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Inactivation (Desensitization) of the Acetylcholine Receptor in *Electrophorus electricus* Membrane Vesicles by Carbamylcholine: Comparison between Ion Flux and α-Bungarotoxin Binding

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Summary. The inactivation (desensitization) of the acetylcholine receptor by carbamylcholine, a stable analogue of acetylcholine, has been investigated in eel Ringer's solution, pH 7.0, 0 °C, by measurements of (i) ion flux and (ii) the kinetics of the reaction of [^{125}I]-α-bungarotoxin with the receptor. The effect of preincubation with carbamylcholine is significantly different in the two types of measurement. In both the receptor-controlled flux of inorganic ions and the toxin-binding kinetics a biphasic process has been observed (Hess, G.P., Lipkowitz, S., Struve, G.E., 1978, *Proc. Nat. Acad. Sci. USA* **75**:1703; Hess, G.P. et al., 1975, *Biochem. Biophys. Res. Commun.* **64**:1018; Bulger, J.E. et al., 1977, *Biochemistry* **16**:684), only the initial fast phase of which is inhibited and the subsequent slow phase persists. However, preincubation with carbamylcholine *per se* has no effect on the toxin reaction. The results obtained are consistent with the proposal of Katz and Thesleff (Katz, B., Thesleff, S., 1957, *J. Physiol. (London)* **138**:65) that the active form of the receptor is converted to an inactive form in the presence of acetylcholine receptor ligands, and with our previous experiments (Hess et al., 1978) which indicated that one receptor form is responsible for the initial fast phase of both the receptor-controlled ion flux and the toxin binding reaction, and that its conversion to the other form results in the slow phases in these two measurements.

Acetylcholine receptor-mediated changes in the transmembrane voltage of nerve and muscle, and the cells from the electric organ of *Electrophorus electricus* (Lester, Changeux & Sheridan, 1975; Del Castillo & Webb, 1977), often last less than 1 sec, even though acetylcholine, or its stable analogue carbamylcholine, which initiated these changes is still present (Katz & Thesleff, 1957). This phenomenon has been ascribed to inactivation (desensitization) of the receptor by ligand. The molecular basis of receptor inactivation is believed to involve the conversion of an active form of the receptor to an inactive form (Katz & Thesleff, 1957).

Desensitization has recently been investigated *in vitro* with membrane vesicles, prepared from the electric organs of *Torpedo* or *E. electricus*, in which acetylcholine receptor-controlled fluxes of inorganic ions could be observed (Sugiyama, Popot & Changeux, 1976; Kasai & Changeux, 1971*a–c*). In experiments with *Torpedo* vesicles, preincubation with carbamylcholine inhibits the receptor-controlled fluxes of inorganic ions (Sugiyama et al., 1976) and the binding of specific snake neurotoxins, to the receptor (Weiland et al., 1977; Barrantes, 1978; Quast et al., 1978). In eel vesicles, preincubation of the eel vesicles with carbamylcholine showed no apparent effect on the receptor-controlled fluxes of inorganic ions (Kasai & Changeux, 1971*a–c*). We have now discovered the reason for the apparent discrepancies between electrophysiological experiments with eel electroplax cells and ion flux experiments with membrane vesicles prepared from eel electroplax and from *T. californica*. Preincubation of *Torpedo* vesicles (Weiland et al., 1977; Barrantes, 1978; Quast et al., 1978) with carbamylcholine affects the kinetics of the reaction of α-bungarotoxin with the receptor. We confirm these results but show that under the same conditions, preincubation of eel vesicles with carbamylcholine has no effect on the toxin reaction. We show the consistency between these results and previous kinetic investigations of the reaction of α-bungarotoxin with the

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receptor in eel vesicles (Hess et al., 1975*b*; Bulger et al., 1977) and of the receptor-controlled fluxes of inorganic ions from the same vesicles (Hess et al., 1978). In both the receptor-controlled flux of inorganic ions and the toxin kinetics, a biphasic process has been observed (Hess et al., 1975*b*, 1978; Bulger et al., 1977), only the initial fast phase of which is inhibited and the subsequent slow phase persists.

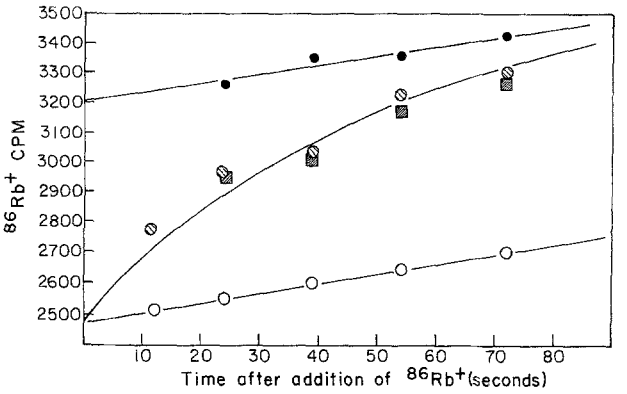

Fig. 1. Effect of preincubation with 1 mM carbamylcholine on the acetylcholine receptor-controlled $^{86}Rb^+$ flux in *E. electricus* electroplax membrane vesicles, pH 7.0, 0 °C. (○) $^{86}Rb^+$ (100 μCi/ml) was added to the vesicles; (●) carbamylcholine and $^{86}Rb^+$ were added simultaneously to the vesicles; (⊗), (▨) the vesicles were preincubated with carbamylcholine for 10 sec or 10 min before addition of $^{86}Rb^+$ (100 μCi/ml). The data points represent the average of 2 measurements. The line connecting the shaded points (⊗, ▨) was computed on the basis that the influx follows a single exponential rate law with a rate coefficient of 0.03 sec^{-1}.

Materials and Methods

Electrophorus electricus and *Torpedo californica* were obtained live from World Wide Scientific Animals and Pacific Bio-Marine, respectively. Carbamylcholine chloride and $^{86}RbCl$ were obtained from Aldrich Chemical Co. and New England Nuclear, respectively. D-Tubocurarine chloride was obtained from Sigma.

Acetylcholine receptor-containing membrane vesicles were prepared from freshly killed animals. *E. electricus* membrane vesicles were isolated as described by Kasai and Changeux (1971*a–c*) with modifications described by Fu et al. (1977). *T. californica* membrane vesicles were isolated as described by Sobel (Sobel, Weber & Changeux, 1977), except that the discontinuous sucrose gradient was centrifuged in a Beckman SW 27 rotor for 17 hr at 25,000 rpm. Measurements were carried out on membrane vesicles resuspended in and equilibrated with: 90 mM KCl, 10 mM NaCl, 1 mM sodium phosphate, pH 7.0 (buffer I); or 169 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.5 mM sodium phosphate, pH 7.0 (buffer II or eel Ringer's solution, *see* references (Keynes & Martins-Ferreira, 1953; Hess et al., 1978). Protein was assayed according to the procedure of Lowry et al. (1951), with bovine serum albumin as the standard.

α-Bungarotoxin was purified from *Bungarus multicinctus* venom obtained from the Miami Serpentarium. The purified α-bungarotoxin was isolated as previously reported (Lee et al., 1972) with a modification of the rechromatography on CM-cellulose CM-52 (Whatman). The rechromatography on a CM-cellulose CM-52 column (1.65 × 40 cm) equilibrated in 0.05 M ammonium acetate, pH 5.0, was performed by elution with a linear gradient of 500 ml of 0.05 M ammonium acetate, pH 5.0, and 0.25 M ammonium acetate, pH 5.8. Fractions of 5 ml were collected and their absorbance at 280 nm and conductivity measured. The major peak containing α-bungarotoxin was eluted with 0.15 M ammonium acetate, and the corresponding fractions were pooled, lyophilized, and relyophilized to a constant weight. 94 mg α-bungarotoxin were applied to the column and gave 66.4 mg after the third lyophilization. Subsequent iodination was performed by using lactoperoxidase to catalyze the reactions (Morrison & Bayse, 1970). A sample of α-bungarotoxin (9.6 mg, 1.25 μmol) was dissolved in 1 ml of 10 mM sodium phosphate, pH 6.5 (buffer III). To the stirred α-bungarotoxin solution, additions were made as follows: 33 μl of 0.1 N HCL; 10 mCi $Na^{125}I$ (Amersham, IMS.300, Batch 12AA, shipped in a NaOH solution of pH 8–11) transferred with the aid of a total of 350 μl of buffer III; 66 μl of 0.1 N HCl; 300 μl of 4.15 mM KI; and 500 μl of a solution of 1.1 mg lactoperoxidase (Sigma, # 1–2005, Lot # 36C–9560) in 2.75 ml of buffer III. To 50 ml of buffer III were added 50 μl of 30% H_2O_2. The reaction was initiated by addition of 200 μl of this solution. After 3 min, the reaction was quenched by addition of 30 μl of 50 mM sodium persulfate. The reaction mixture was then chromatographed on CM-cellulose CM-52 (Bulger et al., 1977) to separate the monoiodinated [^{125}I]-α-bungarotoxin from the unreacted and diiodinated material. The monoiodinated [^{125}I]-α-bungarotoxin was obtained by pooling the appropriate fractions, lyophilization, desalting on a Sephadex G-50 (Pharmacia) column (1.61 × 40 cm) in 10 mM ammonium acetate, pH 6.5, and relyophilization to a constant weight. The monoiodinated [^{125}I]-α-bungarotoxin had a specific activity of 1.65 mCi (61.1 MBq)/mg or 16,000 cpm/pmol when counted on a Beckman Biogamma with 55.2% efficiency. The methods used to demonstrate that the material was the mono-[^{125}I]-α-bungarotoxin have been reported (Bulger et al., 1977).

The binding of [^{125}I]-α-bungarotoxin to the acetylcholine receptor was measured by the method of Kohanski et al. (1977), except that the CM-cellulose minicolumns were equilibrated with 1 mM sodium phosphate, pH 7.2. The concentration of α-bungarotoxin binding sites in the experiments was 50 nM for *E. electricus* vesicles and 100 nM for *T. californica* vesicles. The concentration of [^{125}I]-α-bungarotoxin was 500 nM in all cases. The vesicles were either mixed simultaneously with the [^{125}I]-α-bungarotoxin and carbamylcholine (control) or preincubated with carbamylcholine for predetermined times prior to addition of [^{125}I]-α-bungarotoxin. The background of unspecifically bound toxin was measured by incubating the vesicles with 5 μM α-bungarotoxin for 60 min prior to the addition of the [^{125}I]-α-bungarotoxin. All reactions were carried out at 0°. After addition of [^{125}I]-α-bungarotoxin to each vesicle suspension, aliquots were removed at various times and the extent of toxin binding determined using the CM-cellulose minicolumns with elution with 2.2 ml of 1 mM sodium phosphate, pH 7.2. Experiments with *E. electricus* vesicles required dilution of the aliquots into 5 μM α-bungarotoxin in 1 mM sodium phosphate, pH 7.2 prior to the column assay.

Before the start of the ion flux experiments the vesicles (1 mg membrane protein/ml) were equilibrated with buffer II (eel Ringer's solution) overnight. The influx was stopped at various times (Fig. 1) after the addition of $^{86}Rb^+$ by diluting the vesicles sixfold to give a final concentration of 10 mM D-tubocurarine chloride in buffer II. After 20 min aliquots (80 μg membrane protein) were removed for determination of the $^{86}Rb^+$ content of the vesicles using a Millipore filter assay (Kasai & Changeux, 1971*a–c*; Hess et al., 1975*b*).

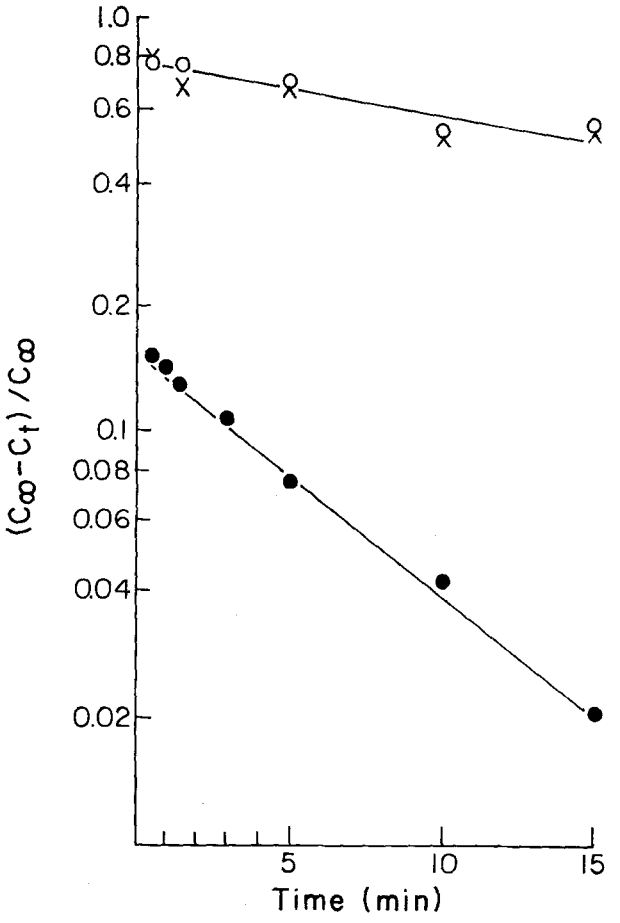

Fig. 2. Time course of [^{125}I]-α-bungarotoxin/receptor complex formation at 0 °C. *E. electricus* vesicles (50 nM binding sites, 10 pmol sites/mg protein, 5.0 mg protein/ml) in buffer II (eel Ringer's solution: 169 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.5 mM sodium phosphate, pH 7.0; *see* Keynes & Martins-Ferreira, 1953) with addition of: (●) 500 nM [^{125}I]-α-bungarotoxin (1.65 mCi/mg toxin protein); (○) 500 nM [^{125}I]-α-bungarotoxin and 1 mM carbamylcholine added simultaneously; and (x) 1 mM carbamylcholine for 10 min and then 500 nM [^{125}I]-α-bungarotoxin.

Results

The graph in Fig. 1 shows acetylcholine receptor-controlled flux of $^{86}Rb^+$ into electroplax membrane vesicles. The solid symbols (●) represent the influx in presence of 1 mM carbamylcholine and the open symbols (○) the influx in absence of carbamylcholine. The vesicles were completely filled within the time resolution of the Millipore filter assay, ~10 sec. The shaded symbols refer to an experiment in which the vesicles were preincubated with 1 mM carbamylcholine for 10 sec (◍) and 10 min (▨). After this preincubation $^{86}Rb^+$ influx was allowed to proceed in the presence of 1 mM carbamylcholine for the times indicated on the abscissa. In both experiments, half of the influx occurred in about 25 sec. The final concentration of $^{86}Rb^+$ in the vesicles was the same as in the experiments in which the vesicles were not preincubated with carbamylcholine.

Figure 2 shows the effects of preincubating the vesicles with 1.0 mM carbamylcholine on the rates of [^{125}I]-α-bungarotoxin binding. The solid symbols refer to experiments in which carbamylcholine was omitted from the solution. In agreement with previous experiments (Hess et al., 1975*b*; Bulger et al., 1977), in which an entirely different method was used to determine the concentration of bound [^{125}I]-α-bungarotoxin, the reaction is biphasic where an initial rapid phase is followed by a slower second phase. The upper line in the figure pertains to α-bungarotoxin binding experiments in presence of 1 mM carbamylcholine. In the experiments indicated by the open symbols, [^{125}I]-α-bungarotoxin and carbamylcholine were added simultaneously. In the experiments indicated by crosses, the vesicles were preincubated with 1 mM carbamylcholine for 10 min before addition of [^{125}I]-α-bungarotoxin. Carbamylcholine drastically affects the initial fast phase of the reaction, which is essentially abolished. It also affects the slow phase of the reaction, consistent with earlier reports that carbamylcholine is a noncompetitive inhibitor of this phase (Hess et al., 1975*b*; Bulger et al., 1977; Bulger & Hess, 1973). Preincubation of the membrane vesicles with carbamylcholine, however, has no additional effect on the reaction (Fig. 2).

We have repeated the toxin binding experiments with *Torpedo* vesicles using the same experimental conditions shown in Fig. 2 and observe that preincubation of the vesicles with carbamylcholine affects the progress curve of the reaction as previously reported (Weiland et al., 1977; Barrantes, 1978; Quast et al., 1978). The differences observed in these experiments are therefore not due to the toxin preparations or the assay technique, but are due to different properties of the receptor in the two membrane preparations.

Discussion

The simple model shown in Fig. 3 is based on: (i) electrophysiological experiments of Katz and Thesleff (1957) which indicated that the receptor exists in two states, an active form (△) which is converted in the presence of receptor ligand to an inactive form (□); or (ii) measurements of receptor-controlled fluxes of inorganic ions with vesicles which suggested that the interconversion between receptor forms does not prevent receptor-controlled fluxes but merely decreases the efficiency of the process (Hess et al., 1978). The model predicts the results shown in Fig. 1: a very fast flux, which is complete within the time resolution of the assay (~10 sec), is converted to a slow

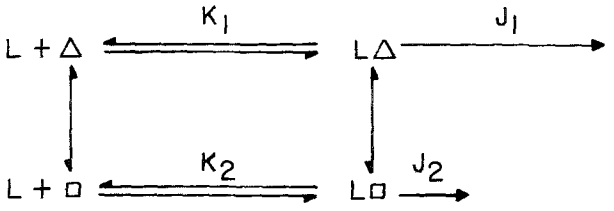

Fig. 3. Minimum mechanism for acetylcholine receptor-controlled ion flux. The conformations referred to as active and inactive by Katz and Thesleff (1957) on the basis of electrophysiological experiments are represented by △ and □, respectively. J is the rate coefficient for ion fluxes associated with the different receptor conformations (△ and □).

flux which is complete within about 1 min when the receptor is preincubated with 1 mM carbamylcholine for 10 sec or 10 min. According to the results shown in Fig. 1, the conversion of the active state of the receptor to the inactive form occurs within the time resolution of the Millipore filter assay (~ 10 sec). The same results are obtained after 10 sec of preincubation with 1 mM carbamylcholine as after 10 min.

The results (Fig. 1) indicate also why desensitization has not been detected in efflux experiments with eel vesicles previously equilibrated with radioactive ions (Kasai & Changeux, 1971 *a–c*). It has been shown (Hess et al., 1975*b*; 1976) that in these preparations the fluxes are of two types. (i) About 85% of the flux is unspecific in that it is not in response to carbamylcholine. The time taken for half of the radioactive ions inside these vesicles to exchange with nonradioactive ions in the external solution, $t_{1/2}$, is on the average 20 min. (ii) About 15% of the flux comes from specific vesicles which do respond to carbamylcholine. We shall refer to this 15% value as the amplitude of the specific flux. In the presence of 1 mM carbamylcholine the whole exchange of inorganic ions across the membrane of the specific vesicles occurs in less than 10 sec (Hess, 1979), has a $t_{1/2}$ value of 25 sec when the receptor is desensitized (Fig. 1), and a $t_{1/2}$ value of about 6 hr in absence of carbamylcholine (Hess & Andrews, 1977). (iii) In earlier experments (Kasai & Changeux, 1971 *a–c*), the $t_{1/2}$ value for the efflux from the whole vesicle population, τ, has been used to assess receptor-controlled fluxes. In the absence of carbamylcholine the specific vesicles do not contribute to the efflux ($t_{1/2} \sim 20$ min). (iv) In the presence of 1 mM carbamylcholine τ will depend mainly on the amplitude of the specific flux. Within less than 10 sec. 15% of the total inorganic ions in the vesicle population has effluxed from the specific vesicles. τ is now determined by the time it takes for the nonspecific vesicles to lose an amount of radioactive ions which correspond to 35% (50%–15%) of the radioactive ions in the total vesicle population. From the $t_{1/2}$ for the efflux from unspecific vesicles one can calculate a value of about 8 min (Hess et al., 1975*b*; 1976) and such values are generally observed with our vesicle preparation. The amplitude of the specific flux is not changed by pre-incubating the vesicles with carbamylcholine (Fig. 1), and the efflux from these vesicles is 60 times faster than the efflux from the unspecific vesicles. Therefore, the τ value is again seen to be determined by the amplitude of the specific flux and the $t_{1/2}$ value of the efflux from unspecific vesicles. Therefore, preincubation of the vesicles with carbamylcholine will have only a small effect on τ, and desensitization of receptor-controlled fluxes may not be observed with eel vesicles. In contrast to these results, experiments with *Torpedo* vesicles indicate that inhibition or desensitization of the receptor decreases the amplitude of the specific flux (Eldefrawi et al., 1978; Bernhardt & Neumann, 1978). Therefore desensitization can be observed with *Torpedo* vesicles even when the experiments are done with a mixture of specific and unspecific vesicles (Sugiyama et al., 1976; Kasai & Changeux, 1971 *a–c*).

Our interpretation of the experiments in Fig. 2 depends on the following points. (i) The data in Fig. 2 indicate that the main effect of carbamylcholine on the toxin reaction is to abolish the initial fast phase, but that the slow phase persists. Preincubation with carbamylcholine has, therefore, the same effect on the kinetics of the toxin reaction as it has on the kinetics of inorganic ion flux, suggesting that the slow phase of the reaction in both cases is associated with the inactivated state of the receptor. (ii) The interconversion of receptor conformations as measured by the toxin reaction at the toxin concentration used has a $t_{1/2}$ value of about 2 min (Bulger et al., 1977) and is therefore much slower than the interconversion of receptor conformations as measured by receptor-controlled fluxes induced by 1 mM carbamylcholine ($t_{1/2} < 10$ sec. Fig. 1). (iii) In the accessible range of toxin concentrations, the dissociation constant of the receptor/toxin complex pertaining to the initial fast phase of the toxin reaction is too large to measure (Hess et al., 1976; Bulger et al., 1977). The dissociation constant of the receptor/carbamylcholine complex as determined in flux measurements is much lower (Hess et al., 1975*b*). When carbamylcholine and α-bungarotoxin are added simultaneously to the vesicles, the kinetics of the interconversion is therefore expected to be determined by the rate of interconversion of the receptor/carbamylcholine complex rather than of the receptor/toxin complex, and occurs in a time interval too short to be measured by the technique used. Therefore, preincubation of the vesicles with carbamylcholine appears to have no effect other than that observed when carbamylcholine and toxin are added simultaneously to the vesicles.

These data support our previous suggestion (Hess et al., 1978; Hess, 1979) that a two-state model (Fig. 3) can account for both the toxin reaction and the kinetics of the receptor-controlled fluxes of inorganic ions. (i) The active conformation of the receptor is responsible for both the fast phase of the receptor-controlled flux of inorganic ions and the fast phase of the toxin reaction. (ii) The interconversion of receptor forms induced by either carbamylcholine or toxin occurs with significantly different rates. (iii) The inactive form of the receptor is associated with the slow phase of both the flux and the toxin binding process. With the assumption that the receptor-controlled fluxes associated with the inactive form of the receptor are of comparable magnitude to the nonreceptor-controlled flux rates of the cell, and therefore do not perturb the transmembrane potential, the model also accounts for the *in vivo* electrophysiological measurements (Katz & Thesleff, 1957).

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