EVIDENCE FOR SEPARATE INITIATION AND INHIBITORY SITES IN THE REGULATION OF MEMBRANE POTENTIAL OF ELECTROPLAX

I. KINETIC STUDIES WITH α-BUNGAROTOXIN

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Received July 30, 1973

Summary: The minimum reaction mechanism for the irreversible reaction of α-bungarotoxin with membrane preparations of Electrophorus electricus involves a rapid reversible phase (K_{diss} = 0.2 μM) followed by an irreversible reaction (k = 0.038 min^{-1}). Compounds which initiate changes in membrane potential of electroplax affect only the rate of reaction but not the binding of toxin to the membrane. d-Tubocurare which inhibits membrane potential changes, as does α-bungarotoxin, is a competitive inhibitor which affects toxin binding but does not affect the rate of reaction. The simplest explanation of this is that membrane potential changes are controlled by two different sites, one for initiators and the other for inhibitors.

The chemical regulation of electrical potential at the nerve synapse and neuromuscular junction has been investigated extensively by Nachmansohn and coworkers using electroplax preparations of Electrophorus electricus (1, 2). Compounds which initiate changes in membrane potential, such as acetylcholine, carbamylcholine, and decamethonium, and compounds which inhibit these changes, such as curare and the snake neurotoxin, α-bungarotoxin, are assumed to bind to the same site in the membrane, the acetylcholine receptor site. This assumption, and the irreversible reaction of α-bungarotoxin with excitable membranes (3) has led to extensive efforts toward isolation of the toxin binding component of the membrane (4, 5, 6, and 7). This paper presents the first evidence that inhibitors and initiators of changes in potential bind to different sites in electroplax membranes. The conclusion drawn in this paper came from a quantitative analysis of the irreversible reaction between α-bungarotoxin and excitable membrane fragments from electric eel (8).

In Figure 1(a), curve (2) shows the amount of [^{125}I]-α-bungarotoxin

bound irreversibly to the membrane preparation as a function of toxin concentration; curve (3) shows the amount of toxin bound when the irreversible toxin binding has been inhibited by 1 x 10^{-4} M d-tubocurare or by prior treatment with 2.2 μM α-bungarotoxin (unlabeled). Measurement of the total amount of toxin bound reversibly and irreversibly to the membrane indicates that the reversibly bound toxin constitutes a major portion of the total (curve (1)). This reversibly bound toxin has not been considered in published estimations of total toxin sites in membranes (9, 10).

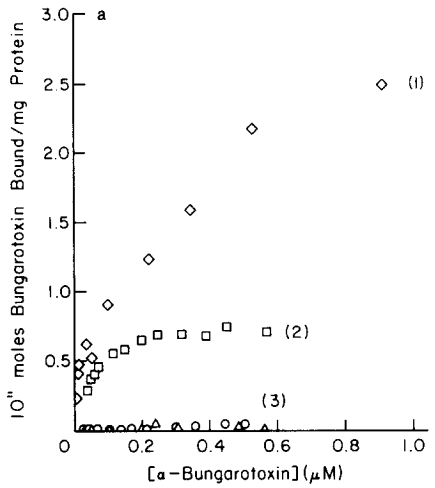
Figure 1(b), in which the fraction of unreacted sites is plotted on a log scale versus time (equation 5(b)), shows the time-dependent reaction of [^{125}I]-α-bungarotoxin with the membrane. The linearity of this plot indicates that the reaction follows a single exponential in the presence and absence of 9.0 μM decamethonium (curves (1) and (2)). The common ordinate intercept of 0.85 indicates that 15% of the reaction had gone to completion before measurements were made, and that the intercept was not affected by this concentration of decamethonium. The observed rate constant, $k_{(obs)}$, for the remaining 85% of the reaction (which is considered subsequently) is affected by decamethonium (curve (2)) and other initiators and inhibitors of changes in membrane potential.

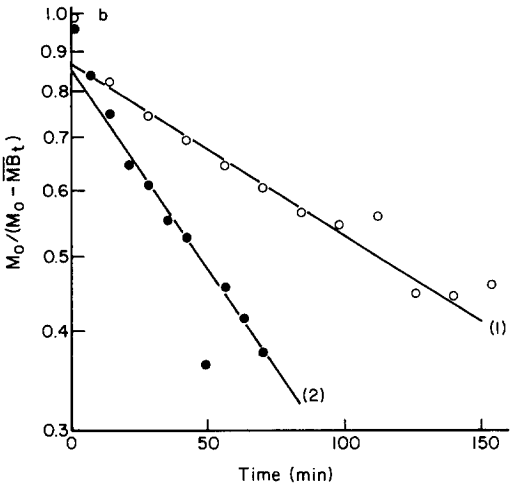
In Figure 1(c), $k_{(obs)}$ for the α-bungarotoxin reaction with the membrane is plotted as a function of initial toxin concentration. A simple bimolecular process, as has been assumed (11) for this reaction, requires that $k_{(obs)}$ increases linearly with increasing toxin concentration (equation (3)). The data, however, indicate that $k_{(obs)}$ reaches a limiting value of 0.038 min^{-1} at high toxin concentrations.

A minimum reaction mechanism describing these data is:

$$M + B \underset{}{\overset{K_B}{\rightleftharpoons}} MB \xrightarrow{k} \overline{MB}. \qquad (I)$$

In this scheme, the toxin, B, reacts with a membrane site, M, to form a reversible MB complex, in a rapid step. K_B is the dissociation constant of





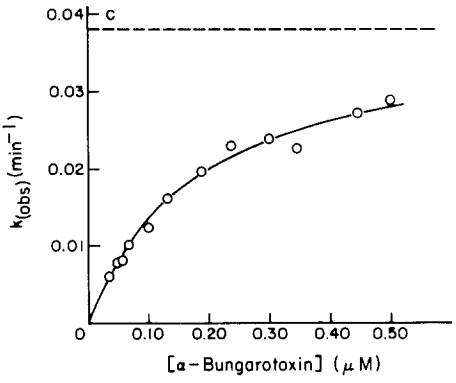


Figure 1: Binding of [^{125}I]-α-bungarotoxin to membrane fragments from Electrophorus electricus.

(a) ◇ , 0.3 ml of membrane (15.1 mg protein/ml) were incubated with various concentrations of [^{125}I]-α-bungarotoxin for 1 hour, then centrifuged for 1 hour at 105,000 x g. The difference in [^{125}I]-α-bungarotoxin concentration before and after centrifugation was used to determine the total bound concentration of toxin. □ , Membranes were incubated at various toxin concentrations for 1 hour at a constant 10-fold excess of [^{125}I]-α-bungarotoxin over membrane sites. The reversibly bound toxin was removed and the irreversible reaction stopped by dilution of the reaction mixture to a final toxin concentration of 5 x 10^{-9} M with a solution of 1.6 x 10^{-4} M decamethonium in eel Ringer solution (15). After standing 15 minutes, the samples were centrifuged as before and the resulting pellets were removed and counted with appropriate controls. Results are expressed in terms of moles of [^{125}I]-α-bungarotoxin irreversibly bound per milligram of protein. O, Incubation in the presence of 100 μM d-tubocurare; Δ , incubation using membrane pretreated with 2.2 μM α-bungarotoxin (unlabeled) for 4 1/2 hours.

(b) The amount of [^{125}I]-α-bungarotoxin irreversibly bound was followed at 0.21 μM toxin as a function of time. ●, No additions; o, with 9.0 μM decamethonium.

the reversible complex, and k the rate constant for the irreversible formation of $\overline{MB}$. The rate equation for this scheme is given by equations (2) and (4).

In Figure 2, $k_{(obs)}$ for the toxin reaction is plotted as a function of toxin concentration according to the linear form of equation (4), in the presence and absence of various reaction inhibitors at the indicated concentrations. A replot of the data in Figure 1(c) is given by curve (1). As predicted by the mechanism (scheme (I)), a straight line is obtained with a slope and intercept corresponding to K_B = 0.18 μM and k = 0.038 min^{-1} respectively.

Values for the dissociation constants of the membrane-toxin complex reported by others (7, 12) were obtained by treatment of the reaction as a reversible process, a treatment which yields values reflecting the dissociation constant, the rate constant, and the total time period of the reaction (equation (6)).

In Figure 2, curve (2) is drawn from data which indicate that curare affects the toxin reaction in a manner characteristic of inhibitors which compete for the same binding site as the reacting compound (equation 4(b)). Although curare affects the slope of the plot, indicating a less favorable toxin binding constant in the presence of curare, it does not affect the rate constant of the reaction at high toxin concentrations, indicating its complete displacement from the reaction site.

In contrast, curves (3) and (4) indicate that decamethonium and carbamylcholine affect the toxin reaction in a manner characteristic of

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(c) Replots of irreversible binding data according to scheme (I). Each experimental point represents the average of measurements obtained from membrane preparations from separate eels. The solid line is calculated from the parameters evaluated from curve (1) in Fig. 2. The dashed line indicates the limiting rate constant of the reaction. All experiments were carried out at 1°C, pH 7.0, in eel Ringer solution (15).

α-Bungarotoxin and the [^{125}I]-derivative were prepared essentially as described by Nirenberg (16). The purity of the preparations was checked by acrylamide gel electrophoresis. The [^{125}I]-derivative contained 1 mole of ^{125}I per mole of toxin. The electrophysiological response to the labeled and unlabeled toxin by electroplax of E. electricus was the same.

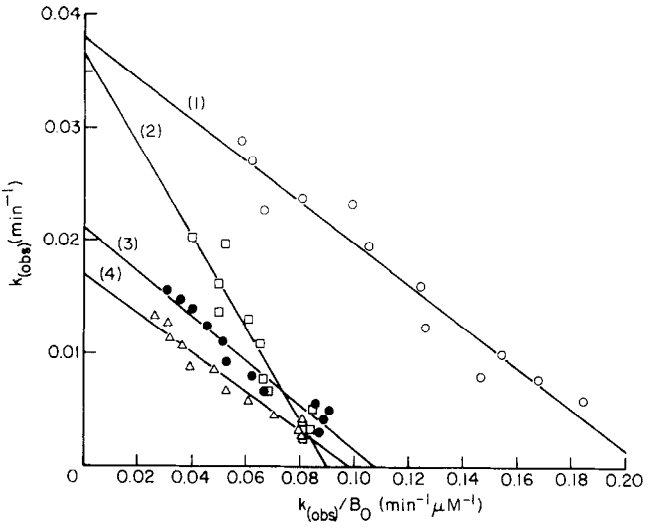

Figure 2: Effect of inhibitors on the irreversible reaction between [^{125}I]-α-bungarotoxin and excitable membranes. Irreversibly bound toxin was determined as described previously.
o, No additions; □, with 0.3 μM curare; ●, with 6.5 μM decamethonium; Δ, with 30 μM carbamylcholine. In the case of the carbamylcholine experiments, either 8×10^{-6} M 3-hydroxyphenyltrimethylammonium iodide or 2×10^{-4} M 0,0,diethyl S-2-diethylaminoethylphosphorothiolate was added to the incubation mixtures to prevent hydrolysis of carbamylcholine by inhibiting the acetylcholinesterase in the preparation. These enzyme inhibitors had a small inhibitory effect on toxin binding, which was not considered in the data presented. The data are plotted according to equation (4). M_0 values are based on experimentally determined values obtained from 3-4 hour incubations of membranes with 0.5 μM toxin and were calculated using a least square computer program. Each experimental point represents the average of measurements obtained from membrane preparations from separate eels. All experiments were carried out at 1°C, pH 7.0 in eel Ringer solution.

inhibitors which do not compete for the same binding site as the reacting compound (equation 4(c)). The slope of the plots is not affected, indicating that toxin binds to the membrane equally well in the presence or absence of these compounds.

Use of equation (4) to evaluate apparent dissociation constants for membrane-inhibitor complexes gave values of 0.2 μM, 24 μM and 8 μM for curare, carbamylcholine, and decamethonium respectively, in good agreement with values previously determined in experiments with electroplax (13, 14).

The data presented in this paper, and its analysis, explain some of the variations in both stoichiometry and reported dissociation constants of the α-bungarotoxin binding reaction. The effects of α-bungarotoxin

concentration and of inhibitors upon $k_{(obs)}$ values for the reaction are analogous to those observed in many enzyme-catalyzed reactions and are interpreted in a manner consistent with the minimum reaction scheme presented.

The major conclusion arising from this treatment of the data is that initiators and inhibitors of membrane potential changes occupy separate, but interacting, sites on the membrane, and that α-bungarotoxin binds at the inhibitory site.

Acknowledgements: We are grateful to Drs E. Bartels-Bernal and W. Niemi, Department of Neurophysiology, Columbia University, for performing the electrophysiology, and to the National Institutes of Health and the National Science Foundation for financial support. J.E.B. is grateful to the National Institutes of Health for the award of a Postdoctoral Fellowship.

APPENDIX

Definitions: All concentrations of membrane-bound toxin are expressed in moles per milligram membrane protein. B_0, initial concentration of α-bungarotoxin; M_0, initial concentration of membrane sites; M, concentration of free membrane sites; MB and MB_t, the concentrations of reversible membrane-toxin complex, and of irreversible membrane-toxin complex at time t respectively; I_0, the initial inhibitor concentration. K_B and k are defined in the text; K_I, the dissociation constant of the membrane-inhibitor complex.

$$K_B = [(M)(B_0)][MB]^{-1};$$
$$K_I = [(M)(I_0)][MI]^{-1}$$

Equations: The equations pertain to experimental conditions with B_0, $I_0 > M_0$, and considered as constants.

(1) $$M + B \xrightarrow{k} \overline{MB}$$

(2) For a bimolecular mechanism, and for the mechanism shown in scheme (I),

$$\overline{MB}_t = M_0 (1 - e^{-k_{(obs)}t})$$

(3) For a bimolecular mechanism,

$$k_{(obs)} = k B_0$$

(4) For the mechanism of scheme (I),

(a) $$k_{(obs)} = \frac{k B_0}{B_0 + K_B}$$

(b) In the presence of a competitive inhibitor,

$$k_{(obs)} = \frac{k B_0}{B_0 + K_B (1 + \frac{I_0}{K_I})}$$

(c) In the presence of a noncompetitive inhibitor,

$$k_{(obs)} = \frac{k B_0 \frac{K_I}{K_I + I_0}}{B_0 + K_B}$$

(5) Evaluation of $k_{(obs)}$ from equation (2),

(a) $$\frac{M_0}{M_0 - \overline{MB}_t} = e^{k_{(obs)}t}$$

(b) $$\ln \frac{M_0}{M_0 - \overline{MB}_t} = k_{(obs)}t$$

(6) Evaluation of $k_{(obs)}$ using the Taylor series,

(a) $$\frac{M_0}{M_0 - \overline{MB}_t} = 1 + k_{(obs)}t + \frac{(k_{(obs)}t)^2}{2!} \cdots$$

(b) When higher terms of the series are small compared to the first two terms, and all the measurements are made at the same time after mixing the reactants,

$$\overline{MB}_t = \frac{kt\Phi M_0 B_0}{B_0 + K_B \Phi}$$

$$\Phi = [1 + k t]^{-1}$$

REFERENCES

1. Nachmansohn, D. (1959) in Chemical and Molecular Basis of Nerve Activity, Academic Press, New York.
2. Nachmansohn, D. (1971) Proc.Nat.Acad.Sci., U.S. 68, 3170-3174.
3. Chang, C.C., and Lee, C.Y. (1963) Arch.Intern.Pharmacodyn. 144, 241-257.
4. Miledi, R., Molinoff, P., and Potter, L.T. (1971) Nature, 229, 554-557.

5. Meunier, J.-C., Olsen, R., Menez, A., Morgat, J.-L., Fromageot, P., Ronseray, A.-M., Boquet, P., and Changeux, J.-P. (1971) C.R.Acad.Sci., Paris D 273, 595-598.
6. Schmidt, J., and Raftery, M.A. (1972) Biophys.Biochem.Res.Comm. 49, 572-578.
7. Bosmann, H.B. (1972) J.Biol.Chem. 247, 130-145.
8. Changeux, J.-P., Gautron, J., Israel, M., and Podleski, T.R. (1969) C.R.Acad.Sci., Paris 269, 1788-1791.
9. Kasai, M., and Changeux, J.-P. (1971) J.Membr.Biol. 6, 58-80.
10. Barnard, E.A., Wieckowski, J., and Chiu, T.H. (1971) Nature, 234, 207-209.
11. Franklin, G.I., and Potter, L.T. (1972) FEBS Letters, 28, 101-106.
12. Raftery, M.A., Schmidt, J., Clark, D.G., and Wolcott, R.G. Biochem.Biophys.Res.Comm. 45, 1622-1629.
13. Changeux, J.-P., and Podleski, T.R. (1968) Proc.Nat.Acad.Sci., U.S. 59, 944-950.
14. Kasai, M., and Changeux, J.-P. (1970) C.R.Acad.Sci., Paris, 270, 1400-1403.
15. Keynes, R.D. (1953) J.Physiol.(London),119, 315
16. Sytkowski, A.J., Vogel, Z., and Nirenberg, M. (1973) Proc.Nat.Acad.Sci., U.S. 70, 270