ALLOSTERIC INTERACTIONS OF THE MEMBRANE-BOUND ACETYLCHOLINE RECEPTOR; KINETIC STUDIES WITH α-BUNGAROTOXIN

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Summary: The specific and irreversible reaction of a snake neurotoxin, α-bungarotoxin, with the acetylcholine receptor of electroplax membrane preparations from Electrophorus electricus proceeds by an initial fast phase followed by a slower one. The fraction of the reaction in the fast phase increases with increasing initial toxin concentrations, while the fraction going slowly decreases correspondingly. Both phases are affected by compounds which initiate or inhibit nerve impulse transmissions. The time course of the reaction can be fitted to the sum of two exponentials. The dependence on initial toxin concentration of the two exponentials, and of the fraction of reaction governed by the exponentials, can be fitted to a minimum reaction mechanism which involves at least two types of toxin binding sites with different dissociation constants and ligand-induced conversion of one type of site into the other. The mechanism is consistent with our previous data which showed that activators and inhibitors of membrane electrical potential changes occupy separate sites, only half of which interact. This type of mechanism has been seen in a number of allosteric regulatory enzymes.

The mechanism by which the binding of chemical mediators to the membrane-bound acetylcholine receptor regulates ion flux across the membrane, and thereby the electrical potential, is being investigated extensively, using intact cells (1,2), membrane preparations (3-6), and isolated receptor (7-14). The complexity of the systems being used (15-18) made it difficult in the past to obtain the quantitative data needed to understand the underlying mechanism of receptor-ligand interaction.

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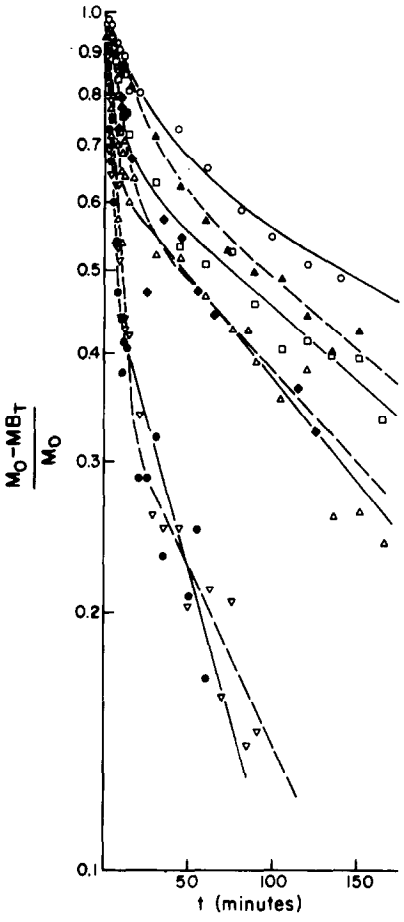
We have recently solved the problems involved in making quantitative measurements in equilibrium and kinetic studies of the interaction of chemical mediators with the membrane-bound acetylcholine receptor (19,20), which constitutes only a small fraction of the total membrane components. We used Electrophorus electricus membrane vesicles which exhibit ion flux regulated by chemical mediators (4,5). We reported (19,20) that the specific and irreversible reaction of a snake neurotoxin, α-bungarotoxin (21), with the membrane-bound receptor proceeds in two phases; an initial fast step followed by a slower one. We described the kinetics of the slow phase of the reaction. The dependence on initial toxin concentration of the reaction indicated (19) that the formation of a reversible, high affinity, toxin-receptor complex preceeds the irreversible step. The data also indicated that activators (carbamylcholine, decamethonium bromide) and inhibitors (d-tubocurarine, α-bungarotoxin) of membrane electrical potential changes occupy separate binding sites on the receptor, and that only half the sites interact with each other. These data can be explained by a number of mechanisms for receptor-ligand interaction, including an allosteric mechanism which has been proposed to account for the action of a number of regulatory enzymes (22,23) and by a mechanism involving two independent classes of binding sites on the membrane. Kinetic investigations which distinguish between the two mechanisms are reported in this paper, where we will describe the kinetics of the fast step of the reaction of [^{125}I]-α-bungarotoxin with the membrane-bound receptor, and the effect of the initial bungarotoxin concentration on the distribution of the reaction between the slow and fast phases.

RESULTS The electroplax membrane vesicles were incubated with [^{125}I]-α-bungarotoxin for various periods of time at 4°C, pH 7.0. The fraction of unreacted membrane sites is plotted on a logarithmic scale as a function of time in Figure 1. The experiments were performed as a function of initial bungarotoxin concentration. The data indicate that the time course of the reaction falls into two steps, an initial fast one followed by a slow step.



The kinetic data were analysed by fitting the time course to the sum of two exponentials:-

$$MB_t = M_0 \left(1 - (1 - \alpha)\, e^{-k_I^o t} - \alpha\, e^{-k_{II}^o t}\right). \qquad (1)$$

MB_t is the irreversibly formed membrane-toxin complex at time t, M_0 the initial concentration of receptor-binding sites, and k_I^o and k_{II}^o are the observed rate constants for the fast and slow phase of the reaction respectively. α is the fraction of the reaction occurring in the slow step. The coordinates of the solid lines in Figure 1 were obtained by using a non-linear least square computer program which gave the values of the two exponentials and of α.

The experiments shown in Figure 2 (a)(b) and (c) were conducted on membrane preparations, each from four different eels. The dependence of k_I^o on initial bungarotoxin concentration is shown in (a). The data are consistent with either a bimolecular reaction, or rapid formation of a reversible, low-affinity toxin-receptor complex preceding the irreversible reaction. The slope of the line gives a value of $7 \pm 1 \times 10^5$ $M^{-1}min^{-1}$ for k_I^o. Experiments in which the initial α-bungarotoxin concentration was kept constant, either 0.2 μM or 0.5 μM, but the concentration of d-tubocurarine or decamethonium was varied, indicated that these compounds inhibit the fast phase of the reaction (Hess et al., manuscript in preparation). Curare, decamethonium, and carbamylcholine were found to be inhibitors for the slow

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Figure 1: The irreversible reaction of [^{125}I]-α-bungarotoxin with electroplax membrane preparations from Electrophorus electricus, pH 7.0, 4°C. The amount of irreversibly bound [^{125}I]-α-bungarotoxin was determined as described below. The data are plotted on a semi-logarithmic scale as log fraction of unreacted membrane sites versus time. Various initial toxin concentrations were used:-

0, 0.05 μM; □ , 0.08 μM; ▲ , 0.07 μM; △ , 0.1 μM; ◆ , 0.18 μM; ▽ , 0.317 μM; ●, 0.49 μM. The observed time course of the reaction was fitted to the sum of two exponentials using a non-linear least square computer program, which yielded the coordinates of the solid lines.

Electroplax membranes were prepared essentially by the procedure of Kasai and Changeux (4, 5). α-Bungarotoxin and the mono-[^{125}I]-derivative were prepared essentially as described by Nirenberg and colleagues (26). The purity of the preparation was checked by acrylamide gel electrophoresis. The derivative contained 1 mole of ^{125}iodine per mole of toxin. The electrophysiological response to labeled and unlabeled toxin by E. electricus electroplax was the same. The membrane preparations were incubated at various initial toxin concentrations and at constant 10-fold excess of [^{125}I]-α-bungarotoxin over membrane sites. After various intervals of time, the irreversibly bound toxin was removed and the irreversible reaction quenched by dilution, approximately 100-fold, of the reaction mixture to a final toxin concentration of 5×10^{-9} M. The diluant eel Ringer (27) solution was 1×10^{-4} M in d-tubocurarine. After standing 15 minutes, the samples were centrifuged at 105,000 x g for 1 hour at 4°C, and the resulting pellets removed and counted with appropriate controls. The radioactivity in the supernatant was counted to determine the concentration of free toxin. The concentration of membrane protein was determined by the method of Lowry et al. (28). Results are expressed in terms of moles of [^{125}I]-α-bungarotoxin irreversibly bound per mg. membrane protein. The total number of receptor sites is determined by reacting the membrane preparation with [^{125}I]-α-bungarotoxin (5×10^{-7} M) under the conditions described until a constant final value of [^{125}I]-α-bungarotoxin incorporation is reached. We have used the value obtained after 5 hr reaction time. The reaction blank is determined for each experiment by quenching the reaction at 0 time and amounts to about 5% of the total number of binding sites.

phase of the reaction (19), with apparent dissociation constants which were in good agreement with values previously determined in electrophysiological experiments on single electroplax (16).

In agreement with earlier reports (19), the concentration dependence of k^{o}_{II} was found to be consistent with a reaction in which a reversibly formed toxin complex, preceeds the irreversible step. The least square line in (b) gives the dissociation constant for the reversible complex, $K_2 \sim 0.1$ μM, and the ordinate intercept gives the rate constant for the irreversible step, $k_{II} = 0.01\ min^{-1}$.

The ordinate intercept, obtained by extrapolating the progress curve of the slow phase (Fig. 1) to zero time, gives the fraction of the total sites which react slowly, α, and the fraction reacting in the fast phase (1-α). The data indicate that the intercept changes as the initial α-bungarotoxin concentration is increased. This indicates that the slow and fast reacting sites are interconvertible, and excludes the possibility that we are observing two independent, parallel, reactions. The unusual feature of the data is that the reaction phase characterized by a high affinity for α-bungarotoxin decreases with increasing initial toxin concentration, while the phase of the reaction characterized by a low affinity for the toxin increases correspondingly. This indicates that the interconversion of the two types of toxin sites is induced by the binding of α-bungarotoxin (22,23).

A minimum reaction scheme consistent with the data is shown in Figure 3, and involves at least two binding sites for α-bungarotoxin.

The differential equations pertaining to the mechanism are solved in the Appendix, and the pertinent parameters are defined. The terms of equation (1), which are consistent with the data shown in Figure 2, become:-

$$k^{\circ}_{I} = \frac{2B_0}{K_1} \quad (k_{23} + k^{\circ}_{I}) \tag{2}$$

$$k^{\circ}_{II} = k^{\circ}_{II} \frac{B_0}{B_0 + 2K_2} \tag{3}$$

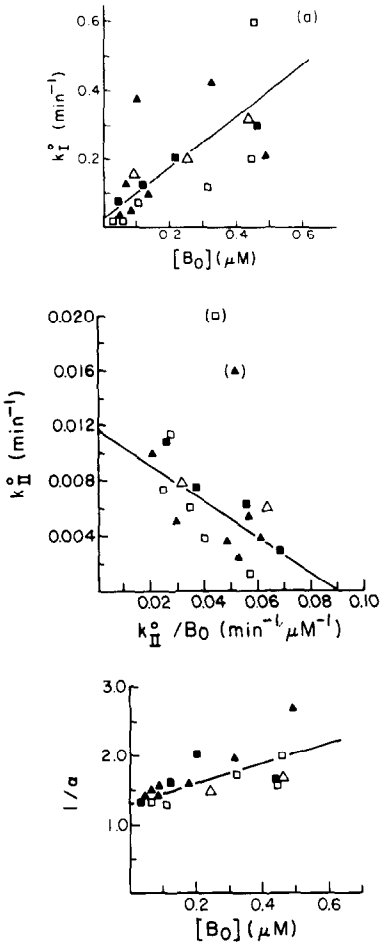

Figure 2: The dependence of k_I°, k_{II}°, and α on initial toxin concentration.

Four different membrane preparations, each from a different eel, were used. Curves such as those shown in Fig. 1 were constructed for each initial concentration of [^{125}I]-α-bungarotoxin used, and the exponentials and the 0 time intercept of the slow phase of the reaction (α) were calculated using a non-linear least square 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 the Figure were obtained by using a linear least square computer program. The four different eels are designated by four different symbols.

(a) The dependence of k_I° on initial toxin concentration. The slope of the line has a value of $7 \pm 1 \times 10^5 M^{-1} min^{-1}$ (see eq. (2)).

(b) The dependence of k_{II}° on initial toxin concentration. The intercept, reflecting k_{II}, has a value of 0.012 ± 0.002 min^{-1}; the slope, reflecting K_2, has a value of 0.12 ± 0.04 μM. The points in parentheses are not included in the least square calculations.

(c) The dependence of α on initial toxin concentration. The slope of the line has a value of 1.5 ± 0.4 μM^{-1} and reflects the value of K_1, k_{23}, and k_I° (see the text). The value of the intercept is 1.3 ± 0.1, which means 80% of the reaction goes by the slow phase at low initial toxin concentration.

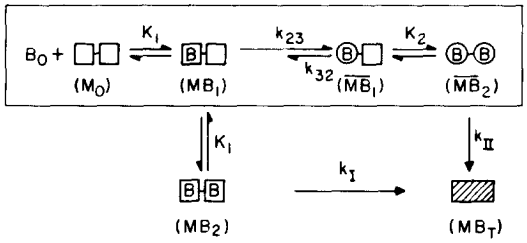

Figure 3: Schematic representation of the allosteric model for the interaction of α-bungarotoxin with the membrane-bound acetylcholine receptor. The minimum model requires that the receptor molecule has two subunits. The squares and circles designate different subunit conformations. B_0 represents the initial bungarotoxin concentration, and M_0 the total concentration of 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 the Appendix), the formation of irreversible MB_1 and $\overline{MB}_1$ complexes is not shown for aesthetic reasons.

$$\alpha = \frac{\frac{2K_1}{B_0}\, k_{23}}{k_I + \frac{2K_1}{B_0}(k_{23} + k_I^*)} \qquad (4)$$

A linear form of equation (4) was used to plot the data in Figure 2(c). As can be seen from the graph, the proposed minimum reaction scheme accounts for the dependence of α on initial bungarotoxin concentration. The intercept of the lines gives a value for $1/\alpha$ of 1.3 ± 0.1, indicating that at the lowest initial toxin concentration, about 80% of the reaction goes by the slow phase and 20% by the initial fast phase, as reported earlier (19).

DISCUSSION The mechanism for membrane-bound receptor-ligand interaction as shown in Figure 3, is the simplest model we can devise which is consistent with all the kinetic data presented and with previous kinetic and equilibrium measurements (19,20) The model, consisting of a sequence of ligand-binding steps coupled to conformational changes, was proposed by Koshland, Nemethy, and Filmer (22), and has been shown to be applicable to a growing number of

regulatory enzymes (23), which show the half-of-the-sites reactivity which we have observed in the interaction of chemical mediators with the membrane-bound receptor (20).

The underlying theory of the mechanism of action of regulatory enzymes has been described in detail (22-25). The methods used to study isolated regulatory enzymes, and the associated theory, has attained a high degree of sophistication (23). The data which we have obtained are of almost the same precision as those obtained previously with isolated regulatory enzymes, thereby bringing investigations of membrane-bound receptor-ligand interaction into a well-studied field. The other important aspect of this report is the evidence for chemical mediator induced changes in conformations of the membrane-bound receptor whose function it is to regulate the flow of ions across the membrane. How these conformational interconversions are affected by chemical mediators, and how they are related to ion flux, are interesting problems for future work.

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APPENDIX The definitions and equations for the mechanism shown in Figure 3 are given below. All concentrations of membrane-bound bungarotoxin are expressed in moles per milligram membrane protein.

Definitions: B_0, initial α-bungarotoxin concentration; M_0, initial concentration of receptor sites. o and □ (*Fig. 3*) *symbolize two conformations of the membrane-bound receptor.* MB_t represents the irreversibly formed receptor-toxin complex. The conversions of MB_1 (Fig. 3) and of $\overline{MB}_1$ to irreversibly formed toxin-receptor complexes are also considered in the equations. The rate constants for the conversions are k_I' and k_{II}' respectively.

Equations: When $B_0 >> M_0$:

$$M_0 = MB_2 \left[\frac{B_0^2 + 2K_1B_0 + K_1^2}{B_0^2}\right] \qquad \text{(App. 1a)}$$

$$MB_1 = MB_2 \frac{2K_1}{B_0} \qquad \text{(b)}$$

$$\overline{MB}_1 = MB_2 \frac{2K_2}{B_0} \qquad \text{(c)}$$

$$\frac{dMB_2}{dt} = -MB_2a_{11} + \overline{MB}_2a_{12} \qquad \text{(App. 2a)}$$

$$\frac{d\overline{MB}_2}{dt} = MB_2a_{21} - \overline{MB}_2a_{22} \qquad \text{(b)}$$

$$\frac{d\overline{MB}_T}{dt} = MB_2[k_I + \frac{2K_1}{B_0}k_I'] + \overline{MB}_2\,[k_{II} + \frac{2K_2}{B_0}k_{II}'] \qquad \text{(c)}$$

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

$$MB_T = M_0(1 - (1-\alpha)e^{-a_{11}t} - \alpha e^{-a_{22}t}) \qquad \text{(App. 3)}$$

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

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

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

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

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

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

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

The assumption that $k_{II} >> k_{32}$ is justified by the observation of two exponentials in the reaction of α-bungarotoxin with the membrane-bound receptor (Fig. 1). The linear dependence of a_{11} on initial bungarotoxin concentration (Fig. 2a) indicates that under the experimental conditions $K_1 >> B_0$ and a_{11} is then k_I° (eq. (2) in the text). The dependence on initial bungarotoxin concentration of a_{22} (Fig. 2b) indicates that $k_{II}\,2K_2/B_0 >> k_{II}$. Under these conditions a_{22} is k_{II}° (eq. (2) in the text). Experimentally it was found that $a_{11} >> a_{22}$ leading to the relationship between B_0 and α shown by eq. (4) in the text.