffer, pH 6.5, before use. After addition of the toxin, a linear gradient was set up from 0.01 to 0.1 M sodium phosphate, pH 6.5. 200 ml of each solution was used. Fractions of 30 min each were collected at a flow rate of approximately 3 drops/min. Samples of these fractions were taken for counting radioactivity (5 μl) and also for measuring absorbance at 280 nm. As noted in Figure 1, several peaks containing radioactivity and protein were obtained. The major peaks correspond to diiodotoxin, cold unreacted toxin, and the monoiodotoxin which was used in the experiments. An Amicon diafiltration apparatus was used to remove the high concentration of sodium phosphate. One-milliliter samples of the toxin were transferred to small glass vials and stored at −20 °C.

The toxin concentration was determined by absorbance at 280 nm. The molar extinction coefficient of the toxin, based on dry weight of lyophilized powder and a molecular weight of 7904 (Clark et al., 1972), is 9500 M^{-1} cm^{-1} at 280 nm.

Samples were taken for determination of the specific activity of the [^{125}I]iodo-α-bungarotoxin. In order to avoid unspecific binding of [^{125}I]toxin to glass, a dilution technique was used to determine specific activity. Five microliters of [^{125}I]toxin were diluted with 25 μl of cold toxin and 2 ml of bovine serum albumin (12 μg/ml). The syringe used for pipetting the [^{125}I]toxin was equilibrated with the labeled toxin by withdrawing the samples at least 50 times. Ten-, twenty-, and thirty-microliter samples, in duplicate, of the diluted solution were taken for counting. The counts per minute and the number of moles of [^{125}I]iodo-α-bungarotoxin provide a linear relationship and the slope is the specific activity. A simulated ^{125}I standard of ^{129}I, ICN model R35B (147 000 disintegrations/min), was used to calculate counting efficiency, taken as the ratio of observed counts per minute to expected disintegrations per minute. Using $t_{1/2}$ for ^{125}I of 60 days, the specific activity could be determined for any time subsequent to the initial determination.

Using the molar extinction coefficient and specific activity of the toxin, it was determined that the [^{125}I]derivative contained 1 mol of ^{125}I/mol of toxin. The purity of the preparations used in the kinetic experiments was checked by electrophoresis on 15% acrylamide gels at pH 4.3 according to Davis (1964). Electrophysiological experiments were conducted[1] and the response to the labeled and unlabeled toxin by electroplax of *E. electricus* was the same.

Measurement of Total Binding of Toxin. The total amount of reversibly *plus* irreversibly bound toxin was determined by an ultracentrifugal technique. Varying amounts of [^{125}I]-iodo-α-bungarotoxin (0.5–15 μl) were added to 0.3–0.5 ml of membrane suspended in eel Ringers' solution (Keynes and Martins-Ferreira, 1953) in a polycarbonate centrifuge tube in the presence or absence of various inhibitors of the binding reaction. Membrane protein concentrations ranged from 5.3 to 17 mg/ml in the various experiments. Toxin and membrane were allowed to incubate on ice for the appropriate time period, usually 60 min, during which duplicate 25-μl samples were removed using Centaur pipets with disposable plastic tips. The plastic tip and sample were counted together in the same sample vial in a Nuclear-Chicago γ counter. At the end of the incubation period, the reaction mixture was centrifuged for 1 h at 40 000 rpm in a Beckman No. 40 rotor. Triplicate samples of the supernatant were counted as above. Sedimentation of membrane protein was >97%. From the difference in the concentration of [^{125}I]iodo-α-bungarotoxin before and after centrifugation and the amount of protein sedimented, the concentration of bound toxin was determined and expressed as moles of toxin bound/milligram of protein. The concentration of free toxin was obtained from the reaction mixture *after* centrifugation.

Measurement of the Specific Irreversible Reaction of [^{125}I]Iodo-α-bungarotoxin in the Kinetic Experiments. About 18 mg of membrane protein was used to determine the concentration of irreversibly bound [^{125}I]iodo-α-bungarotoxin. An important aspect of the method is that in an experimental run the milligrams of membrane protein and moles of [^{125}I]-iodo-α-bungarotoxin were the same in each reaction vessel. Different volumes of eel Ringers' solution (against which the membrane preparation had been dialyzed) were added to each reaction tube. The volumes used varied from 3 to 30 ml to obtain initial toxin concentrations in the 0.05–0.5 μM range. The concentration of [^{125}I]iodo-α-bungarotoxin was always tenfold greater than the total number of α-bungarotoxin sites in the solution, so that an approximately constant free toxin concentration was maintained throughout the reaction. The

[1] We are grateful to Drs. E. Bartels-Bernal and W. Niemi, Department of Neurophysiology, Columbia University for performing the electrophysiological experiments.

reaction was initiated by addition of approximately 50 μl of [^{125}I]iodo-α-bungarotoxin to the membrane suspension at 4 °C. At appropriate time intervals (more frequent at the beginning in order to obtain as many points during the initial fast phase as during the slower phase), aliquots containing approximately 0.6 mg of protein and 8×10^{-11} mol of [^{125}I]-iodo-α-bungarotoxin were transferred from the reaction mixture, each to a quench solution of 1×10^{-4} M *d*-tubocurarine in eel Ringers' solution in polycarbonate centrifuge tubes. Either glass pipets or Centaur pipets with disposable tips were used. This dilution to a final volume of 8.5 ml resulted in a concentration of the free toxin in the quench solution of approximately 7×10^{-9} M. The tubes were centrifuged for 1 h at 4 °C at 40 000 rpm (105 000*g*) in the Beckman preparative centrifuge. The supernatant was poured into a γ-counting vial and the concentration of free toxin was determined. Experiments in which the free toxin concentration was found to be lower than 90% of the theoretical amount were discarded. The 10% loss is attributed to toxin bound specifically to the membrane-bound receptor sites and to experimental error. When control experiments were run with concentrations of 0.1 μM [^{125}I]iodo-α-bungarotoxin but without membrane suspension, we found that, after 1.5 h, 60% of the toxin was lost from the solution. In the absence of membrane suspensions or bovine serum albumin (see above), the toxin binds to glass and to the polycarbonate centrifuge tubes.

The amount of toxin bound per pellet was determined also by counting and the amount of protein per pellet was determined according to Lowry et al. (1951). The amount of [^{125}I]iodo-α-bungarotoxin trapped in the pellet but that had not reacted with the membrane was determined. Aliquots of the membrane preparation, containing approximately 0.6 mg of membrane protein, were added to a quench solution of final volume of 8.5 ml of eel Ringers' solution, 1×10^{-4} M *d*-tubocurarine, and 7×10^{-9} M [^{125}I]iodo-α-bungarotoxin. The amount of [^{125}I]iodo-α-bungarotoxin per mg of protein in the pellet was determined as above, and this blank, usually about 5% of the total number of binding sites, was subtracted from each experimental determination of [^{125}I]iodo-α-bungarotoxin bound per mg of protein. The total number of toxin-binding sites was determined for each eel membrane preparation by incubating the membrane preparation in 0.5–0.8 μM [^{125}I]-iodo-α-bungarotoxin at 4 °C for 5 h. Control experiments lasting for 24 h and experiments conducted in the presence of decamethonium and *d*-tubocurarine, in concentrations used in the experiments, indicated that 5 h was adequate.

We have shown previously (Bulger and Hess, 1973) that the quench solution prevents contamination of the membrane by reversibly bound toxin, which binds to the membrane with low affinity and constitutes a major portion of the total toxin bound. We also ascertained that the irreversible reaction of 0.5 μM [^{125}I]iodo-α-bungarotoxin (the highest initial toxin concentration used in the kinetic experiments) with membrane sites is prevented during a 5-h reaction period by the presence of 1×10^{-4} M *d*-tubocurarine. The same result was obtained when *d*-tubocurarine was replaced by 1.6×10^{-4} M decamethonium (Bulger and Hess, 1973).

To show irreversibility, the following experiments were performed. (1) The membranes were incubated with 0.5 μM [^{125}I]iodo-α-bungarotoxin for 3 h and the amount of irreversibly bound toxin was determined as described under Experimental Procedures. Three other experiments were run in which after 3 h of treatment with 0.5 μM [^{125}I]iodo-α-bungarotoxin the reaction mixture was diluted 100-fold with eel Ringers' solution containing (2) 2.2 μM α-bungarotoxin, (3) 1.6×10^{-4} M decamethonium, and (4) 2.2 μM α-bungarotoxin and 1.6×10^{-4} M decamethonium. The solutions were allowed to stand for 15 h and the amount of irreversibly bound toxin was determined as described. Picomoles of toxin bound per milligram of membrane protein were (1) 4.8, (2) 4.7, (3) 4.5, and (4) 4.7.

For all experiments in which an effector of the toxin-membrane reaction was used, the membrane suspension and the ligand were preequilibrated. If a series of experiments was designed to vary the concentration of decamethonium while maintaining constant toxin concentration, the decamethonium and membrane suspension were equilibrated in the reaction tube for at least 30 min at 4 °C prior to the addition of toxin. When the toxin concentration was varied with constant effector concentration, membrane suspensions with appropriate concentrations of *d*-tubocurarine or decamethonium were dialyzed against eel Ringers' solution with the same effector concentration at 4 °C for at least 10 h before initiation of the toxin reaction. Reactions in which *d*-tubocurarine was used as an effector always involved preequilibration in the latter manner.

Results

The data in Figure 1 illustrates some of the properties of [^{125}I]iodo-α-bungarotoxin. The final step in purification of the iodinated toxin is illustrated in Figure 1a, the elution profile of the toxin from a CM-cellulose chromatographic column. The major peaks correspond to [^{125}I]diiodo-α-bungarotoxin (peak 1), unreacted toxin (peak 2), and [^{125}I]iodo-α-bungarotoxin (peak 3). The monoiodo compound used in the experiment moved as a single band in disc gel electrophoresis at pH 4.3 on 15% polyacryamide gels, and electrophysiological experiments with monocellular electroplax preparations gave results identical to those obtained with the noniodinated toxin.

The number of [^{125}I]iodo-α-bungarotoxin sites per milligram of membrane protein was determined for each kinetic experiment by allowing the reaction to proceed for 5 h. These infinity points were found not to vary by more than 10% during a 24-h period. In studies with membrane preparations from 13 eels, the number of specific, irreversible [^{125}I]iodo-α-bungarotoxin membrane sites thus found was $0.9 \pm 0.2 \times 10^{-11}$ mol/mg of membrane protein, which corresponds, within experimental error, to the number of moles of binding sites of a specific reversible chemical mediator, decamethonium (Fu et al., 1974).

The data in Figure 1b show that the reaction blank depends on the concentration of membrane protein in the reaction. The experimental conditions were chosen so that the reaction blank corresponded to no more than 5% of the infinity point, or to less than 0.5% of the [^{125}I]iodo-α-bungarotoxin used in the experiments.

The time-course of the reaction of [^{125}I]iodo-α-bungarotoxin with membrane sites, at 4 °C, pH 7.0, is shown in Figure 2. The fraction of unreacted membrane sites is plotted on a logarithmic scale as a function of time. Each curve corresponds to a different initial toxin concentration which was always ten times greater than that of the toxin sites. The upper curve corresponds to the lowest initial toxin concentration, 0.05 μM, and the lowest curve to the highest initial toxin concentration used, 0.50 μM. The data indicate that the reaction falls into two steps, an initial fast phase and a subsequent slower one. The ordinate intercept, obtained by extrapolating the progress curve of the slow phase of the reaction to zero time, gives the fraction of sites which react slowly (α) and the fraction reacting

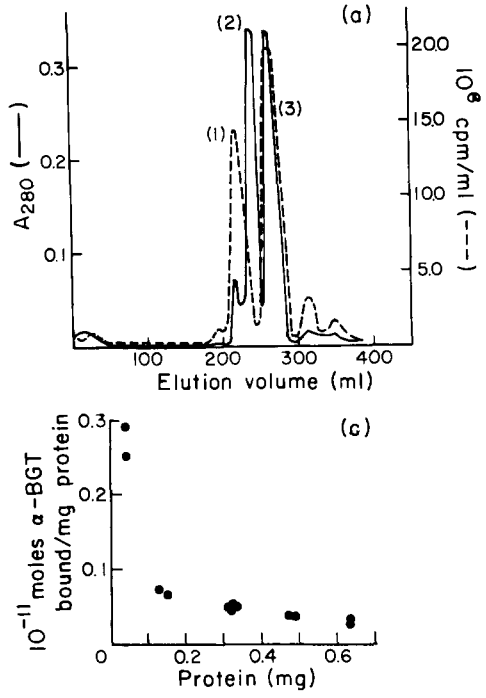


FIGURE 1: (a) Gradient elution profile of α-bungarotoxin reacted with ^{125}ICl. 10.4 mg of the dialyzed products from the ^{125}ICl reaction with α-bungarotoxin was added to a 0.9 × 40 cm carboxymethylcellulose column and eluted with a 400-ml linear, pH 6.5, sodium phosphate gradient from 0.01 M to 0.1 M. A_{280} was recorded for each fraction (volume 4.1 ml) and radioactivity was determined in a Nuclear-Chicago γ counter. (b) Dependence of the pellet blank on the size of the pellet. The amount of [^{125}I]iodo-α-bungarotoxin trapped in the pellet but that had not reacted specifically with the membrane was determined by the addition of an aliquot of membrane preparation and sufficient [^{125}I]iodo-α-bungarotoxin to give a final concentration of 7×10^{-9} M in the quench solution (see text). After centrifugation at 105 000g for 1 h, the amounts of toxin and protein in the pellet were determined. The experiments reported in this paper were designed so that each pellet contained 0.6 mg of protein.

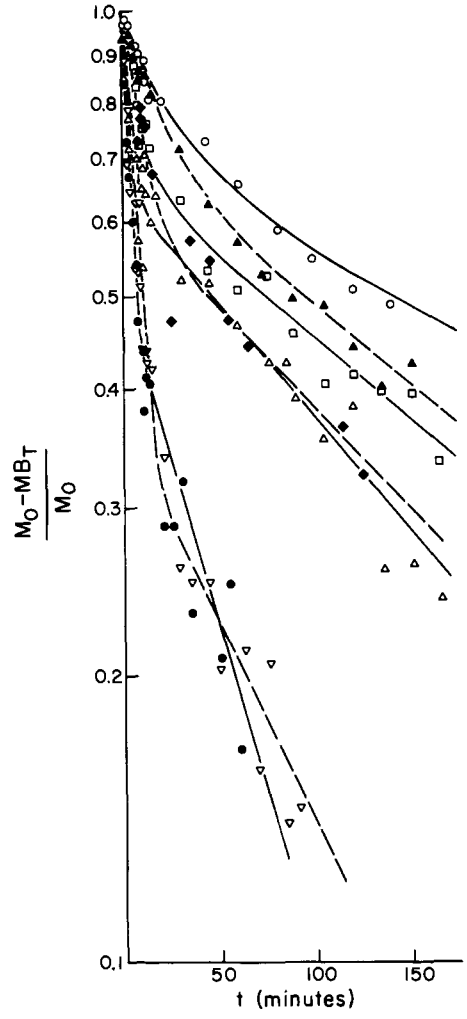


FIGURE 2: The irreversible reaction of [^{125}I]iodo-α-bungarotoxin with electroplax membrane preparations from *Electrophorus electricus*, pH 7.0, 4 °C, eel Ringers' solution. The amount of irreversibly bound [^{125}I]-iodo-α-bungarotoxin was determined as described in the text. The data are plotted on a semilogarithmic scale as fraction of unreacted membrane sites vs. time. Various initial toxin concentrations (μM) were used: (○) 0.05; (▲) 0.07; (□) 0.08; (△) 0.1; (♦) 0.18; (▽) 0.32; (●) 0.50 μM. The observed time course of the reaction was fitted to the sum of two exponentials using a nonlinear least-squares computer program, which yielded the coordinates of the solid lines.

in the fast phase ($1 - \alpha$). The kinetic data were analyzed by fitting the time-course of the reaction to the sum of two exponentials (eq I, Figure 5). In this equation, MB_T represents the irreversibly formed membrane site–toxin complexes at time t, and M_0 the initial concentration of receptor-binding sites. k_I^o and k_{II}^o are the observed rate constants for the fast and slow phase of the reaction, respectively. The coordinates of the solid lines in the Figure were obtained by using a nonlinear least-squares computer program, which gives the values of the two exponentials and α. The data show that both phases of the reaction, and the fraction of the reaction which goes by the slow phase, depend on the initial α-bungarotoxin concentration, B_0. The concentration dependence of k_I^o, k_{II}^o, and α is illustrated in Figure 3. The data shown were obtained with membrane preparations from four different eels, each designated by a different symbol. For each initial concentration of [^{125}I]-iodo-α-bungarotoxin used in the reaction, the amount of irreversible reaction was determined as a function of time, as shown in Figure 2, and k_I^o, k_{II}^o, and α were evaluated at each initial toxin concentration used. Figure 3a shows the dependence of k_I^o on initial toxin concentration. The data are consistent with either a bimolecular reaction, or the rapid formation of a reversible, low-affinity toxin–receptor complex preceding the irreversible reaction. We can not resolve these alternatives, since we are limited experimentally in the initial α-bungarotoxin concentrations which we can use. In both cases, the observed rate constant has the same dimensions and can be expressed as $k_I^o = k_I'' B_0$ (see Figure 5, eq II). The slope of the line in Figure 3a gives a value of 7×10^5 M^{-1} min^{-1} for k_I''. The parameter k_{II}^o does not increase linearly with increasing toxin concentration but reaches a limiting value. A minimum mechanism describing the data is shown in eq 1.

$$M_0 + B_0 \underset{}{\overset{K_2}{\rightleftharpoons}} \overline{MB} \xrightarrow{k_{II}} \overline{MB_t} \qquad (1)$$

In this equation, $\overline{MB}$ represents a reversibly formed α-bungarotoxin–membrane site complex with dissociation constant K_2. The formation of the complex is considered to be rapid compared to formation of the irreversible complex $\overline{MB_t}$, a process characterized by rate constant k_{II}.

For the mechanism shown in eq 1, the exponential k_{II}^o has the form given by Figure 5, eq III. In Figure 3b, the data are replotted according to a linear form of eq III in Figure 5. As predicted by the mechanism, a straight line is obtained with slope and intercept corresponding to $2K_2 = 0.1$ μM and $k_{II} = 12 \times 10^{-3}$ min^{-1}, respectively. In order to test the mechanism further, we have determined k_{II}^o values using a different experimental technique (Figure 4e).

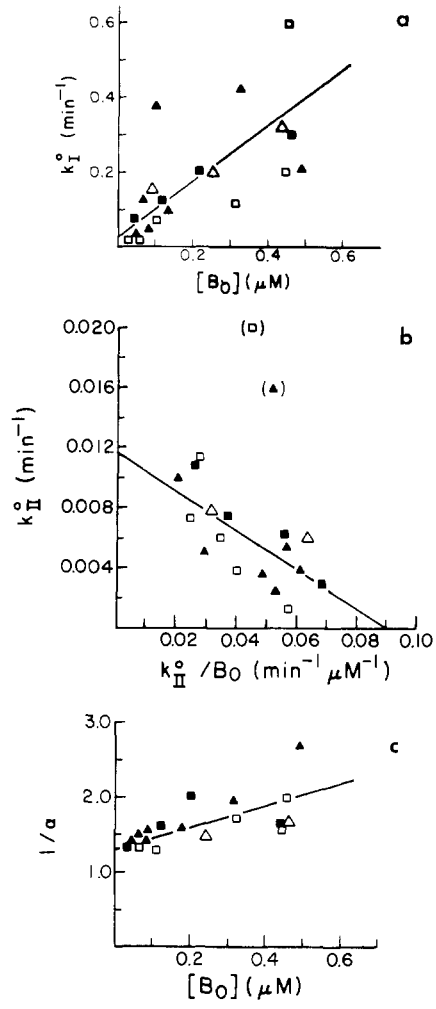


FIGURE 3: The dependence of k_I°, k_{II}°, and α on initial toxin concentration. Four different membrane preparations, each from a different eel, were used. Curves, such as those shown in Figure 2, were constructed for each initial concentration of [^{125}I]iodo-α-bungarotoxin used, and the exponentials and the 0 time intercept of the slow phase of the reaction (α) were calculated using a nonlinear least-squares computer program. Shown in a, b, and c are the parameters plotted as a function of initial toxin concentration. The parameters are defined in the Appendix. The coordinates of the solid lines in a, b, and c were obtained by using a linear least-squares computer program. The four different eels are designated by four different symbols. (a) The dependence of k_I° on initial toxin concentrations. The slope of the line has a value of 7×10^5 M^{-1} min^{-1}. (b) The evaluation of K_2 and k_{II} from the dependence of k_{II}° on initial toxin concentration. The intercept, reflecting k_{II}, has a value of 0.012 min^{-1}; the slope, reflecting K_2, has a value of 0.1 μM. The points in parentheses are not included in calculating the parameters of the solid line. (c) The dependence of $1/\alpha$ on initial toxin concentration. The slope of the line has a value of 1.5 μM^{-1}. The value of the intercept is 1.3.

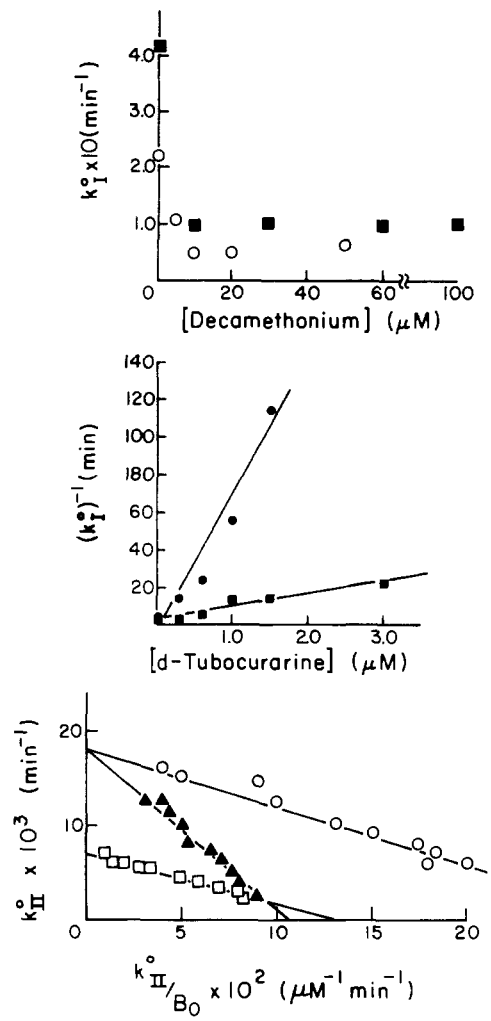


FIGURE 4: The effect of inhibitors on the fast and slow phase of the reaction; pH 7.0, 4 °C eel Ringers' solution. (a, top) (○) 0.25 μM α-bungarotoxin; (■) 0.5 μM α-bungarotoxin. Points are for membrane preparations from three different eels. (b, middle) (●) 0.2 μM α-bungarotoxin; (■) 0.5 μM α-bungarotoxin. Values at 0 and 1.5 μM *d*-tubocurarine concentrations represent the averages of two points. Membrane preparations from two different eels were used. (c, bottom) The k_{II}° values were determined as described in the text using Appendix eq 2. (○) No additions; (▲) plus 0.3 μM *d*-tubocurarine; (□) plus 6.5 μM decamethonium.

Figure 3c shows a plot of α^{-1} as a function of initial α-bungarotoxin concentration. This plot demonstrates an unusual feature of the data: the fraction of the reaction which proceeds by the slow phase, α, and which is characterized by a high affinity for α-bungarotoxin decreases with increasing initial toxin concentration; the fraction of the reaction ($1 - \alpha$) which proceeds rapidly and which is characterized by a low affinity for the toxin increases correspondingly.

The effects of decamethonium, a compound which initiates changes in membrane potential, and *d*-tubocurarine, an inhibitor of these changes, on k_I° and k_{II}°, are shown in Figure 4. Figure 4a shows the effect of various concentrations of decamethonium on k_I° at two different initial concentrations of α-bungarotoxin, 0.25 and 0.5 μM. k_I° decreases to a limiting value of 33 and 25% of the value obtained in the absence of inhibitor, respectively. The inhibitory effect is seen at a ratio of decamethonium concentration to its dissociation constant, K_D (determined in equilibrium measurements (Fu et al., 1974)), of 10, and no further effect is seen when the ratio reaches 100. For a simple inhibition mechanism, k_I° is expected to decrease, as the inverse of the decamethonium concentration, towards zero at infinite inhibitor concentration. The effect of *d*-tubocurarine on k_I° is shown in Figure 4b. A plot of $1/k_I^\circ$, as a function of *d*-tubocurarine concentration, is shown at two initial α-bungarotoxin concentrations, 0.2 and 0.5 μM. The intercept of the two lines, computed by a least-squares method, was found to be just in the first quadrant. For a simple mechanism, an intercept of the two lines in the second quadrant is expected (see Appendix, eq 1a). Interpretation of these data is further complicated by the effect of ligand concentration on α. The significance of the data is that *d*-tubocurarine, in contrast to decamethonium, causes a linear decrease in k_I°.

The effects of decamethonium and *d*-tubocurarine on the slow phase of the reaction (Figure 4c) could be analyzed in

TABLE I: Parameters for Reaction of [^{125}I]Iodo-α-bungarotoxin with Electroplax Membrane Preparations, Eel Ringers' Solution: (Keynes and Martins-Ferriera, 1953) pH 7.0, 4 °C.

Parameters Measured	Parameters Evaluated	
k_I^o	k_I'' [a]	7×10^5 M^{-1} min^{-1}
k_{II}^o	$2K_2$	0.1 μM
		0.1 μM [b]
	k_{II}	12×10^{-3} min^{-1}
		18×10^{-3} min^{-1} [b]
	K_I competitive *d*-tubocurarine	0.2 μM
	K_I noncompetitive decamethonium	2.5 μM
	K_I noncompetitive carbamylcholine	24 μM [c]

[a] See eq II, Figure 5. [b] By determining the concentration of irreversibly reacted receptor sites at two different time intervals (Fig. 4c; Appendix eq 2). [c] Bulger and Hess, 1973.

more detail. The k_{II}^o values were evaluated by determining the concentration of irreversibly reacted receptor sites at two time intervals, both long enough for the first exponential of eq 1 to decay (see Appendix, eq 2). When the k_{II}^o values are plotted according to a linear form of Figure 5, eq III, straight lines are obtained in absence of inhibitor (open circles), in presence of 0.3 μM *d*-tubocurarine (filled triangles), and in presence of 6.5 μM decamethonium (open squares). The intercept of the upper line indicates a k_{II} value of 18×10^{-3} min^{-1} and the slope a $2K_2$ value of 0.1 μM, in agreement with the parameters obtained by direct evaluation of k_{II}^o (Figure 3b and Table I). On the basis of inhibitory mechanisms commonly observed in enzyme reactions, the data indicate that *d*-tubocurarine is a competitive inhibitor of the α-bungarotoxin reaction. From the slope of the line (solid triangles) and Appendix equation 1b, a K_I value of ~0.2 μM was obtained. In contrast, the lowest line indicates that 6.5 μM decamethonium affects the toxin reaction in a way characteristic of noncompetitive inhibitors. From the intercept of the line, and Appendix equation 1c, a K_I value of ~2.5 μM was obtained. A different approach used by us earlier (Bulger and Hess, 1973) to evaluate k_{II}^o values indicated that both decamethonium and carbamylcholine were noncompetitive inhibitors of the inhibitors of the α-bungarotoxin reaction, with noncompetitive inhibition constants for carbamylcholine of 24 μM and for decamethonium of 8 μM; *d*-tubocurarine was a competitive inhibitor with a K_I value of 0.2 μM. The values are in good agreement with values previously determined with monocellular electroplax (Changeux and Podleski, 1968). In these earlier experiments (Bulger and Hess, 1973), we observed the total time course of the reaction of α-bungarotoxin with membrane sites at only a few different initial toxin concentrations and were unaware that α depends on initial toxin concentrations. The data shown in Figure 4c are the same data corrected for the concentration dependence of α. The parameters pertaining to the reaction of [^{125}I]iodo-α-bungarotoxin with electroplax membrane preparations are summarized in Table I.

Discussion

The difficulties inherent in investigating the ligand-binding properties of a receptor which comprises less than 1% of the total membrane proteins have been discussed in the preceding paper of this issue (Fu et al., 1977). The corrections which need to be made in equilibrium measurements are avoided by a kinetic approach similar to one used previously by us in investigations of chymotrypsin- and lysozyme-catalyzed reactions (Hess, 1971; Holler et al., 1975a,b), in which we used specific chromophoric inhibitors with high affinity constants to measure interactions with the substrate-binding site of colorless substrates which bind only poorly to the enzyme.

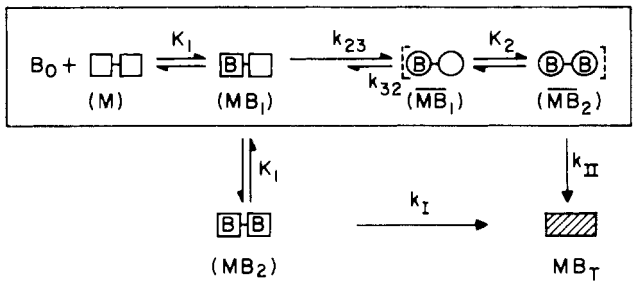
In the reaction of [^{125}I]iodo-α-bungarotoxin with the membrane-bound receptor, several important factors need to be considered and corrected for: (1) reversibly and unspecifically bound toxin (Bulger and Hess, 1973), and (2) the tendency of α-bungarotoxin to stick to surfaces. The reversibly bound toxin, which constitutes a major portion of total toxin binding (Bulger and Hess, 1973), has not been considered by a number of authors (Kasai and Changeux, 1971; Barnard et al., 1971; Miledi and Potter, 1971; Miledi et al., 1971) in determining the total number of toxin sites. This could explain why the number of toxin molecules bound appears larger than the number of decamethonium molecules displaced (Kasai and Changeux, 1971), whereas in our experiments the ratio of toxin sites to decamethonium sites is one to one (Fu et al., 1974). It could also explain why *d*-tubocurarine seems to block the α-bungarotoxin sites only incompletely (Miledi and Potter, 1971; Miledi et al., 1971), since *d*-tubocurarine is not expected to compete with the unspecifically bound toxin.

The method we used results in low and reproducible blanks and allows one to determine the material balance of both protein and radioactivity. Removal of the toxin–receptor complex by filtration (Schmidt and Raftery, 1973; Fulpius et al., 1972) gives blanks almost tenfold higher and does not allow one to determine the protein concentration of the receptor–toxin complex. The method used to determine the k_{II}^o values shown in Figure 4c gives reproducible results, requires only small amounts of membrane preparation, and the effect of toxin concentration on k_{II}^o can be measured with the same membrane preparation in 1 day. The method does not allow one to determine k_I^o and α. To obtain these parameters the progress curve of the whole reaction has to be determined for each initial toxin concentration used. The experiments usually require several membrane preparations and 2 to 3 days of experiments. Because of the importance of the concentration dependence of α, the experiment shown in Figure 2 was done with a single membrane preparation. Additionally, four more membrane preparations were used to establish this concentration dependence (Figure 3c). All measurements which gave a satisfactory material balance are shown in the graphs.

The following information deduced from equilibrium experiments described in the preceding paper of this issue (Fu et al., 1977) is consistent with the kinetic data described here: (1) different receptor binding sites for activators and inhibitors of neural transmission and (2) activators and inhibitors compete for only half of the sites available to them.

(i) The data shown in Figure 4c indicate that decamethonium, an activator of neural transmission, and α-bungarotoxin, an inhibitor, do not occupy the same high-affinity toxin-binding site; that is, decamethonium is a noncompetitive inhibitor of the slow phase of the α-bungarotoxin reaction. The same result has been obtained earlier with carbamylcholine (Bulger and Hess, 1973). On the other hand, *d*-tubocurarine behaves like a competitive inhibitor of the slow phase of the reaction.

(ii) The fast phase of the reaction is only partially inhibited by decamethonium (Figure 4a) even at decamethonium concentrations 100 times greater than the constants for dissociation from its binding site. This indicates that α-bungarotoxin



(I) $MB_T = M_0(1-(1-\alpha)e^{-k_I^o t} - \alpha e^{-k_{II}^o t}$

(II) $k_I^o = \frac{2B_0}{K_1}(k_{23} + k'_I) = k''_I B_0$

(III) $k_{II}^o = k_{II}\frac{B_0}{B_0 + 2K_2}$

(IV) $\alpha = \frac{k_{23}\frac{2K_1}{B_0}}{k_I + \frac{2K_1}{B_0}(k_{23} + k_I)}$

FIGURE 5: Schematic representation of the allosteric model for the interaction of α-bungarotoxin with the membrane-bound acetylcholine receptor. The minimum model proposed previously (Hess et al., 1975) requires that the receptor molecule has at least two subunits. The squares and circles designate different subunit conformations. B_0 represents the initial bungarotoxin concentration, and M the concentration of free receptor sites. K_1 is the dissociation constant of the low affinity site and K_2 the dissociation constant of the high affinity site. k_I and k_{II} represent the rate constants for the formation of the irreversible receptor–toxin complexes, and k_{23} and k_{32} are the rate constants for the protein isomerization. Although considered in the development of the rate equation (see Appendix), the irreversible formation of MB_1 and $\overline{MB}_1$ complexes is not shown for aesthetic reasons.

and decamethonium can occupy a fraction of their respective binding sites simultaneously and without interaction. Evidence that carbamylcholine can also occupy parts of its sites in the presence of α-bungarotoxin has been obtained previously (Fu et al., 1974). *d*-Tubocurarine, however, acts as an inhibitor of both phases of the reaction (Figure 4b,c).

(iii) Additional evidence for different receptor binding sites of the membrane-bound receptor for activators (decamethonium, carbamylcholine) and an inhibitor (*d*-tubocurarine) was obtained from kinetic measurements of $^{22}Na^+$ efflux of excitable electroplax membrane vesicles (Hess et al., 1976). Evidence of half-of-the-site occupancy of acetylcholine in the presence of *d*-tubocurarine was also obtained with *Torpedo* membranes (Eldefrawi, 1974).

Recently, evidence has accumulated that the receptor contains different types of binding sites, in agreement with the earlier conclusion of Bulger and Hess (1973). Among these are the reports (Gordon et al., 1974) that the subunits of the receptor are not equivalent and that the toxin-binding site is associated with only one of the subunits. Eldefrawi and Eldefrawi (1973) have reported the isolation of an α-bungarotoxin binding protein from *T. marmorata* which does not bind acetylcholine.

A number of explanations are often considered for partial interactions between activators and inhibitors as found in our studies: different receptor molecules, preexisting nonequivalent binding sites, or an allosteric mechanism involving ligand-induced conformational changes. Reports of the interconversion of reversibly formed receptor–toxin complexes (Klett et al., 1973) and of inhomogeneity of binding sites or receptor molecules have appeared (Eldefrawi et al., 1972; O'Brien and Gibson, 1974; Chang, 1974; Brockes and Hall, 1975a,b).

Additional information obtained from the kinetic experiments, which has a bearing on these points, is discussed below.

(iv) The experimental conditions used, $B_0 \gg M_0$, require that in bimolecular reactions the rate coefficients are directly proportional to initial toxin concentration. The experiments shown in Figures 3b and 4c indicate that k_{II}^o reaches a limiting value with increasing toxin concentrations. These results are consistent with the formation of a reversible toxin–membrane site complex which precedes the irreversible slow phase of the reaction.

The reaction of α-bungarotoxin with membrane preparations of *Torpedo marmorata* has been treated as a bimolecular process by Franklin and Potter (1972), and these authors observed an initial fast phase in the reaction. The limited concentration range of reactants used by these authors precluded observation of the complexity of the reaction.

(v) An unusual feature of the reaction is that the fraction of the reaction associated with the slow phase of the reaction (α) decreases with increasing toxin concentration (Figures 2 and 3c). This is unusual because the data indicate (Figure 3a,b) that the affinity for the reversible toxin–receptor complex associated with this phase of the reaction is considerably higher than for a similar complex associated with the fast phase. The fraction of the reaction associated with the fast phase ($1 - \alpha$), however, increases with increasing toxin concentrations.

We have derived the rate equations for a number of models which are excluded by these data and present a simple model which is not excluded in Figure 5. The model involves at least two types of receptor sites, M and $\overline{M}$, and the binding of at least two toxin molecules per molecule of receptor protein.

The reversible steps of the minimum reaction scheme are inside the rectangle. They consist of sequential ligand-binding steps and ligand-induced changes in the dissociation constants of the binding sites. The squares and circles in Figure 5 signify different forms of the receptor, each characterized by a different dissociation constant for the reversible toxin–receptor site complex. K_1 and K_2 represent the two dissociation constants. The rate constants k_{23}, k_{32} pertain to isomerization steps involved in the conversion of the receptor forms which are slow compared to the reversible toxin-binding steps. Since it is not necessary for the model to specify the sequences of steps involved in the conversion of receptor forms, brackets are placed around the forms indicated by circles. Also considered in the model but not shown for aesthetic reasons is the formation of irreversible complexes involving only one toxin molecule per receptor.

The rate equation for this model is given in Figure 5 (see Appendix, eq 3–5) and was derived to show that the model predicts the decrease of α with increasing toxin concentration. The data in Figure 3c are plotted according to a linear form of equation IV in Figure 5 to show the relation between α and initial toxin concentration predicted by the model. In view of the difficulties involved in determining α, the agreement between theory and experiment is good. The model is essentially an extension of the Monod et al. (1965) model, proposed by Koshland et al. (1966) to account for the mode of action of a number of allosteric enzymes. The constants K_1 and K_2 used in the model can easily be related to those used by Koshland et al. (1966). Although the proposed model considers a receptor with only two subunits, a simple expansion of the model accommodates a multisubunit receptor (Koshland, 1970; Hammes and Wu, 1974).

Some of the alternative models which we have considered and which are excluded by the data are the following. The rapid interconversions between different receptor forms and

reversible toxin–receptor complexes are excluded because the resulting rate equation involves a single exponential. The interconversion between receptor forms in the absence of toxin is excluded; if the interconversion is fast compared to the toxin-dependent interconversion, the rate equation involves only a single exponential; if the interconversion is rate-limiting, the model does not predict the relationship between k_{II}^{o} and B_0. Models in which the high affinity sites are present in the absence of toxin do not predict the relationship between α and B_0. All the models discussed, which involved only a single, reversible toxin-binding site per receptor, also do not account for the relationship between α and B_0. Different receptor molecules or preexisting nonequivalent binding sites are excluded. The existence of two different receptor molecules in our membrane preparation or the existence of nonequivalent binding sites in one receptor molecule reacting differently with the toxin requires that the fraction of reaction associated with the slow step (α) and the fast step ($1 - \alpha$) remains constant and is independent of toxin concentration. The data in Figures 2 and 3c, however, indicate that α depends on toxin concentration.

One good feature of the proposed model is that it is simple and consistent with all the kinetic data and equilibrium data (Fu et al., 1977). A useful feature of the model is that it is based on the well-understood mechanisms (Monod et al., 1965; Koshland et al., 1966; Eigen, 1967; Hammes and Wu, 1974), which adequately account for the mode of action of well characterized regulatory enzymes. Therefore, the theory is available for modifying this model when additional measurements are made. A noteworthy feature of the model is the requirement of ligand-induced conformational changes. This feature is attractive because of the phenomena to be explained: the passage of ions through nerve and muscle membranes produced by the interaction of acetylcholine with the receptor.

Appendix

The definitions and equations for various mechanisms are given below. All concentrations of membrane-bound α-bungarotoxin are expressed in moles per milligram of membrane protein.

Definitions: B_0, initial α-bungarotoxin concentration; M_0, initial concentration of receptor sites. MB_T represents the irreversibly formed receptor–toxin complexes at time t. The conversions of MB_1 (Figure 5) and of $\overline{MB}_1$ to irreversibly formed toxin–receptor complexes are also considered in the equations. The rate constants for these conversions are k_I' and k_{II}', respectively. The equations are derived assuming that the reversibly formed complexes are in equilibrium with reactants, and $\overline{MB}_1$ and MB_2.

In the presence of an inhibitor, when $B_0 I_0 \gg M_0$, eq III Figure 5 can be written for a competitive inhibitor as:

$$\frac{1}{k_{II}^{o}} = \frac{1}{k_{II}}\left(1 + \frac{2K_2}{B_0}\right) + I_0\left[\frac{2K_2}{B_0}\left(\frac{1}{k_{II}K_I}\right)\right] \quad (1a)$$

$$k_{II}^{o} = k_{II} - \frac{k_{II}^{o}}{B_0} 2K_2\left(1 + \frac{I_0}{K_I}\right) \quad (1b)$$

For a noncompetitive inhibitor:

$$k_{II}^{o} = k_{II}\left(\frac{K_I}{K_I + I_0}\right) - \frac{k_{II}}{B_0} 2K_2 \quad (1c)$$

When measurements of [^{125}I]iodo-α-bungarotoxin incorporations are made at two different time intervals, both long enough for the first exponential of eq 1 in the text ($\exp - k_I^{o}t$) to have decayed, k_{II}^{o} can be evaluated:

$$\ln\left(\frac{M_0 - [MB_{T_{t1}}]}{M_0}\right) - \ln\left(\frac{M_0 - [MB_{T_{t2}}]}{M_0}\right) = k_{II}^{o}(t_2 - t_1) \quad (2)$$

The following equations pertain to the mechanism shown in Figure 5. MB_1 and $\overline{MB}_1$ refer to different conformations of the subunits indicated by □ and ○, respectively. The subscript indicates whether one or two of the binding sites are occupied.

When $B_0 \gg M_0$:

$$M = [MB_2]\left[\frac{B_0^2 + 2K_1B_0 + K_1^2}{B_0^2}\right] \quad (3a)$$

$$[MB_1] = [MB_2]\left(\frac{2K_1}{B_0}\right) \quad (3b)$$

$$[\overline{MB}_1] = [MB_2]\left(\frac{2K_2}{B_0}\right) \quad (3c)$$

$$\frac{d[MB_2]}{dt} = -[MB_2]a_{11} + [\overline{MB}_2]a_{12} \quad (4a)$$

$$\frac{d[\overline{MB}_2]}{dt} = [MB_2]a_{21} - [\overline{MB}_2]a_{22} \quad (4b)$$

$$\frac{d[\overline{MB}_T]}{dt} = [MB_2]\left[k_1 + \frac{2K_1}{B_0}k_1'\right] + [\overline{MB}_2]\left[k_{II} + \frac{2K_2}{B_0}k_{II}'\right] \quad (4c)$$

When $k_{II} \gg k_{32}$, the differential equations defining the minimum mechanism are readily integrated:

$$[MB_T] = M_0(1 - (1 - \alpha)e^{-a_{11}t} - \alpha e^{-a_{22}t}) \quad (5)$$

$$a_{11} = \left[\frac{2K_1}{B_0}(k_{23} + k_1') + k_1\right]\Phi_1$$

$$a_{12} = \left[2k_{32}\frac{K_2}{B_0}\right]\Phi_1$$

$$a_{21} = \left[2k_{23}\frac{K_1}{B_0}\right]\Phi_2$$

$$a_{22} = \left[\frac{2K_2}{B_0}(k_{32} + k_{II}') + k_{II}\right]\Phi_2$$

$$\alpha = \left[\frac{2K_1}{B_0}k_{23}\frac{a_{11}}{a_{11} - a_{22}}\right]\left[k_1 + \frac{2K_1}{B_0}(k_{23} + k_1')\right]^{-1}$$

$$\Phi_1 = \frac{B_0^2}{B_0^2 + 2K_1B_0 + K_1^2}$$

$$\Phi_2 = \frac{B_0}{B_0 + 2K_2}$$

The assumption that $k_{II} \gg k_{32}$ is justified by the observation of two exponentials in the reaction of α-bungarotoxin with the membrane-bound receptor (Figure 2). The linear dependence of a_{11} on initial bungarotoxin concentration (Figure 3a) indicates that under the experimental conditions $K_1 \gg B_0$ and a_{11} is then k_I^{o} (eq II, Figure 5). The dependence on initial bungarotoxin concentration of a_{22} (Figure 3b) indicates that $k_{II}'(2K_2/B_0) \ll k_{II}$. Under these conditions, a_{22} is k_{II}^{o} (eq III Figure 5). Experimentally, it was found that $a_{11} \gg a_{22}$ leading to the relationship between B_0 and α shown by eq IV in Figure 5.

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