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Comparison of the Interactions of a Specific Neurotoxin (α-Bungarotoxin) with the Acetylcholine Receptor in *Torpedo californica* and *Electrophorus electricus* Membrane Preparations†

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ABSTRACT: α-Bungarotoxin, a snake neurotoxin, binds irreversibly and specifically to the acetylcholine receptor isolated from the electroplax of *Electrophorus electricus* and *Torpedo* species and has been an important tool in the study of the receptor–ligand binding mechanism. Two distinct kinetic processes have been observed in studies with membranes from *E. electricus*. A minimum mechanism for the toxin reaction involves (i) the reversible binding of two toxin molecules to the receptor prior to the irreversible formation of toxin:receptor complexes and (ii) a toxin-induced conformational change of the receptor which leads to an increase in the affinity of the receptor binding sites for toxin [Hess, G. P., Bulger, J. E., Fu, J.-j. L., Hindy, E. F., & Silberstein, R. J. (1975) *Biochem. Biophys. Res. Commun. 64*, 1018–1027]. Only one process has been detected in *Torpedo* membranes. Here, we determine whether the receptors in *Torpedo californica* and *E. electricus* membranes have different properties or whether the measurements and their interpretation were responsible for the different results. Two methods which are frequently used in binding studies to separate free and bound toxin, a CM-52 cellulose minicolumn assay and DE-81 filter disk assay, have been compared. The results obtained indicate that the interaction of toxin with receptor from *T. californica* is similar to that observed with receptor from *E. electricus*. The apparent differences which have been reported in the literature are shown to have arisen from the design of the experiments in which *T. californica* membranes were used.

The binding of an activating ligand to the acetylcholine receptor protein is a triggering event for the initiation of electrical signals in nerve and muscle cells. Many studies have been directed toward the characterization of this interaction, and snake neurotoxins that specifically inhibit the nicotinic acetylcholine receptor have been essential tools in this field (Chang & Lee, 1963; Weiland et al., 1976; Blanchard et al., 1979; Weber & Changeux, 1974; Maelicke et al., 1977; Brockes & Hall, 1975; Bulger & Hess, 1973; Hess et al., 1975; Bulger et al., 1977; Quast et al., 1978). In many studies, a toxin from *Bungarus multicinctus*, α-bungarotoxin, is used because it binds irreversibly to the receptor of muscle cells and of the electric organ of *Electrophorus electricus* and *Torpedo* species, and it competes with receptor–ligands for this binding (Albuquerque et al., 1979). Many different results and interpretations have been reported for the interaction of this toxin with the receptor in *E. electricus* and *Torpedo* species membrane fractions.

On the basis of electrophysiological measurements, Katz & Thesleff (1957) suggested that exposure of the receptor to acetylcholine induces a conformational change and results in an increase in the affinity of the receptor toward this ligand. A reaction that consists of two phases and results in an increase in the affinity of the receptor toward the ligand has been observed in the binding of α-bungarotoxin to both soluble and membrane-bound receptor protein isolated from *Electrophorus electricus* [Hess et al., 1975; for a review, see Heidmann & Changeux (1978) and Eldefrawi & Eldefrawi (1979)], and in the receptor-controlled translocation of inorganic ions across the plasma membrane of vesicles prepared from *E. electricus* (Hess et al., 1978, 1979, 1980; Aoshima et al., 1980; Cash & Hess, 1980; Cash et al., 1980). A preliminary investigation of the reaction of α-bungarotoxin with the receptor in *T. marmorata* membranes indicated a simple bimolecular process preceded by a fast initial phase of low amplitude (Franklin & Potter, 1972).

The exception to these results appeared to be the reaction of α-bungarotoxin with the membrane-bound acetylcholine receptor isolated from the electric organ of *T. californica* (Quast et al., 1978). In these investigations covering a range of initial toxic concentrations, only a simple first-order reaction

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was observed, and evidence for a multiphasic reaction was not obtained (Blanchard et al., 1979). Similarly, while the stoichiometry of activating ligand-binding sites and α-bungarotoxin-binding sites appears to be 1:1 in electroplax membranes of *E. electricus* (Bulger & Hess, 1973; Fu et al., 1974, 1977, Hess et al., 1975; Bulger et al., 1977), a different stoichiometry in *T. californica* has also been reported (Raftery et al., 1975).

Because this toxin reaction forms the basis of many biochemical studies of the acetylcholine receptor, we investigated the reaction of α-bungarotoxin with the receptor in *T. californica* membrane preparations to determine whether the reported differences in results are due to differences in the receptor protein or the iodinated α-bungarotoxin preparation, in the methods used for measuring the receptor–toxin reaction, in the experimental design, or in the interpretation of the results.

Measurements of the reaction of the toxin with the receptor involve the separation of free, labeled toxin (generally the mono-^{125}I form) from the labeled toxin:receptor complex. At least four different methods have been used for this purpose: ultracentrifugation (Bulger & Hess, 1973), Millipore filtration (Weber & Changeux, 1974), and chromatography on CM-52 cellulose minicolumns (Kohanski et al., 1977) or DEAE filter disks (Schmidt and Raftery, 1973). Blanchard et al. (1979) stressed that the results obtained in the DEAE disk assay and the ultracentrifugation assay are very different. The latter method has been used to investigate the reaction of the toxin with membrane-bound receptor from *E. electricus* (Bulger & Hess, 1973; Bulger et al., 1977). Two methods which are frequently used to separate free and bound toxin, a CM-52 cellulose minicolumn assay and a DE-81 filter disk assay, have been compared here. We can now demonstrate that the reaction of the toxin with membrane-bound receptor from *E. electricus* and from *T. californica* is similar and that the apparent differences which have been reported arose because of the design of the experiments in which *T. californica* membrane preparations were used.

Experimental Procedures

T. californica (from Pacific Biomarine, Venice, CA) vesicles were prepared according to the method of Sobel et al. (1977). α-Bungarotoxin purified from *Bungarus multicinctus* venom (from Miami Serpentarium, FL) was labeled with ^{125}I by the peroxidase method (Morrison & Bayse, 1970). Di- and monoiodinated forms were separated as described previously (Bulger et al., 1977), only the monoiodinated form being used in the experiments. The monoiodinated toxin was stored at −20 °C and was periodically rechromatographed on a CM-52 minicolumn to remove any impurities generated during storage. The concentration of toxin protein was determined by the Fluram (from Pierce Chemical Co.) method (Böhlen et al., 1973) using unlabeled α-bungarotoxin as a standard. The protein concentration of this standard was verified by measuring the molar extinction coefficient of the toxin at 280 nm (Bulger et al., 1977). The concentration of the α-bungarotoxin-binding sites in each preparation of *T. californica* membranes was determined by either of the two methods employed (see below). *T. californica* vesicles (0.04–0.05 μM α-bungarotoxin-binding sites) and α-[^{125}I]bungarotoxin (0.1–0.5 μM) were incubated at 0 °C in the appropriate buffer in a silanized vial. Two 100-μL samples of the incubation mixture were taken at the same time, one of which was applied to a CM-52 cellulose minicolumn and the other to a DE-81 filter disk as follows.

Minicolumn Assay. After complete equilibration with 1 mM sodium phosphate buffer, pH 7.2, a 1.25-mL bed volume of CM-52 cellulose (Whatman) was deposited into a silanized Pasteur pipet plugged with glass wool. The 100-μL sample was injected onto an almost dry bed top so that the mixing of sample and resin was almost instantaneous. This is important in order to quench completely the receptor–toxin reaction. The column was eluted with 2.2 mL of 1 mM phosphate buffer, pH 7.2, and the eluate was counted in a Beckman Biogamma counter. Other details of the method have been published (Kohanski et al., 1977).

Disk Assay. The disk assay was performed as described by Blanchard et al. (1979): A 100-μL sample was applied to a DE-81 filter disk (2.5 cm) (Whatman) which was then washed 3 times for 10 min each time in a bath of 50 mM NaCl, 0.1% Triton X-100, and 10 mM phosphate buffer, pH 7.4, and then transferred to a Biogamma vial and counted.

In both assays, the amount of unspecific binding was determined by incubating the vesicles for 1 h before initiating the reaction of α-[^{125}I]bungarotoxin with unlabeled α-bungarotoxin which was of a concentration 10-fold greater than that of the toxin-binding sites in the solution. The unspecific binding was found to be proportional to the amount of labeled toxin present in the incubation mixture (Bulger & Hess, 1973; Figure 2). For a given toxin concentration, unspecific binding also increased with the total amount of protein in the sample (Bulger et al., 1977; Kohanski et al., 1977) and reflected the remaining free toxin which was not removed by whichever separation method was used.

Results

The reaction between α-bungarotoxin and the acetylcholine receptor is known to be dependent on salt concentration, and this is apparent both in the rate of the reaction and in the total amount of toxin bound (specific and unspecific binding) (Weber & Changeux, 1974; Schmidt & Raftery, 1974). The performance of the CM-52 cellulose ion-exchange column is very dependent on ionic strength, and a low salt concentration must be used to obtain an efficient separation of free and bound toxin. On the other hand, *Torpedo* Ringer's solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate buffer, pH 7.5) was used in the incubation reaction in most of the studies in which DE-81 filters were used (Blanchard et al., 1979; Quast et al., 1978). In order to compare the results of the two assays, it was, therefore, necessary to alter the concentration of salts in the samples applied to the minicolumn. Figure 1A shows the binding of α-[^{125}I]bungarotoxin to *T. californica* vesicles in *Torpedo* Ringer's solution observed by the two techniques. In the experiment shown, samples for the toxin-binding assay were taken from the same reaction mixture. However, it was necessary to decrease by a large factor the concentration of salts in the sample applied to the minicolumn. This was achieved by diluting 200-fold a sample of the reaction mixture with 1 mM phosphate buffer, pH 7.5, containing a 50-fold excess of unlabeled α-bungarotoxin to quench the toxin-binding reaction. Two 150-μL samples of this solution were applied to two separate minicolumns and eluted as described above, and the mean value of bound toxin is shown in the figure. For the parallel filter assay, a sample was similarly diluted in *Torpedo Ringer*'s solution containing a 50-fold excess of unlabeled α-bungarotoxin. A 100-μL sample of this solution was then applied to the filter and washed. Both assays gave essentially the same results (Figure 1A). During the total 15-min reaction period shown, about 33% of the reaction had gone to completion before the first measurement could be made by either of the methods used. This initial fast phase is followed by a slower phase which occurs within the time resolution of

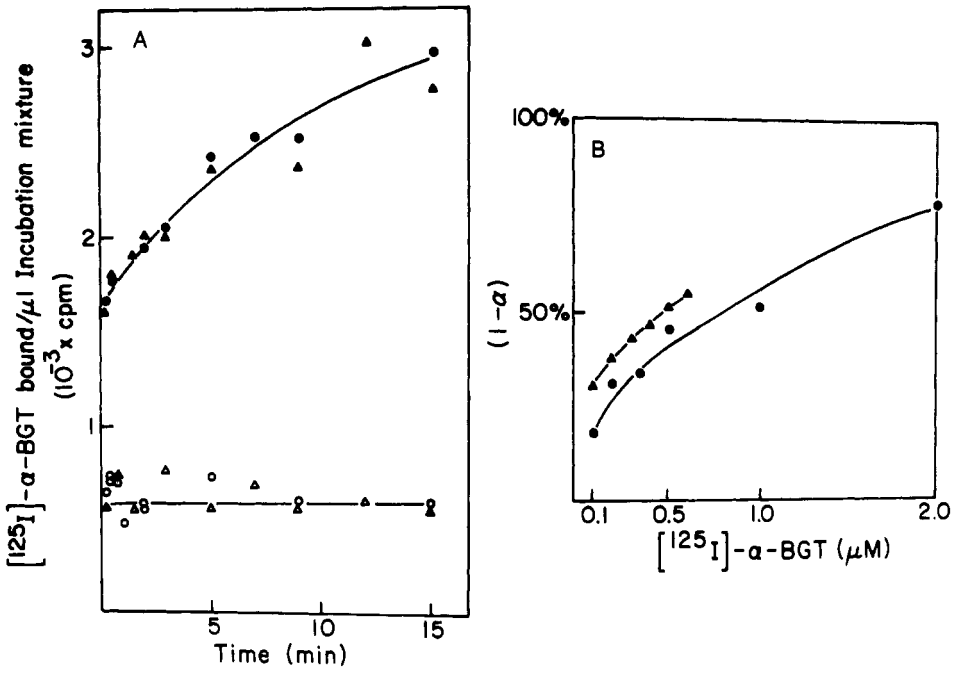

FIGURE 1: Binding of α-[^{125}I]bungarotoxin to acetylcholine receptor from *T. californica* vesicles in *Torpedo* Ringer's solution, pH 7.5, 0 °C. (A) Time course of toxin binding. *T. californica* vesicles (40 nM toxin sites) were incubated with 100 nM α-[^{125}I]bungarotoxin. The vesicles were preincubated for 1 h with (open symbols) or without (closed symbols) a 10-fold excess of cold α-bungarotoxin before the labeled toxin was added. At the time shown, aliquots of the incubation mixture were removed, diluted, and applied to the separation medium. (●, ○) A 20-μL aliquot of the incubation mixture was diluted with 380 μL of *Torpedo* Ringer's solution containing α-bungarotoxin (250 nM). After the solution was mixed, 100 μL was applied to a DE-81 filter and washed as described above. (▲, △) a 10-μL aliquot of the incubation mixture was diluted with 1990 μL of a 1 mM phosphate buffer, pH 7.5, containing α-bungarotoxin (25 nM). Two 150-μL samples were obtained to separate CM-52 cellulose columns and eluted as described above. The results shown represent the mean values obtained. (B) Dependence of the extent of the fast phase (1-α) on initial toxin concentration. Data were taken from Bulger et al. (1977) for *E. electricus* (▲) and from *Torpedo* experiments (●) similar to the ones described in (A).

the methods used. A simple bimolecular reaction scheme (Blanchard et al., 1979) is ruled out by these results which are essentially similar to those obtained with an *E. electricus* preparation (Hess et al., 1975; Bulger et al., 1977). Previously, we have reported (Bulger & Hess, 1973; Fu et al., 1974; Hess et al., 1975) that in the case of receptor-containing vesicles prepared from the electroplax of *E. electricus* the specific and irreversible reaction of α-bungarotoxin with the receptor proceeds in two phases: an initial fast step followed by a slower one. The dependence of the reaction on initial toxin concentration indicated (Bulger & Hess, 1973) that the reversible formation of a toxin:receptor complex precedes the irreversible step and that two toxin molecules bind to the receptor before the irreversible reaction occurs. The fraction of the reaction which proceeds by the initial fast phase ($1 - \alpha$) depends on the toxin concentration which is used. Figure 1B shows the dependence of $1 - \alpha$ on toxin concentration in experiments with vesicles prepared from *E. electricus* (▲) and *T. californica* (●). The measurements on which this graph was based are shown in Figure 2. The data in Figure 2 show that the total amount of specifically bound toxin is independent of the initial toxin concentration, whereas the amount of toxin bound unspecifically increases with initial toxin concentration. The dependence of the fast phase of the reaction on toxin concentration (Figure 1B) appears very similar in the two preparations. The quantitative relationship between $1 - \alpha$ and the minimum reaction mechanism for the reaction of toxin with the *E. electricus* membrane preparation (Figure 4b) has been published (Hess et al., 1975; Bulger et al., 1977).

In order to exclude differences that could originate from the properties of the iodinated toxin used or the *Torpedo* membrane preparations, we have used the procedure described by Blanchard et al. (1979) to present our data. The same data from a typical binding experiment were plotted according to

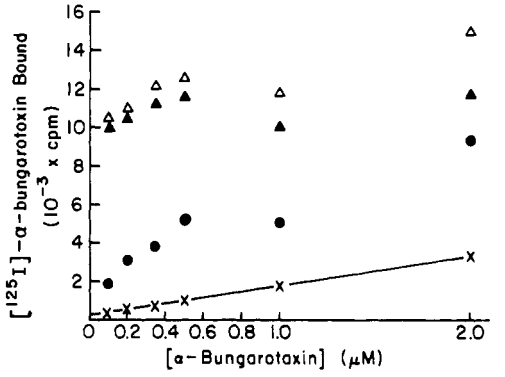

FIGURE 2: Influence of toxin concentration on the binding of α-[^{125}I]bungarotoxin with *Torpedo* membranes. Toxin sites (~40 nM) were incubated after the vesicles were incubated for 6 h with the toxin concentration indicated on the abscissa. with the concentration of labeled toxin indicated in the graph, and the DE-81 filter assay was used as described above; 10^4 cpm represents 35 nM toxin. (×) Unspecific binding: The vesicles were preincubated for 1 h with a 10-fold excess of unlabeled α-bungarotoxin before the labeled toxin was added. (▲, △) The open symbol represents both specific and unspecific toxin binding. The closed symbol represents specific toxin binding. (●) C_b: This value is obtained by extrapolating to zero time the time-dependent toxin reaction, which is uncorrected for unspecific binding and is represented by Δ on this graph. Note that $1 - \alpha = (C_b - [\text{reaction blank}])/C_\infty$, where C_b includes the initial fast phase of the reaction as well as the unspecific binding at $t = 0$ and C_∞ represents specific binding when the reaction has gone to completion.

Blanchard et al. (1979) (Figure 3A). The solid lines are data obtained with our *Torpedo* membrane and toxin preparations. The dotted lines are obtained from Figure 5 in the paper by Blanchard et al. (1979). It can be seen that the results are similar. Only a single slow phase of the reaction is observed. When a reaction blank is subtracted from the toxin-binding data, however, both a fast and a slow phase of the reaction

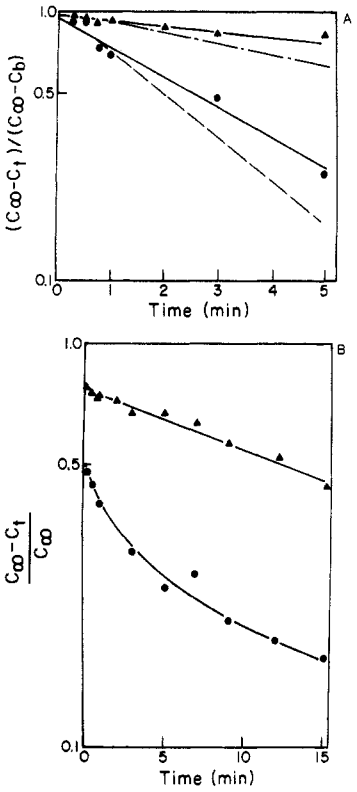

FIGURE 3: Binding of α-[^{125}I]bungarotoxin to acetylcholine receptor in *T. californica* vesicles in *Torpedo* Ringer's solution, pH 7.5, 0 °C. *T. californica* vesicles (50 nM toxin-binding sites) were incubated, for the time shown, in *Torpedo* Ringer's solution with 100 (▲) or 500 nM (●) α-[^{125}I]bungarotoxin. Free and bound toxins were separated on a DE-81 filter. (A) Plot of the time course of the toxin according to Blanchard et al. (1979). The background value, C_b, was obtained by extrapolating the time-dependent toxin reaction to zero time. C_t and C_∞ represent the total binding at a time t and after 6 h of reaction, respectively. The coordinates of the solid line are determined by our measurements. For purposes of comparison, the data of Blanchard et al. (1979) are also shown. The dashed line was obtained with 500 nM toxin and the dashed and dotted line with 125 nM toxin. (B) Replot of the time course of the reaction shown above. Unspecific binding, measured after incubation with a 10-fold excess of unlabeled toxin, has been subtracted from these curves.

can be observed (Figure 3B). It should be noticed that the slow part of the reaction appears to consist of two phases.

Discussion

The results presented in Figures 1A and 3A show that the assay technique or the membrane or toxin preparations per se do not explain the different results obtained with *E. electricus* and *Torpedo* membrane preparations. The results of the toxin-binding assays using columns or DEAE filters appear to be identical and are in agreement with the ultracentrifugal assay used by Bulger et al. (1977). The minicolumn method has the advantage of not being very restricted in sample volume or protein concentration, making it more suitable than the disk assay for studies using material with fewer α-bungarotoxin-binding sites than are present in the electroplax of *Torpedo* species, such as muscle cells or *E. electricus* electroplax.

One important point in the experiments of Bulger and colleagues (Bulger & Hess, 1973; Bulger et al., 1977) was the exact evaluation of the unspecific binding of toxin to the membrane fragments (Bulger & Hess, 1973). The effects of both toxin concentration and membrane concentration on unspecific toxin binding were evaluated (Bulger & Hess, 1973; Bulger et al., 1977; Kohanski et al., 1977). The results obtained with the CM-52 minicolumn assay of *E. electricus* membrane preparations were then compared to the ultracentrifugal assay using the same membrane preparation. Here, we show that when the initial concentration of toxin is greater than that of the toxin-binding sites, the total amount of specifically bound toxin is constant (Figure 2). This result would be obtained either if the amount of toxin bound unspecifically is independent of the initial toxin concentration used or when the unspecifically bound toxin depends on initial toxin concentration (Bulger & Hess, 1973; Bulger et al., 1977; Kohanski et al., 1977) but appropriate corrections are made. After appropriate corrections for unspecific toxin binding were made, a 1:1 stoichiometry of activating ligand-binding sites and α-bungarotoxin-binding sites has been determined in electroplax membranes from *E. electricus* (Bulger & Hess, 1973; Fu et al., 1977; Bulger et al., 1977). The same stoichiometry has recently been observed in *T. californica* membranes (Neubig & Cohen, 1979; Delegeane & McNamee, 1980). Unless data are corrected to take account of the toxin which is bound unspecifically, stoichiometries other than 1:1 for the ratio of binding sites for an activating ligand to those for α-bungarotoxin may be observed (Raftery et al., 1975).

The difference between the results illustrated in Figure 3A and those in Figure 3B, as well as those obtained previously (Hess et al., 1975; Bulger et al., 1977), arises from the use of a correction for unspecific binding in our experiments. In the experiments of Blanchard et al. (1979) (Figure 3A), such a blank was not used, but the slow phase of the reaction was extrapolated to 0 time, and this interpolated value was subtracted from all measurements. Using this procedure, one can notice that the amount of toxin finally bound depends on the initial toxin concentration used, even when this concentration is greater than that of the toxin-binding sites. Such findings indicated that an appropriate reaction blank was not used. The blank obtained by Blanchard et al. (1979) is always larger than the experimentally determined blank (Figure 1). The initial phase of the reaction, which is too fast to be followed by the DEAE filter assay, is thereby subtracted, and the incorrect conclusion is reached that the reaction is monophasic in *Torpedo* membranes. The difference between the experimentally determined blank and the blank obtained by the method of Blanchard et al. (1979) corresponds to the initial fast phase of the toxin reaction. The amplitude of this initial phase of the reaction depends on the initial concentration of toxin in a similar way in *Torpedo* species and *E. electricus* membranes (Figure 1B). In addition, if the reaction is followed for a longer period of time than was the case in the experiments of Blanchard et al. (1979), a third and slower phase is observed (Figure 3B).

It should be noticed from the corrected data in Figure 1B that at low concentrations of toxin the reaction of the toxin with the receptor, in both *E. electricus* and *Torpedo* membranes, proceeds almost completely by the slow phase. In the case of *E. electricus*, this result is predicted by the equations based on the minimum reaction (Hess et al., 1975) (Figure 4). In the case of *T. californica* membranes, this result is in agreement with results obtained in other laboratories (Weiland et al., 1976; Delegeane & McNamee, 1980). At the low concentration of toxin used in these studies, only one phase of the reaction is expected to be observed. Also, the concentrations of toxin-binding sites and receptor sites were nearly equal in these experiments so that the free toxin concentration changed during the measurements. Interpretations of the kinetic measurements of the reaction of toxin with *E. electricus* membranes were facilitated by (i) using an excess concentration of toxin over that of the receptor sites so that the free toxin concentration during the measurements remained

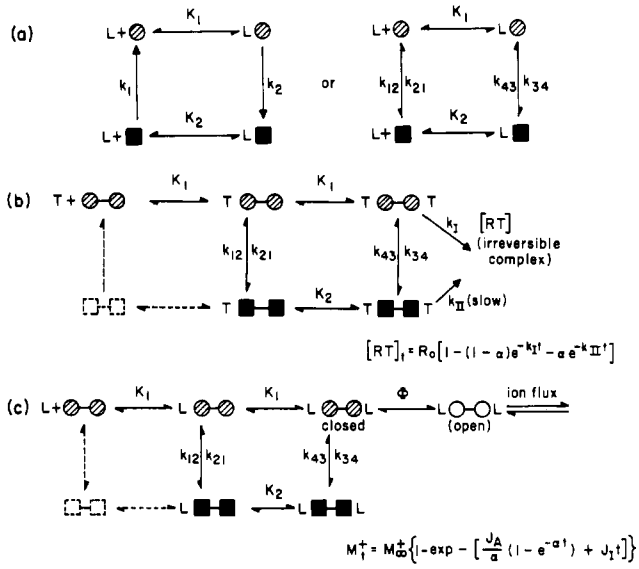

FIGURE 4: Minimum mechanisms which have been proposed to account for properties of the acetylcholine receptor. Forms of the receptor directly involved in its physiological function, the active forms, are indicated by (○) and (⊘) and other forms by (■). Capital K's and Φ are equilibrium constants, and lower case k's are rate constants for receptor inactivation of reaction of the receptor with toxin. J_A and J_I are flux rate constants associated with the active conformation or equilibrium mixtures of active forms, respectively, and α is the observed rate constant for receptor inactivation. L represents acetylcholine or carbamoylcholine concentration. The concentrations of the other reactants are represented as follows: T, α-bungarotoxin; R_0, receptor; [RT], irreversible receptor-toxin complex; M^+, metal ion inside a cell or membrane vesicle. The subscripts t and ∞ indicate the time at which the measurement was made. The dotted receptor forms may exist but are not necessary to account for the measurements. The integrated rate equations which account for the reaction of the receptor over a wide range of toxin concentrations, or for the receptor-controlled ion translocation over a wide range of carbamoylcholine or acetylcholine concentrations, are also shown. The values of the constants pertaining to the various models have been published (Katz & Thesleff, 1957; Bulger et al., 1977; Cash & Hess, 1980; Hess et al., 1980). (a) Mechanism suggested by Katz & Thesleff (1957) on the basis of electrophysical measurements with the neuromuscular junction. (b) Minimum mechanism suggested by Hess et al. (1975) on the basis of kinetic measurements of the reaction of α-bungarotoxin with acetylcholine receptor containing membrane vesicles isolated from the electric organ of *E. electricus*. In the equation defining the concentration of the toxin:receptor complex at time t, α is the fraction of sites which bind α-bungarotoxin slowly. (c) Minimum mechanism suggested on the basis of kinetic measurements of the acetylcholine receptor controlled ion flux in membrane vesicles isolated from the electric organ of *E. electricus* (Cash & Hess, 1980; Aoshima et al., 1980). In the equation defining the concentration of a specific inorganic ion in the vesicles at time t, α is the first-order rate constant for inactivation (desensitization) of the receptor.

essentially constant (Bulger & Hess, 1973; Bulger et al., 1977) and (ii) investigating the reaction over a wide range of initial toxin concentrations (Bulger et al., 1977).

The initial time course of the reaction of toxin with *Torpedo* species membranes is too fast to be measured by presently available techniques, and a detailed analysis of models for this reaction is, therefore, not possible. The important point to be made here is that a comparison of the methods and toxin used by Blanchard et al. (1979) and by us indicates that the toxin reaction with *T. californica* membranes is multiphasic (Figure 1A,B, Figure 3B), is similar to the reaction with *E. electricus* membranes, and does not disprove the existence of cooperative interactions of the *T. californica* receptor with α-bungarotoxin.

Correlations between binding measurements using reversible ligands and kinetic measurements of functional properties of a protein have only been possible when the protein under investigation has been obtained in a high degree of purity (Dixon & Webb, 1979). The receptor, however, constitutes only a small fraction of the total compounds in the membrane preparation, and correction for unspecific ligand binding is a very difficult problem. The results obtained by using different techniques to measure the binding of reversible ligands to the membrane-bound receptor are not yet consistent with each other (Dunn et al., 1980). Kinetic measurements of a specific irreversible reaction are somewhat simpler because as we have demonstrated here and previously (Bulger & Hess, 1973; Bulger et al., 1977; Kohanski et al., 1977) it is possible to correct for reversible unspecific binding. As we have shown here, when the measurements are corrected for unspecific toxin binding, kinetic measurements from a number of laboratories using different membrane preparations give consistent results (Weiland et al., 1976; Delegeane & McNamee, 1980; Blanchard et al., 1979).

It is instructive to compare the information obtained from kinetic measurements of the toxin reaction with that obtained by measuring the effect of acetylcholine concentration on the electrical response of muscle cells and on the receptor-controlled ion translocation in *E. electricus* membrane vesicles. The minimum mechanisms which have been proposed to account for various measurements of the properties of the acetylcholine receptor are presented in Figure 4. Conformational changes that regulate the function of the acetylcholine receptor, triggered by the interaction of ligands with the receptor, were first suggested over 20 years ago (Nachmansohn, 1955; Katz & Thesleff, 1957). The general features of the binding mechanism suggested by Katz & Thesleff (1957), on the basis of electrophysiological measurements with the frog neuromuscular junction (Figure 4a), have been retained by most later models. Kinetic investigations of the reaction of the toxin with receptor from *E. electricus* led to a model which required the binding of two ligands before significant formation of irreversible complexes occurred (Figure 4b) (Hess et al., 1975; Bulger et al., 1977). Quench-flow measurements in the millisecond to second time region of the receptor-controlled ion translocation in *E. electricus* membrane vesicles activated by carbamoylcholine (Cash & Hess, 1980) or acetylcholine (Hess et al., 1980; Cash et al., 1980) led to a model similar to that deduced from the toxin reaction (Figure 4c). The only evidence which was incompatible with these models appeared to be the reaction of the toxin with *T. californica* membranes. A multiphasic kinetic behavior for the interaction of α-bungarotoxin with *T. californica* acetylcholine receptor is apparent from the results of this present study, in agreement with similar studies with *E. electricus* membranes (Hess et al., 1975; Bulger et al., 1977) and rat diaphragm muscle (Brockes & Hall, 1975). The apparent differences which have been reported in the literature arose from the design of the experiments in which *T. californica* membranes were used.

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Protein Involvement in Structural Transitions of Erythrocyte Ghosts. Use of Thermal Gel Analysis To Detect Protein Aggregation†

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ABSTRACT: In this study, it is shown that systematic temperature-induced protein aggregation occurs on the erythrocyte membrane by intermolecular disulfide bond formation. Specific protein bands disappear from acrylamide gel profiles over rather narrow temperature regions. The aggregation appears to be the result of irreversible structural transitions of the membrane, which can be seen in a sensitive scanning calorimeter. When this method of thermal gel analysis is used, the results suggest that spectrin is a participant in the A transition, that bands 2.1, 4.1, and 4.2 and the cytoplasm portion of 3 are involved in the B transition, and that the transmembrane portion of band 3 may undergo changes in the C transition, previously shown to occur in the anion transport domain of the membrane. The aggregation of specific proteins in the narrow temperature region of these transitions persists as the transitions are moved around on the temperature axis by varying solution conditions. The assignment of particular proteins to specific transitions is reinforced by selective extraction of membrane proteins. Large variations in both the calorimetry and the aggregation pattern occur as salt concentration is increased from 77 mosm to 310 mosm, which is manifested in the splitting of the B transition into two separate transitions, B_1 and B_2. It is speculated that this occurs as the result of a structural change which may involve components of the cytoskeletal network.

When an ultrasensitive scanning calorimeter is used, five distinct thermal transitions can be seen to occur in human erythrocyte membranes over the temperature region 45-80 °C (Jackson et al., 1973; Brandts et al., 1978). Each of these transitions presumably occurs as the result of "melting" or disorganization of a cooperative domain on the membrane. One of these transitions (the A transition) was previously shown (Brandts et al., 1977) to be due to the partial unfolding of the spectrin complex and results in the loss of the characteristic biconcave shape of the ghosts. Another (the C transition) is now known to occur in the membrane domain which

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