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Mechanism of Inactivation (Desensitization) of Acetylcholine Receptor. Investigations by Fast Reaction Techniques with Membrane Vesicles†

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ABSTRACT: Exposure of the acetylcholine receptor to acetylcholine, or its stable analogue carbamylcholine, inactivates (desensitizes) the receptor. Inactivation of receptor-controlled ion ($^{86}Rb^+$) flux in the presence of different concentrations of carbamylcholine (12.5 μM to 28 mM) was measured in the millisecond to minute time region, using a quench flow technique and membrane vesicles prepared from the electric organ of *Electrophorus electricus*. Three different kinetic measurements were made to establish the relationship between carbamylcholine concentration and the ion translocation process: (i) the rate of inactivation of the ion translocation process; (ii) the rate of recovery of the inactivated receptor upon removal of carbamylcholine; and (iii) the rate of the ion flux mediated by equilibrium mixtures of active and inactive receptor forms. The kinetics of these three processes follow single-exponential rate laws, and simple analytical expressions for their ligand concentration dependence could be used. Therefore, it was possible to determine the value of the rate constants in a scheme relating the ligand binding steps to ion translocation, and to predict the dependence of these rate constants on carbamylcholine concentration over the 200-fold range investigated.

Electrical signals play an important role in the function of nervous systems and in muscle contraction. Such signals are determined by the concentrations of inorganic ions on both sides of a cellular membrane and by the rates with which specific inorganic ions move through the membrane (Hodgkin & Huxley, 1952). The rates of the ion movements through the membranes are controlled by membrane-bound proteins, receptors, and are determined by the concentration of specific small molecules which bind to these receptors (Nachmansohn & Neumann, 1975). The important relationship between the ligand binding processes and the transmembrane ion fluxes, which determine the amplitude and duration of electrical signals and thereby the transfer of information between cells, is not fully understood. Determining this relationship for the acetylcholine receptor is the purpose of the experiments described.

Measurements of electrical signals controlled by the acetylcholine receptor indicated that the ligand binding process may be complex (Katz & Thesleff, 1957). Addition of acetylcholine or carbamylcholine to muscle cells produces an electrical signal which subsequently disappears even when the concentration of the ligand remains constant. Two different ligand-induced conformational changes of the receptor have been proposed to account for these observations: (i) Nachmansohn (1952, 1955) suggested that a conformational change which results in channel opening causes the signal, and (ii) Katz & Thesleff (1957) suggested a subsequent conformational change which leads to inactivation (desensitization) of the receptor and disappearance of the signal. More recently, evidence for the complexity of the ligand binding mechanism and of the ligand-induced isomerization of receptor forms has come from two types of studies: (i) the reaction of specific neurotoxins (Chang & Lee, 1963) with receptor-rich membrane preparations from *Electrophorus electricus* (Bulger & Hess, 1973; Bulger et al., 1977; Maelicke et al., 1971; Hess et al., 1975b); (ii) investigations of the binding of various ligands to both the isolated and the membrane-bound receptor (Weiland et al., 1977; Grünhagen & Changeux, 1978; Barrantes, 1978; Heidmann & Changeux, 1978; Rübsamen et al., 1978; Hess, 1979; Quast et al., 1978, 1979; Eldefrawi & Eldefrawi, 1979; Jürss et al., 1979; Dunn et al., 1980). Recently, it has become possible to investigate the correlation between ligand binding processes and the receptor-controlled ion translocation (Hess et al., 1979).

The measurements reported here were made with receptor-rich membrane vesicles, chosen because they allow one to investigate receptor-mediated ion translocation under controlled conditions which are not limited by the metabolic restrictions present in intact cells. The internal and external ion

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‡ Recipient of a Muscular Dystrophy Association Investigator's Fellowship. On leave from Yamaguchi University, Japan.
§ Supported by Center Grant CA 14454 from the National Institutes of Health.

0006-2960/81/0420-3467$01.25/0

concentrations can be varied and accurately known. The number of receptor sites, associated with a definite internal volume of solution and exposed to a known concentration of ligand, can be determined accurately. The vesicles were prepared from membranes of the electric organ of *E. electricus*. The membrane contains acetylcholine receptors which have antigenic properties in common with those of mammalian muscle cells (Patrick & Stallcup, 1979). Kasai & Changeux (1971) demonstrated receptor-controlled ion flux with a similar preparation in which a small fraction of the vesicles had functional receptors (Hess et al., 1975a,b). Subsequently, techniques for measuring directly and quantitatively the effect of ligand concentration on the receptor-controlled ion translocation process, in vesicles of uniform size and containing functional receptors, were developed (Hess et al., 1975a, 1979; Hess & Andrews, 1977; B. Lenchitz, R. L. Noble, and G. P. Hess, unpublished experiments). Recently, a flow quench method (Fersht & Jakes, 1975) was adapted for measurements of the ion translocation processes in the same vesicles in the millisecond to minute time region (Hess et al., 1979).

In our studies of the effect of carbamylcholine concentration on the receptor-controlled flux (Cash & Hess, 1980; Aoshima et al., 1980), we found that three additional kinetic measurements could be made to establish the relationship between this flux and the ligand binding process. We show that the model previously proposed is supported by these new measurements which we report here: (i) the rate of inactivation (desensitization) of the receptor-controlled ion translocation process, (ii) the rate of recovery of the inactivated receptor, and (iii) the rate of the ion flux mediated by equilibrium mixtures of active and inactive receptor forms. The kinetics of each of these processes is simple and follows a single-exponential rate law. This leads to a simple analytical expression for the concentration dependence of each process. Consequently, it is possible to determine the value of the rate constants in a scheme that relates the ligand binding steps to ion translocation.

Experimental Procedures

The preparation of membrane vesicles from the electric organ of *E. electricus* and the chemicals used have been described in previous papers (Hess et al., 1979). All the experiments were carried out in eel Ringer's solution (169 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 1.5 mM sodium phosphate, pH 7.0) at 1 °C. The vesicles were allowed to equilibrate for at least 12 h with eel Ringer's solution, pH 7.0, before use. Protein concentration was determined by the method of Lowry et al. (1951). The application of the flow quench technique (Fersht & Jakes, 1975) to measurements of inorganic ion flux in membrane vesicles has been described (Hess et al., 1979; Cash & Hess, 1980; Aoshima et al., 1980), including the quenching of the receptor-controlled flux of inorganic ions by *d*-tubocurarine and the determination of the $^{86}Rb^+$ content of the vesicles (Hess et al., 1979; Cash & Hess, 1980).

The rate of receptor inactivation was measured by continuous quench flow for times less than 400 ms and by pulsed quench flow (Fersht & Jakes, 1975) for longer times. The vesicle suspension (800 μg of membrane protein/mL, 0.225 mL) was first mixed with an equal volume of carbamylcholine solution at various concentrations. After incubation (for 57 ms to 20 s), the vesicles were mixed with an equal volume of $^{86}Rb^+$ solution (100 μCi/mL, 0.225 mL) containing carbamylcholine for the influx measurement (for 1.2 s). The carbamylcholine concentration in the influx measurement was at least 5 mM. When the carbamylcholine concentration during incubation was higher than 5 mM, that used in the influx measurement was 20 mM. Two types of control experiments were performed; both followed the same protocol except that carbamylcholine was omitted (i) from all solutions or (ii) from the first incubation.

The recovery of the inactivated receptor was observed by the pulsed quench flow technique. Aliquots (0.11 mL) of the vesicle solution (1.5 mg of membrane protein/mL), 70 μM in carbamylcholine, were incubated for 1 h and diluted 7-fold with eel Ringer's solution. The solutions were then allowed to stand for various periods of time (1–20 s) before being mixed with 0.45 mL of a solution which was 15 mM in carbamylcholine and contained 150 μCi of $^{86}Rb^+$. Influx was allowed to proceed for 1.2 s. In the control experiment, the same protocol was followed, but carbamylcholine was omitted from (i) all solutions or (ii) the first incubation.

The Ion Flux Rate of Inactivated Receptor. The flux of $^{86}Rb^+$ into vesicles after equilibration of receptor with carbamylcholine was measured in out-of-machine experiments. Vesicle suspensions (800 μg of protein/mL) which were equilibrated with different concentrations of carbamylcholine for at least 1 h were then mixed with an equal volume of solution containing $^{86}Rb^+$ (100 μCi/mL) and carbamylcholine at the same concentration as was used during incubation. At appropriate time intervals, 0.5 mL of the solution was mixed with 1.7 mL of 3 mM *d*-tubocurarine. After exactly 15 min, two 1-mL aliquots of the solution were taken. Each aliquot was placed on a Millipore filter (HAWP 025). The filters were washed, and the $^{86}Rb^+$ content of the vesicles was determined as previously described (Hess et al., 1975a). In the control experiment, carbamylcholine was omitted from the solution.

Theory

A scheme which relates the ligand binding processes to receptor-controlled flux rates is shown in Figure 1. It consists of the minimum number of intermediates and constants which are needed to account for the receptor-controlled influx of metal ions over the 200-fold concentration range of carbamylcholine we investigated. The scheme was first suggested on the basis of influx measurements alone (Cash & Hess, 1980). Here, we present three additional measurements to test this model. The rate and equilibrium constants pertaining to the model are defined as follows: $K_1 = 2[A][L]/[AL] = [AL][L]/2[AL_2]$; $K_2 = [IL][L]/2[IL_2]$; $K_{c1} = [\overline{AL}]/[IL] = k_{21}/k_{12}$; $K_{c2} = [\overline{AL_2}]/[IL_2] = k_{43}/k_{34}$; $[\overline{AL_2}]/[\overline{AL_2}] = \Phi$; J_m has the unit of s^{-1} and is the maximum flux rate obtained if all the receptor is converted to the open-channel form. The species symbols are taken from the scheme (Figure 1).

With the assumption that the structural transition corresponding to inactivation is slow compared to the ligand binding steps and channel opening, the fractions of the receptor in the open-channel form induced by ligand, $[\overline{AL_2}]_0$, after time t, $[\overline{AL_2}]_t$, and when equilibrium is reached, $[\overline{AL_2}]_\infty$, are given by eq 1–3. L represents the initial concentration of ligand, and it is assumed that, under the conditions used, the changes in free ligand concentration during equilibration are negligible.

$$[AL_2]_0 = \frac{L^2}{L^2(1 + \Phi) + L(2K_1)\Phi + K_1^2\Phi} \quad (1)$$

$$[AL_2]_\infty = \frac{L^2K_{c2}}{K_{c2}[L^2(1 + \Phi) + L(2K_1)\Phi + K_1^2\Phi] + \Phi[L^2 + L(2K_2)]} \quad (2)$$

$$[AL_2]_t - [AL_2]_\infty = ([AL_2]_0 - [AL_2]_\infty)e^{-\alpha t} \quad (3)$$

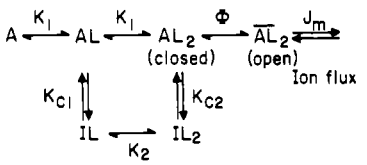

FIGURE 1: Model which relates the ligand binding processes to the receptor-controlled ion translocation process. The model consists of the minimum number of intermediates and constants needed to account for the effect of different carbamylcholine concentrations on the following measurements: (i) the rate of receptor inactivation, (ii) the rate of recovery of the inactivated receptor, (iii) the flux rate mediated by the equilibrium concentration of active receptor, and (iv) the flux rate mediated by the initial concentrations of active receptor. Three receptor states are involved: an active form, A; an open-channel form, $\bar{A}$; and an inactive receptor form, I. Two microscopic ligand dissociation constants are involved, K_1 pertaining to the A state, and K_2 pertaining to the I state. Two conformational equilibrium constants are required, pertaining to the equilibria between the A and I states, K_c, and one, Φ, pertaining to the equilibrium between the closed (A) and open-channel ($\bar{A}$) active forms. Conversions between active and inactive forms occur with first-order rate constants, k. The method adopted to arrive at the model consists of accounting for experimental results in terms of the minimum number of intermediates and of using the minimum number of constants (Cash & Hess, 1980). The following assumptions were made: (1) Ligand binding and channel-opening processes are fast compared to ion flux and receptor inactivation. (2) Ligand binding sites of the active conformation are identical. The model incorporates the following observations: (i) The effect of carbamylcholine over a 200-fold concentration range on the observed influx rate coefficient, $J_m[\overline{AL}_2]_o$, indicates the requirement that two (only) ligand molecules be bound before channel opening occurs. (ii) The maximum value of $J_m[\overline{AL}_2]_o$ and the observed cooperativity of ion flux are greater in the presence of acetylcholine than of carbamylcholine (Hess et al., 1980), suggesting that the channel-opening equilibrium constant, $1/\Phi$, is responsible for both phenomena. (iii) The value of α (Figure 2d) reaches a maximum value, α_{max}, at a concentration at which $J_m[\overline{AL}_2]_o$ has not yet reached its maximum value, indicating that the receptor is inactivated when only one carbamylcholine molecule is bound to the active form, while channel opening requires two bound ligand molecules. (iv) Increasing the carbamylcholine concentration beyond that required to produce α_{max} does not result in a decrease in the inactivation rate, indicating that AL_1 and AL_2 are inactivated with comparable rates. (v) By consideration of the large difference between the rate coefficients associated with the active form ($J_m[\overline{AL}_2]_o$) and equilibrium mixtures of active and inactive receptor forms ($J_m[\overline{AL}_2]_\infty$), the reactivation rate (Figure 4) is large compared to the inactivation rate (Table I). This is accommodated by reactivation involving conversion between IL and AL (rather than between IL_2 and AL_2) and requires that on losing a ligand molecule IL_2 is directly converted to IL (rather than via AL).

The active form of the receptor, A, is converted to the inactive form, I, with a first-order rate constant for inactivation, α.

$$\alpha = \frac{k_{43}L + k_{21}2K_2}{L + 2K_2} + \Phi\left(\frac{k_{34}L^2 + k_{12}L(2K_1)}{L^2(1+\Phi) + L(2K_1)\Phi + K_1^2\Phi}\right) \quad (4)$$

The rate equation for the receptor-controlled influx of metal ions, M, into vesicles is (Hess et al., 1975)

$$\frac{d[M]}{dt} = J_m[AL_2]_t([M]_o - [M]) \quad (5)$$

[M] represents the metal ion concentration inside the membrane vesicles, and $[M]_o$ denotes the metal ion concentration outside the membrane vesicles. Substitution of eq 3 into eq 5 and integration give eq 6, where the subscripts refer to the time of measurements.

The term α can be obtained by use of eq 3 since the relative concentrations of the species $\overline{AL}_2$ can be obtained by the procedure outlined below. The following comments are pertinent to the procedures used: (i) After incubation with carbamylcholine, influx is measured in the presence of high concentrations of carbamylcholine. The data in Figure 2b indicate that, at these high concentrations of carbamylcholine ($[\overline{AL}_2]_o \gg [\overline{AL}_2]_\infty$), the terms containing $[\overline{AL}_2]_\infty$ in eq 6 can be neglected. (ii) After various periods of incubation with different concentrations of ligand, influx is measured during a constant period of time so that eq 6 can be written as eq 7

$$-\ln\left(\frac{[M]_\infty - [M]_t}{[M]_\infty}\right) = J_m\left\{([\overline{AL}_2]_o - [\overline{AL}_2]_\infty)\frac{1 - e^{-\alpha t}}{\alpha} + [\overline{AL}_2]_\infty t\right\} \quad (6)$$

$$[\overline{AL}_2]_t = -\ln\left[\frac{([M]_\infty - [M]_{\text{constant time}})K}{[M]_\infty}\right] \quad (7)$$

where K contains the constants J_m, α, and the time for which influx is allowed to proceed. This time interval is the same in all experiments. $[\overline{AL}_2]_t$ represents the concentration of the active form of the receptor after incubation with carbamylcholine for time t. $[\overline{AL}_2]_t$ after preincubation time, t (in eq 7), is $[\overline{AL}_2]_o$, at the time $^{86}Rb^+$ influx is initiated (eq 6).

The observed rate coefficient characteristic of the equilibrium concentration of the open-channel form, $J_m[\overline{AL}_2]_\infty$, was evaluated (see eq 6) from the following expression:

$$([M]_\infty - [M]_t)([M]_\infty)^{-1} = \exp(-J_m[\overline{AL}_2]_\infty t) \quad (8)$$

The observed rate coefficient characteristic of the receptor before inactivation, $J_m[\overline{AL}]_o$, was evaluated from influx data as has been described previously (Cash & Hess, 1980; Aoshima et al., 1980). From a knowledge of these constants, one can estimate K_2 and K_{c2}:

$$\frac{1}{J_m[\overline{AL}_2]_\infty} - \frac{1}{J_m[\overline{AL}_2]_o} = \frac{\Phi 2K_2}{J_m K_{c2}}\frac{1}{L} + \frac{\Phi}{J_m K_{c2}} \quad (9)$$

Results

Exposure of the receptor to carbamylcholine reduces the rate of transmembrane ion flux mediated by this protein (Figure 2a). This inactivation depends on both the concentration of carbamylcholine and the length of exposure. At the carbamylcholine concentration used, 0.25 mM (▲) (Figure 2a), the initial influx was reduced by one-half in less than 2 s. On prolonged exposure of the receptor to carbamylcholine, the receptor-controlled flux rates decreased to a finite value which was independent of incubation time but was dependent on carbamylcholine concentration (Figure 2b). We conclude, therefore, that the flux rate after incubation is determined by the equilibrium concentration of the open-channel form, $[\overline{AL}_2]_\infty$. Exposure to 50 μM carbamylcholine for 1 h inactivated the receptor by 50%. α was evaluated by plotting the data according to eq 3 (Figure 2c). The relative concentration of $[\overline{AL}_2]_t$, the open-channel form, after incubation with carbamylcholine for a certain period of time, was calculated by using eq 7 and the measurements shown in Figure 2a. The measurements in Figure 2b were used to calculate the relative concentration of $[\overline{AL}_2]_\infty$. It can be seen (Figure 2c) that the inactivation of the receptor-controlled flux follows a first-order rate law for more than 90% of the reaction. The value of α, obtained from the slope of the lines, is 0.9 s^{-1} when the carbamylcholine concentration is 0.25 mM (▲). The values of α in the carbamylcholine concentration range investigated (125

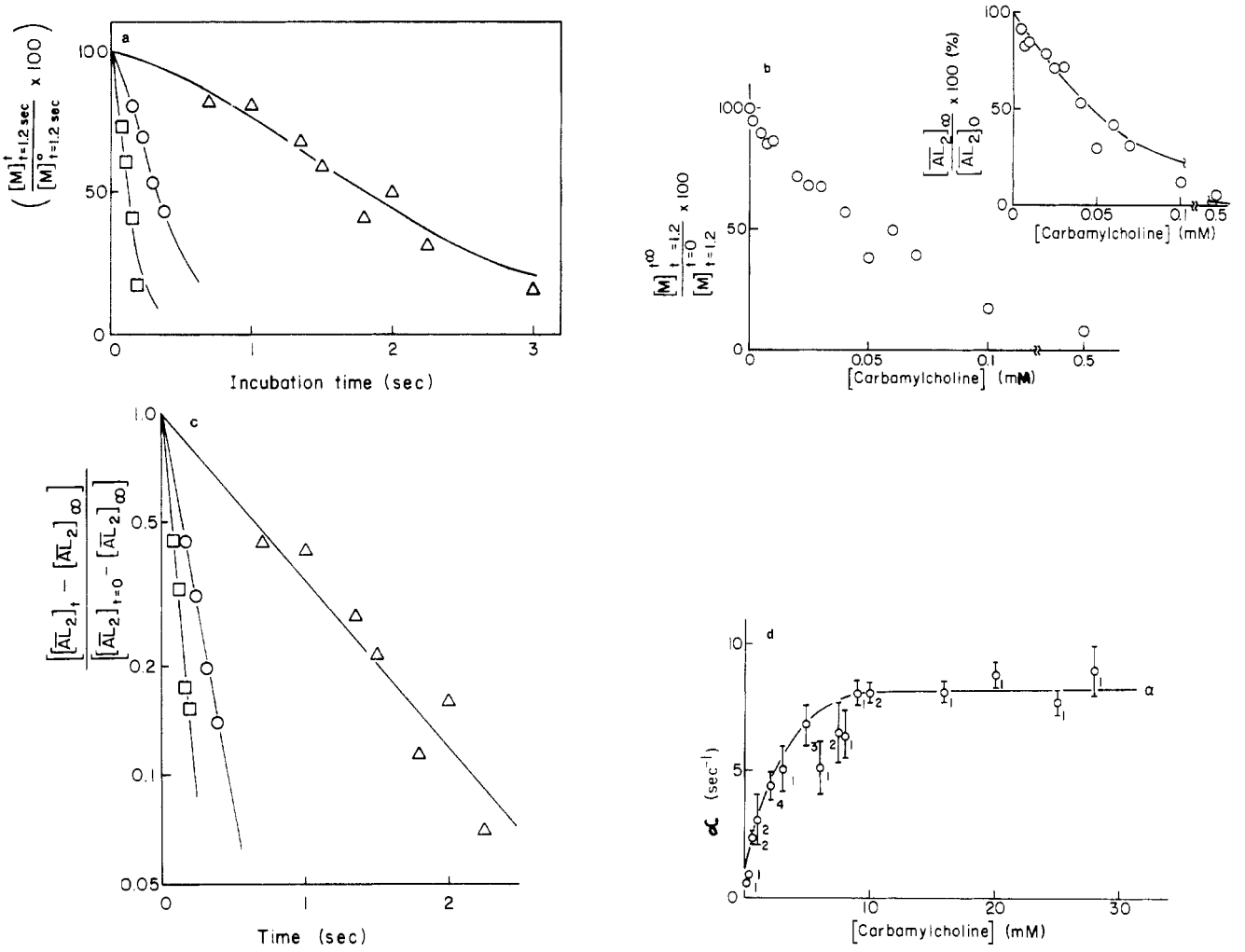

FIGURE 2: Effect of carbamylcholine concentration on the rates of receptor inactivation. $[M]_{t=1.2}^{t}$ refers to the concentration of $^{86}Rb^{+}$ inside the vesicles 1.2 s after influx was initiated by addition of $^{86}Rb^{+}$ and carbamylcholine. The superscript refers to the time of incubation with carbamylcholine. (a) Inhibition of receptor-controlled flux by incubation with carbamylcholine for various periods of time: (△) 0.25, (○) 2, and (□) 10 mM carbamylcholine. Each point is the mean of three determinations. The solid lines were calculated with eq 3 and 7 and the values of α (determined in experiments illustrated in Figure 2). (b) Influx due to equilibrium concentrations of the open-channel form of the receptor. The vesicles were incubated with different concentrations of carbamylcholine for 1 h. Each point is the mean of three determinations. Inset: Plot of $[\overline{AL_2}]_\infty/[\overline{AL_2}]_0$ vs. carbamylcholine concentration. The values of $[\overline{AL_2}]$ were calculated from the measurements of $[M]_{t=1.2s}$ by use of eq 7 (see the text). (c) Evaluation of the rate constant for the inactivation reaction. The data shown in Figure 2a,b were plotted according to eq 3. The values of α obtained from the slope of the lines, at the different concentrations of carbamylcholine, are as follows: 0.25 mM carbamylcholine (△), 0.9 ± 0.1 s^{-1}; 2 mM carbamylcholine (○), 4.9 ± 0.1 s^{-1}; 10 mM carbamylcholine (□), 8.8 ± 0.4 s^{-1}. (d) Concentration dependence of α. (○) Each point is the mean of the three determinations made in each experiment. The numbers next to the points refer to the number of experiments. The line was computed by using eq 4 and the values of the constants given in Table I. The experimental errors are given as mean deviations.

μM–28 mM) are shown in Figure 2d. The $t_{1/2}$ values for the inactivation process are in the range 85 ms–1.2 s.

The influx controlled by the equilibrium concentration of the open-channel form of the receptor, $[\overline{AL_2}]_\infty$, is comparatively slow and follows a single-exponential rate law (Aoshima et al., 1980). The observed rate constants for ion flux which are characteristic of $[\overline{AL_2}]_\infty$, determined over a wide range of carbamylcholine concentrations (10 μM–2 mM), are shown in Figure 3. One-half of the maximum rate is obtained at a carbamylcholine concentration of 100 μM. The $t_{1/2}$ values for influx are in the range 46 s–12 min.

After prolonged incubation with 70 μM carbamylcholine, for 1 h in the experiment shown in Figure 4, the inactivated receptor is reconverted to the active form when the carbamylcholine concentration is reduced to 10 μM. This is accomplished by diluting the receptor and carbamylcholine solution 7-fold in the flow quench apparatus. In agreement with the data in Figure 2b, 30% of the receptor is found to be in its active state after exposure to 70 μM carbamylcholine for 1 h (Figure 4, ○). After 8 s in dilution buffer which is 10 μM in carbamylcholine, the relative concentration of the open-channel form reaches a final value which is 85% of that found in control experiments in which the first incubation step, with 70 μM carbamylcholine, was omitted. The agreement of these results with those shown in Figure 2b, which show the equilibrium concentrations of receptor forms as a function of carbamylcholine concentration, indicates that, within the precision of the measurements, the inactivation process is completely reversible. The amount of receptor reactivated was measured as a function of time in the dilution buffer, using the same technique as was used for measuring receptor inactivation. The reactivation process follows a single-exponential rate law, as is shown in Figure 4b. From the slope of the line, an α value of 0.46 s^{-1} is obtained at a carbamylcholine concentration of 10 μM.

Discussion

In general, a correlation of thermodynamic, structural, and

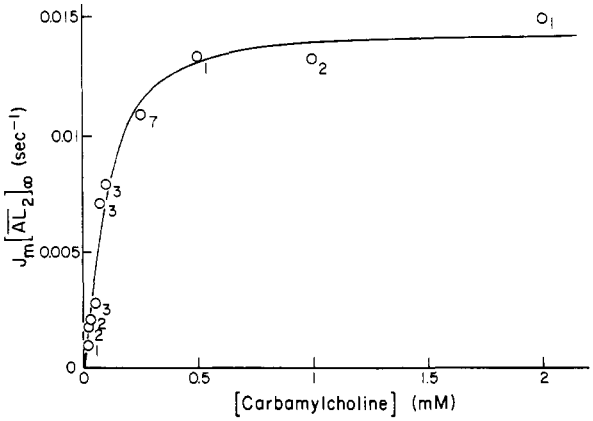

FIGURE 3: Effect of carbamylcholine concentration on the rates of influx mediated by $[\overline{AL_2}]_\infty$, the equilibrium concentration of the open-channel form of the receptor. Each point is the mean of three determinations made for each experimental point. The numbers next to the points reflect the number of experiments. The rate constants were normalized to the mean value obtained at a carbamylcholine concentration of 0.25 mM. The line was computed by use of eq 2 and the values of the constants given in Table I.

kinetic information is required to propose a complete molecular mechanism. Previously, such correlations 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 membrane preparations. In our investigations, we ask a relatively simple question: can we account for the effect of a wide range of carbamylcholine concentrations on the rate of the receptor-controlled ion translocation? The techniques for making these measurements in the physiologically significant millisecond time region have been developed only recently (Hess et al., 1979; Cash & Hess, 1980; Cash et al., 1981).

In earlier studies, we have shown that the integrated rate equation (eq 6) predicts the receptor-controlled ion translocation process over a 200-fold concentration range of carbamylcholine (Cash & Hess, 1980; Aoshima et al., 1980) and a 160-fold acetylcholine concentration range (Hess et al., 1980; Cash et al., 1981). The model in Figure 1 represents the simplest interpretation of our previous influx measurements. Here, we show that eq 6 and the minimum model also account for the effect of carbamylcholine, over a 200-fold concentration range, on three different processes affecting ion translocation: (1) the rates of inactivation of ion translocation as measured by α (eq 4, Figure 2); (2) the rates of ion flux mediated by equilibrium mixtures of active and inactive receptor forms (eq 8, Figure 3); and (3) the rate of recovery of the inactivated receptor upon removal of carbamylcholine (eq 4, Figure 4). The processes measured follow a simple-exponential rate law, making it possible to evaluate the observed rate constants directly. The observed rate constant for the ion translocation process before inactivation, $J_m[\overline{AL_2}]_o$, now becomes the only unknown term in the integrated rate equation (eq 6) which relates the ligand binding steps to ion translocation. This rate coefficient was evaluated by a nonlinear, least-squares fitting computer program for the influx curves which were measured over a 200-fold range of carbamylcholine concentration (Cash & Hess, 1980; Aoshima et al., 1980). From the dependence of $J_m[\overline{AL_2}]_o$ on carbamylcholine concentration, J_m, Φ, and K_1 were evaluated by a procedure which has been described (Aoshima et al., 1980). From the values of $J_m[\overline{AL_2}]_o$ and $J_m[\overline{AL_2}]_\infty$, one can obtain K_2 and K_{c2} (eq 9). From the limiting value of α at high ligand concentration, one obtains k_{34},

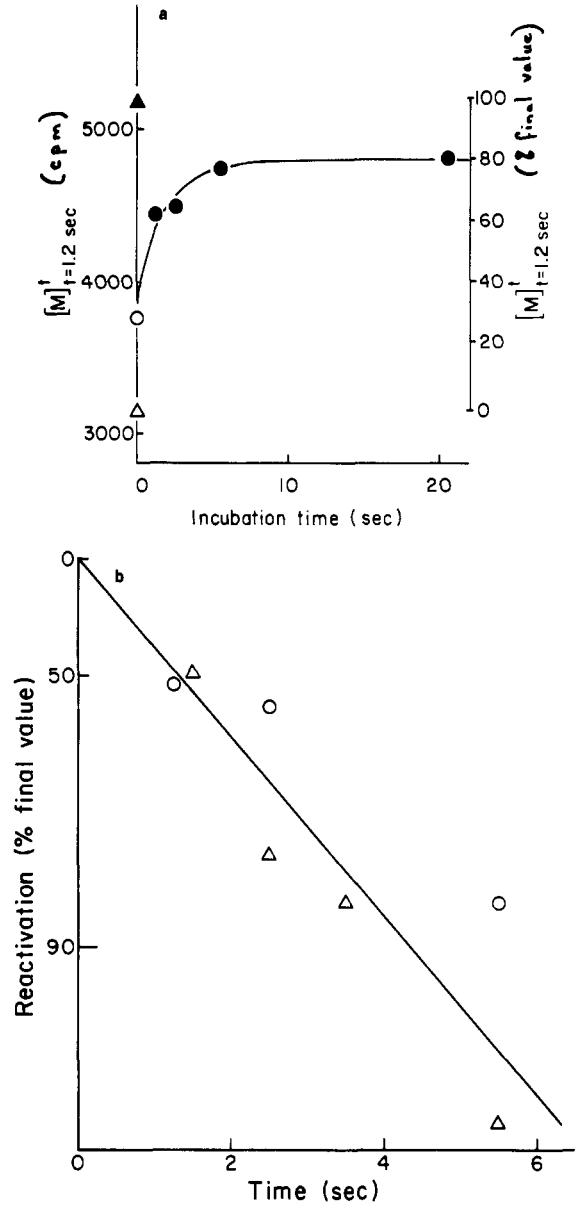

FIGURE 4: Reactivation of the inactivated receptor on decreasing the carbamylcholine concentration from 70 to 10 μM in the flow quench apparatus. (a) The concentration of $^{86}Rb^+$ is given in terms of counts per minute. $[M]_{t=1.2s}$ refers to the concentration of $^{86}Rb^+$ (in terms of counts per minute) inside the vesicles 1.2 s after initiation of influx by addition of tracer and carbamylcholine (5 mM final concentration). Each point is the mean of three determinations. Before the influx measurements were begun, the vesicles were exposed to 70 μM carbamylcholine for 1 h. (●) The vesicles were in the dilution buffer before initiation of influx for the time indicated on the abscissa. The solid line was computed by using an α value of 0.46 s^{-1}. (○) Dilution buffer had the same concentration of carbamylcholine, 70 μM, as the incubation solution. (▲) Vesicles were not exposed to carbamylcholine before addition of $^{86}Rb^+$. (△) Control experiment: influx in the absence of carbamylcholine. (b) Evaluation of α by measuring the rate of reactivation. The data shown in the figure were plotted on a semilogarithmic scale according to eq 3. Percent reactivation corresponds to $[([\overline{AL_2}]_\infty - [\overline{AL_2}]_t)/([\overline{AL_2}]_\infty - [\overline{AL_2}]_0)] \times 100$. The values of $[\overline{AL_2}]$ were calculated from the measurements $[M]_{t=1.2s}$ with eq 7 (see text). From the slope of the line, an α value of 0.46 ± 0.05 s^{-1} was calculated: (△) experiment in Figure 4a; (○) another experiment.

and from the value of α at lower ligand concentration, k_{21}. The value of K_{c1} is determined by the values of K_1, K_2, and K_{c2}. From the values of K_{c1} and K_{c2}, one can now estimate k_{12} and k_{43}. Therefore, it has become possible to estimate the value of all constants included in the scheme. The values obtained at pH 7.0, 1 °C, are listed in Table I. The theoretical curves

Table I: Values of Constants in the Scheme in Figure 1 (Experiments at 1 °C, pH 7.0, in Eel Ringer's Solution)[a]

intrinsic ligand dissociation constant (Cash & Hess, 1980) pertaining to the active (A) state	$K_1 = \frac{2[A][L]}{[AL]} = \frac{[AL][L]}{2[AL_2]} = 1.9$ mM
maximum flux rate obtained if all the receptor is converted to the open-channel form (Hess et al., 1980)	$J_m = 37\ s^{-1}$
conformational equilibrium constant pertaining to the equilibrium between the closed, active (A) state and the open-channel ($\bar{A}$) state (Hess et al., 1980)	$\Phi = \frac{[AL_2]}{[\bar{A}L_2]} = 2.8$
intrinsic ligand dissociation constant pertaining to the inactive (I) state	$K_2 = \frac{[IL][L]}{2[IL_2]} = 21\ \mu M$
conformational equilibrium constant pertaining to the equilibrium between the active (A) and inactive (I) states, both with one ligand molecule bound	$K_{c1} = \frac{k_{21}}{k_{12}} = \frac{[AL]}{[IL]} = 0.10$ $k_{12} = 4.6\ s^{-1}$ $k_{21} = 0.46\ s^{-1}$
conformational equilibrium constant pertaining to the equilibrium between the active (A) and inactive (I) states, both with two ligand molecules bound	$K_{c2} = \frac{k_{43}}{k_{34}} = \frac{[AL_2]}{[IL_2]} = 0.001$ $k_{34} = 11.2\ s^{-1}$ $k_{43} = 0.012\ s^{-1}$

[a] Average error in rate constants ±20% and in equilibrium constants ±40%.

for the concentration dependence of α and $J_m[\overline{AL_2}]_\infty$, computed from the values in Table I, are shown in Figures 2d and 3. Thus, we can explain our results in terms of the minimum scheme (Figure 1) with the values in Table I.

At present, the mechanism which relates ligand binding to ion translocation over the 200-fold concentration range of carbamylcholine investigated is the simplest one, accommodating all the kinetic and equilibrium data available. Each intermediate or constant is required by the measurements (see the legend to Figure 1). The observed cooperativity is accounted for by a process which is known to occur, the opening of the channel. The equilibrium pertaining to channel opening in the model provides an increase in ligand binding affinity, with increasing ligand concentration, which is analogous to the allosteric effects observed in protein–ligand interactions (Monod et al., 1965; Hammes & Wu, 1974). The presence of free I is not included in the model (Figure 1) although its presence in low concentrations is not excluded by our measurements. Future studies may require an elaboration of the simple model, for example, the presence of an inactive receptor form, I, in the absence of ligand, and the abandonment of other simplifying assumptions.

We will now briefly compare the results obtained in our kinetic experiments with the results of ligand binding experiments, structural information, and electrophysiological measurements with animal cells. The general features of a ligand binding mechanism were first suggested by Katz & Thesleff (1957) on the basis of electrophysiological measurements with the frog neuromuscular junction. This mechanism also involves protein isomerization steps in the interconversion of active and inactive receptor forms. Subsequently, direct measurements of ligand binding with *Torpedo* membrane preparations in the millisecond time region were monitored by (i) fluorescent changes of the receptor (Bonner et al., 1976), (ii) fluorescent changes of reversible (Grünhagen & Changeux, 1978) or covalent bound probes (Dunn et al., 1980), and (iii) use of fluorescent acetylcholine analogues (Heidmann & Changeux, 1978; Jürss et al., 1979). The results obtained so far by these different techniques are not yet consistent with each other (Dunn et al., 1980) and have not yet led to a unique extension of the original model (Katz & Thesleff, 1957).

A minimum reaction mechanism relating interconversion of receptor forms to ligand binding steps, requiring the binding of two toxin molecules, which is essentially identical with the scheme in Figure 1, was proposed on the basis of kinetic measurements of the irreversible reaction of the toxin with the receptor in electroplax membrane vesicles of *Electrophorus electricus* (Hess et al., 1975a,b; Bulger et al., 1977). A much simpler mechanism was suggested by studies of the toxin reaction with *Torpedo* membranes (Weiland et al., 1976; Blanchard et al., 1979). Subsequently (Leprince, 1980), the reaction with *Torpedo* membranes was found to be similar to that with eel membranes (Hess et al., 1975a,b; Bulger et al., 1977) and rat diaphragm muscle (Brockes & Hall, 1975). It was shown (Leprince, 1980) that the oversimplification of the toxin reaction with *Torpedo* membranes occurred because in one study only a very low and limited concentration range of toxin was used (Wieland et al., 1976), and in the other study (Blanchard et al., 1979) the initial fast phase of the reaction was subtracted as a reaction blank.

The relationship between the channel opening equilibrium constant and the observed cooperativity is based on the assumption that the two acetylcholine binding sites of the active, A, conformation are equivalent. Studies of the acetylcholine receptor from *Torpedo* species indicate that it contains two apparently identical subunits which bind the ligand (Reynolds & Karlin, 1978; Lindstrom et al., 1979).

Two different inactivated receptor states have been detected in frog muscles in kinetic studies of the formation of single channels (Sakmann et al., 1980). At least in the range of carbamylcholine concentrations and reaction times we have studied, we did not find evidence for a second receptor inactivation process in eel electroplax vesicles.

Using the value of J_m (Table I) and the moles of receptor sites and inorganic ions per unit internal volume of the membrane vesicles in eel Ringer's solution, it was calculated that 6×10^3 ions/ms are translocated per receptor at pH 7.0, 1 °C (Hess et al., 1980, 1981). Analysis of electrical noise in frog muscle cells at temperatures above 8 °C (Neher & Stevens, 1977) gave a value of about 1×10^4 ions/ms per receptor channel. Thus, each technique gives essentially the same results. Therefore, it becomes possible to correlate the results obtained when receptor function is measured in two different ways in membrane vesicles and in muscle cells: (i) chemical kinetic measurements, using membrane vesicles, which relate the ligand binding and ion translocation processes, and (ii) analysis of acetylcholine noise in muscle cells (Katz & Miledi, 1972), which allows one to measure elementary steps in the formation of ion channels through the cell mem-

brane.

Techniques of rapid mixing have been used extensively to follow reactions mediated by proteins in solution which occur in the millisecond to second range (Roughton & Chance, 1963; Chance et al., 1964). Insight into many important biological processes, including oxygen transport, editing in protein synthesis, and regulation of metabolism has been gained by this approach (Hammes & Wu, 1974; Dixon & Webb, 1979; Fersht, 1977). Many interesting and important reactions involve membrane-bound proteins, receptors, enzymes, transport proteins, etc., which control the transfer of molecules across the cell membrane. The advantages of investigating these transmembrane process with membrane vesicles have been well documented (Kaback, 1970; Racker, 1970). Many of these reactions occur in the subsecond time region. The techniques which we have developed (Hess et al., 1979; Cash & Hess, 1980) show that in the case of the acetylcholine receptor containing vesicles one can measure the effect of ligand concentration on the different molecular processes involved in ion translocation in milliseconds, individually, in isolation, and together with specific factors which perturb the individual processes.

Changes in concentration of receptor activators (such as acetylcholine) at the junction between cells are thought to play an important part in expressing the intensity of external stimuli to an organism, and in learning processes in simple animals (Kandel, 1979). Here, we have demonstrated that the effect of carbamylcholine concentration on receptor function can be investigated quantitatively. Whether aspects of the proposed mechanism are general features of the functions of other receptors is an interesting question for future research. The successful application of the approach described to the elucidation of one particular problem suggests that a similar approach to other receptor systems may lead to equally clear-cut answers.

Acknowledgments

We are grateful to Laura Tenney for making the vesicle preparations. We thank Professor Aaron Lewis for use of his computing facility and Gregory Perreault for instruction in its use.

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Kinetics of Hydrogen Ion Diffusion across Phospholipid Vesicle Membranes†

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ABSTRACT: The membrane-impermeant, pH-sensitive fluorescence probe 8-hydroxy-1,3,6-pyrenetrisulfonate can be entrapped within the internal aqueous compartment of unilamellar phospholipid vesicles, where it serves as a reliable indicator of internal aqueous hydrogen ion concentration [Clement, N. R., & Gould, J. M. (1981) *Biochemistry 20*, 1534-1539]. When the external (medium) pH of a suspension of soybean phospholipid vesicles was rapidly changed from 8.2 to 6.65, the rate of subsequent H^+ influx into the vesicles, measured as the change in pyranine fluorescence, was limited (in KCl media) by the rate of charge-compensating counterion redistributions. The half-time for the pyranine fluorescence change (corresponding to an internal pH change from 8.2 to 7.43), which was several minutes in the absence of valinomycin, could be decreased to ~300 ms, but not further, by the K^+ ionophore valinomycin. Proton ionophores such as gramicidin or bis(hexafluoroacetonyl)acetone (1799), on the other hand, decreased the time required for transmembrane H^+ equilibration to <1 ms. These findings indicate that the intrinsic permeability of unilamellar vesicle membranes to hydrogen ions is surprisingly high and much greater than the observed permeabilities of other small ions.

The maintenance and regulation of ion gradients across biological membranes are crucial factors in proper cellular function. The need for specific electrolyte imbalances in such varied functions as nerve impulse transmission, enzyme function, and osmotic balance has long been established. In recent years, primarily as a result of the theories of Mitchell (1968), the central role of transmembrane hydrogen ion fluxes in cellular energetics has also become recognized.

By themselves, phospholipid bilayer membranes are generally considered to be relatively impermeable to most ions, including protons. However, several recent studies of hydrogen ion movements across unilamellar phospholipid vesicle membranes have led to the conclusion that these membranes are several orders of magnitude more permeable to protons and/or hydroxyl ions than to other small, monovalent ions (Nichols et al., 1980; Nichols & Deamer, 1980; Clement & Gould, 1981a), with a net H^+/OH^- permeability of $\sim 10^{-4}$ cm/s, compared with permeabilities of 10^{-10}-10^{-14} cm/s for other monovalent cations.

Utilizing a hydrophilic, pH-sensitive fluorescence probe trapped within the inner aqueous compartment of unilamellar vesicles, Clement & Gould (1981a) were able to directly monitor (in real time) changes in intravesicular pH following an abrupt change in the pH of the external medium. In that study, it was found that transmembrane H^+ equilibration was complete in <1 s when electrically compensating counterion fluxes were enhanced with valinomycin plus K^+. Unfortunately, those experiments were limited in their kinetic resolution by the sample mixing time, so that transmembrane pH equilibrations occurring in <1 s were not resolved.

In this paper we report the results of experiments similar to those of Clement & Gould (1981a) but performed by utilizing a rapid-mixing procedure in order to allow millisecond time resolution of the transmembrane proton fluxes.

Experimental Methods

Preparation of Vesicles. Unilamellar vesicles were prepared from purified (Kagawa & Racker, 1971) soy phosphatides (asolectin,[1] Associated Concentrates, Woodside, NY) by a

† From the Program in Biochemistry and Biophysics, Department of Chemistry, University of Notre Dame, Notre Dame, Indiana 46556. *Received December 1, 1980.* This research was supported by the Science and Education Administration of the U.S. Department of Agriculture under Grants No. 5901-0410-8-0109-0 and 78-59-2185-0-1-109-1 from The Competitive Research Grants Office and by grants from the Indiana Kidney Foundation and Miles Laboratories, Elkhart, IN.

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[1] Abbreviations used: asolectin, purified soybean phospholipids; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate; DMPC, L-α-dimyristoylphosphatidylcholine; 1799, bis(hexafluoroacetonyl)acetone; DLPC, L-α-dilaurylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine.

0006-2960/81/0420-3474$01.25/0