Molecular mechanism of acetylcholine receptor-controlled ion translocation across cell membranes

(Electrophorus electricus)/quench flow technique/carbamoylcholine/d-tubocurarine chloride/membrane vesicles

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ABSTRACT Two molecular processes, the binding of acetylcholine to the membrane-bound acetylcholine receptor protein and the receptor-controlled flux rates of specific inorganic ions, are essential in determining the electrical membrane potential of nerve and muscle cells. The measurements reported here establish the relationship between the two processes: the acetylcholine receptor-controlled transmembrane ion flux of 86Rb+ and the concentration of carbamoylcholine, a stable analog of acetylcholine. A 200-fold concentration range of carbamoylcholine was used. The flux was measured in the millisecond-to-minute time region by using a quench flow technique with membrane vesicles prepared from the electric organ of Electrophorus electricus in eel Ringer’s solution at pH 7.0 and 1°C. The technique makes possible the study of the transmembrane transport of specific ions, with variable known internal and external ion concentrations, in a system in which a determinable number of receptors is exposed to a known concentration of ligand. The response curve of ion flux to ligand was sigmoidal with an average maximum rate of 84 sec⁻¹. Carbamoylcholine induced inactivation of the receptor with a maximum rate of 2.7 sec⁻¹ and a different ligand dependence so that it was fast relative to ion flux at low ligand concentration but slow relative to ion flux at high ligand concentration. The simplest model that fits the data consists of receptor in the active and inactive states in ligand-controlled equilibria. Receptor inactivation occurs with one or two ligand molecules bound. For channel opening, two ligand molecules bound to the active state are required, and cooperativity results from the channel opening process itself. With carbamoylcholine, apparently, the equilibrium position for the channel opening step is only one-fourth open. The integrated rate equation, based on the model, predicts the time dependence of receptor-controlled ion flux over the concentration range of carbamoylcholine investigated. The values of the constants in the rate equation form the basis for predicting receptor-controlled changes in the transmembrane potential of cells and the conditions leading to transmission of signals between cells.

Changes in the transmembrane potential of the cell are determined by protein receptors in the membrane that, upon binding appropriate small molecules, change the permeability of the membrane to specific inorganic ions (1-4). Determining the relationship between the ligand-binding reaction and the receptor-controlled fluxes of specific inorganic ions through the cell membrane is the purpose of the experiments described here.

The receptor studied is the acetylcholine receptor in the excitable membrane of the electric organ of Electrophorus electricus (3). Nachmansohn (1-4) suggested that the action of acetylcholine is an intramembranous process controlling ion permeability in both synaptic and axonal membranes. Acetylcholine induces a conformational change that results in the opening of ion-conducting channels through the membrane (1-4). Since then, the acetylcholine receptor has been isolated in many laboratories and its ligand binding properties have been thoroughly investigated (for recent reviews see refs. 5-9). The relationship of these ligand-binding processes to transmembrane ion flux and the resulting changes in transmembrane potential is not fully understood.

Development of the technique of studying transmembrane processes with vesicles (10-12), the preparation of vesicles rich in acetylcholine receptor in the native membrane (13-15), and the resolution of the receptor-controlled ion flux from other processes (16-19) now allow direct measurement of the effect of acetylcholine receptor ligands on the rates of ion translocation through the membrane in a well-defined system. We found (20) that the receptor controlled flux is biphasic with an initial phase too fast to be measured by available techniques. Subsequently, we developed (21) a method for measuring the rapid ion flux in the millisecond-to-minute time region. Here we report the ligand concentration dependence of the acetylcholine receptor-mediated transmembrane ion flux over a 200-fold concentration range of carbamoylcholine, a stable analog of acetylcholine.

MATERIALS AND METHODS

Electric eels (Electrophorus electricus) were obtained from World Wide Scientific Animals, Ardsley, NY. The vesicles were prepared by a modification (22) of the procedure of Kasai and Changeux (13) and were resuspended in eel Ringer’s solution (186 mM NaCl/5 mM KCl/3 mM CaCl2/1.5 mM MgCl2/1.5 mM sodium phosphate, pH 7.0) and allowed to equilibrate for at least 12 hr before use. Protein concentration was determined by the method of Lowry et al. (23). Vesicle concentration, expressed as protein, was measured by nephelometry, measuring OD at 520 nm and calibrating the almost linear plot of optical density against protein concentration measured by the method of Lowry et al. (23). Carbamoylcholine chloride and d-tubocurarine chloride were obtained from Sigma. 86RbCl was obtained from New England Nuclear. All other chemicals were reagent grade.

The ion flux measurements were made by using a quench flow apparatus with the pulse mode modification of Fersht and Jakes (24). The vesicle suspension (≈800 μg of protein per ml, 0.225 ml) was mixed with an equal volume of the carbamoylcholine solution containing radioactive tracer ion (86RbCl, 100 μCi/ml). After a predetermined time the reaction mixture was quenched by mixing with a solution of d-tubocurarine chloride (30 mM, 0.225 ml), the vesicles were immediately removed from the mixture with a Millipore filter (HAWP 025), and the quantity of 86Rb+ in the vesicles was determined by scintillation

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counting. Complete quenching of the reaction by d-tubocurarine was demonstrated by the absence of receptor-mediated flux with very short reaction times or when curare was added simultaneously with carbamoylcholine. For each measurement with carbamoylcholine, a control measurement in the absence of carbamoylcholine was made. All determinations were made in triplicate. The differences between the normalized Δcpm values with and without carbamoylcholine are presented in terms of fraction of total influx, \( M_f^+ / M_o^+ \), where \( M^+ \) refers to the metal ion concentration inside the vesicles and the subscript refers to the time of measurement.

**RESULTS**

Two typical influx experiments are shown in Fig. 1. With 1 mM and 2 mM carbamoylcholine (Fig. 1a), a progressive slowing of the rate was observed, which we attribute to ligand-induced inactivation of the receptor. With higher concentrations of this ligand, the ion flux was substantially complete within 100 msec, before inactivation was significant. With lower concentrations (Fig. 1b), inactivation was complete before ion flux, the initial flux rate constant decreased to a constant value, and the influx was completed in several minutes at a rate characteristic of the inactivated receptor.

The first-order rate constants for ion flux, \( J \), and inactivation, \( \alpha \), were obtained by a curve-fitting procedure over a wide range of carbamoylcholine concentrations (0.1–20 mM). With various preparations, at a given ligand concentration, \( \alpha \) was constant within our experimental error, but \( J \) showed variations with the preparation. With eight different vesicle preparations from six different eels, \( J \) at 1 mM carbamoylcholine was 3.1 ± 1.4 sec⁻¹. We consider the variation in \( J \) to reflect the variation of the number of receptor sites capable of forming channels per unit internal volume in different vesicle preparations. Influx rates from different preparations were compared after normalization of \( J \) by using values determined at 1 mM carbamo-

![Fig. 2. Rate of acetylcholine receptor-mediated ion translocation, \( J \), before inactivation of receptor, as a function of carbamoylcholine concentration (L). \( J \) was obtained from analysis of the time progress of ion influx as measured by using \(^{86}\text{Rb}^+ \) tracer with the vesicles described (examples in Fig. 1). As a first approximation, \( J \) was evaluated from the initial flux rates. A final evaluation of \( J \) and \( \alpha \) involved a computer program that gave the best values of \( J \) and \( \alpha \) that fitted the influx curves. \( J \) values are normalized to a value of 3.1 sec⁻¹ at 1 mM carbamoylcholine (see the text).](image)

moylcholine. The dependence of influx rate constant, \( J \), on ligand concentration shows a sigmoidal shape (Fig. 2). This curve indicates that two ligand molecules per channel are required for ion flux.

The method we have adopted to arrive at a model consists of accounting for experimental results in terms of the minimum number of intermediates and using the minimum number of constants. The simplest model to which we can fit our data is shown in Fig. 3. This scheme comprises receptor in the active state, \( A \), and in the inactive state, \( B \), as suggested by Katz and Thelev (25) on the basis of electrophysiological experiments. Transmembrane ion flux occurs with the open channel form, \( A_L \). The integrated rate equation pertaining to this model for receptor-controlled transmembrane ion flux with vesicles is

![Fig. 3. Minimum mechanism to account for the observed rates of acetylcholine receptor-mediated ion translocation and receptor inactivation as a function of carbamoylcholine concentration. The active forms of the receptor, \( A \), and the inactive forms, \( B \), bind ligand, \( L \), in rapidly achieved equilibrium denoted by the equilibrium constants, \( K \). Active receptor with two bound ligands, \( A_L \), can rapidly convert to an open channel, \( A_L \), where \( 1/\Phi \) is the equilibrium constant for opening the channel. \( A_L \) permits ion flux with a first-order rate constant \( J_m \). Conversions between the active and inactive forms of receptor occur with first-order rate constants \( k \). For derivation of equations, see Appendix.](image)
where the terms of this equation are defined in the Appendix. In deriving Eq. 1, we assumed that the ligand-binding and channel-opening processes are fast compared with ion flux and inactivation, which follow first-order kinetics. We make the simplest assumption that the ligand-binding sites are identical and only a single dissociation constant, $K_1$, is involved in the binding of ligands to the active, A, conformation. Channel opening, which must involve some change in the receptor, occurs only when two ligand molecules are bound. The channel opening equilibrium, increasing with $1/\Phi$, displaces the second ligand binding equilibrium, giving rise to a cooperative effect of ligand binding which increases with $1/\Phi$.

It follows from the scheme that:

$$\frac{J_m - 1}{J} = \frac{K_1 \Phi^{1/2}}{L} + \Phi^{1/2}. \quad (2)$$

A plot of the data according to this equation, shown in Fig. 4, is consistent with the model and the binding of two ligand molecules to the A conformation prior to channel opening. From this graph we obtain the dissociation constant $K_1 = 1.7 \text{ mM}$ and the channel opening constant $1/\Phi = 0.3$ with $J_m = 84 \text{ sec}^{-1}$. The inactivation rate constant, $\alpha$, reaches a maximum of $2.7 \text{ sec}^{-1}$ at a ligand concentration at which $J$ has not reached its saturation value. This is accommodated by the model in which the binding of only one ligand molecule to the active form of the receptor is required for inactivation to occur, but two bound molecules are required for channel opening. Consequently, inactivation is fast relative to ion flux at low ligand concentration (Fig. 1). Thus, at low ligand concentrations we can determine the flux rate of inactivated receptor, that is, of the equilibrium mixture of active and inactive receptor which depends on the ligand concentration (Fig. 1b). From the second equation in the Appendix, using a computer program, the values of $K_2$ and $K_C$ and hence $K_{C1}$ were obtained, which are listed in the Appendix. The observation that the maximum value of $\alpha$ does not change significantly when the carbamoylcholine concentration, and consequently the concentration of Alg, is increased implies that the active forms of receptor, A, with one or with two carbamoylcholine molecules bound are converted to the inactive form, B, at rates that are not significantly different. From a knowledge of $\alpha$, $K_{C1}$, $K_{C2}$, and $J_m$, the isomerization rate constants $k_{12}$, $k_{31}$, $k_{34}$, and $k_{43}$ can be estimated (Appendix).

**DISCUSSION**

Use of the quench flow technique to measure transmembrane ion flux with vesicles in times down to 20 msec has allowed us to measure ion translocations characteristic of the active form of the acetylcholine receptor before its conversion to the inactive form on incubation with ligand. This inactivation occurs rapidly, well within 1 sec with $>1 \text{ mM}$ carbamoylcholine. The course of the inactivation reaction also can be followed.

In contrast, other techniques involve exposure of the receptor to ligands for longer times. In noise analysis (26), at present, 11 sec are required for the measurements. Inactivation of the eel acetylcholine receptor occurs well within this time. When cells are mixed with solutions containing acetylcholine or its analogs according to conventional electrophysiological techniques, inactivation becomes a major problem in the subsequent measurements.

There are additional advantages in working with membrane vesicles. The rate of movement of a specific inorganic ion across the membrane can be determined. Measurements can be made in controlled conditions not limited by the metabolic restrictions present in intact cells. Internal and external ion concentrations can be varied and accurately known. The number of receptors exposed to a known concentration of ligand can be determined.

When ligands are applied to the surface of cells iotopophoretically, it is difficult to specify the number of receptor sites exposed to a definite concentration of ligand.

Electrical noise analysis of acetylcholine receptor-mediated conductance changes in cells measures the initial transfer rate of ions per receptor-formed channel (26, 27). So far, these measurements have been limited to low concentrations of receptor ligands because complexities of interpretation at high ligand concentrations have not been solved. In experiments vesicles, not only can measurements be made over a wide range of ligand concentration, but one can also observe the time-course of the complete equilibration of inorganic ions across the membrane. As we have shown, the time-resolution of the quench flow technique is sufficient to measure this equilibration process. The rate constants that we evaluate allow one not only to determine the initial ion flux rates that can be obtained in electrical noise analysis but also to predict the time course of the receptor-controlled flux over a wide range of ligand concentration.

The molecular mechanism of receptor-controlled ion flux can be evaluated by many different types of quench flow measurements in addition to those described here. The value of $\alpha$ can be obtained from direct measurements of the rate of inactivation; the values of $K_{C1}$, $K_{C2}$, and $K_2$ can be obtained directly from measurements of the flux after inactivation over a wide range of ligand concentration, and the rate of recovery of the inactive receptor can be determined and gives further information about the mechanism.

We have expanded the original model of Katz and Thesleff (25). Our model requires the binding of two ligand molecules for channel opening, and cooperativity results from the channel opening process itself. Two ligand-binding sites were also required for the reaction of the specific snake neurotoxin, $\alpha$-bungarotoxin, in experiments with similar eel vesicle preparations, and the minimum reaction schemes are essentially similar (28, 29). A number of electrophysiological experiments (25, 30–35) have indicated a requirement of more than one ligand molecule for receptor-mediated conductance changes.

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![Fig. 4. Plot of $(J_m - 1)/2$ against reciprocal carbamoylcholine concentration. The points represent the data of Fig. 2. The line, drawn through the points, satisfies Eq. 2, where $J_m = 84 \text{ sec}^{-1}$, $K_1 = 1.7 \text{ mM}$, and $\Phi = 3.4$. This plot confirms the square dependence of transmembrane ion flux on ligand concentration and can be used to determine the values of $K_1$ and $\Phi$. The slope gives $K_1 \Phi^{1/2}$, the ordinate intercept gives $\Phi^{1/2}$, and the abscissa intercept gives $1/K_1$.](image-url)
although the unequivocal assignment of two molecules per receptor is not simple for these results. If we consider the receptor to be a dimer of units with equivalent binding sites, they will change conformation in a concerted manner after binding ligand to open the channel (36). Assuming equivalent binding sites, the equilibrium constant for channel opening, $K_0$, could be determined from our data (Fig. 4). If this interpretation of $\Phi$ is correct, only one-fourth of the complex of active receptor with two bound carbamoylcholine molecules gives rise to an open channel. Therefore, at saturating carbamoylcholine concentration the value of $j$ is one-fourth of the maximum value, $J_m$. Presumably, different ligands stabilize the open form to different extents, giving rise to different degrees of channel opening and allosteric effect. This suggestion is supported by measurements of conductance fluctuation in cells (25), which indicated that the rate of channel closing varies with the ligand. A further test of our model will be whether the dependence of the channel opening constant, $K_0^{-1}$, on different ligands as determined in our measurements is correlated with that determined by electrophysiological methods.

In contrast to channel opening, inactivation occurs with only one, as well as with two, bound ligand molecules per active receptor. That the ligand dependencies of channel opening and inactivation are different may have a profound effect on the functional behavior of the receptor in the physiological system. As the ligand concentration decreases, inactivation becomes more important relative to ion transport and the resulting propagation of an electrical signal. Prior exposure of the receptor to low ligand concentrations would result in an equilibrium between active and inactive receptor forms. This would modify the receptor-controlled ion translocation process caused by subsequent larger ligand concentration and, hence, the conditions under which a signal is propagated.

We have indicated (19–21) the possibility that transmembrane channels can be formed by the inactive or B state. Although this possibility is not excluded, we now make the simpler assumption that only the active conformation gives rise to open channels. In the model of Katz and Thesleff (25), the postulation of free B was required to account for the relatively fast recovery from inactivation after removal of ligand (25). With the scheme presented here, a difference in the rates of recovery from LgB and LB is predicted. Thus, the postulation of free B under these conditions may not be necessary, although we do not preclude its formation.

In general, the use of the quench flow technique with membrane vesicles as a model for cellular membrane processes enables one to study phenomena with short time resolutions free of interference by other cellular processes, and free of the practical limitations due to indigenous cellular composition. The freedom to define and vary the various concentrations of ions and ligands in this system allows us to perform some experiments that have not been possible with whole cells. The results suggest that our approach may have general use for establishing the relationship between the receptor–ligand binding process and receptor-controlled flux. The relationship between the binding of carbamoylcholine to the receptor and the resulting receptor-controlled flux is represented by the model in Fig. 3. The model is consistent with the results obtained and can be tested by further experiments. The integrated rate equation, based on the model, predicts the time dependence of receptor-controlled flux over a 200-fold concentration range of carbamoylcholine and allowed the evaluation of the rate and equilibrium constants pertaining to the model. The values of these constants form the basis for predicting receptor-controlled changes in the transmembrane potential of cells and the conditions leading to transmission of signals between cells (19).

### APPENDIX

The terms in Eq. 1 derived from this scheme are:

\[
(\text{AL}_2)_0 = \frac{R_0 L^2}{(L^2 + 1) + 2K_1 L + K_1^2},
\]

\[
(\text{AL}_2)_e = \frac{R_0 L^2 K_c}{K_c [L^2 (1 + F) + 2K_0 \Phi L + K_0^2 F] + \Phi L (1 + 2K_0^2)},
\]

\[
\alpha = \Phi \left[ \frac{L_{k34} + 2K_kL_{k34} + L_{k34} + 2K_kL_{k34}L_{k34}}{(L + 2K_0)\Phi + (L + 2K_0)\Phi F + K_0^2 L + K_0^2 F} \right],
\]

where the subscripts 0 and e denote equilibrium concentrations before and after isomerization between receptor forms has commenced. $R_0$ represents the total receptor concentration and $K_1$ and $K_2$ are dissociation constants.

The values that were determined from our results and were used to calculate the solid curves in Figs 1, 2, and 4 are:

\[
K_1 = \frac{2(L)(A)}{(AL)_0} = \frac{(L)(AL)}{(2AL)_0} = 1.7 \text{mM};
\]

\[
K_2 = \frac{(L)(BL)}{2(BL)_0} = 80 \mu \text{M};
\]

\[
K_{C1} = \frac{(AL)}{(BL)} = 4 \times 10^{-2},
\]

\[
K_{C2} = \frac{(AL)_2}{(BL)_0} = 2 \times 10^{-3},
\]

\[
\Phi = \frac{(AL)_2}{(AL)_0} = 3.4,
\]

and the channel opening constant $1/\Phi = 0.3$;

\[
\alpha_{\max} = 2.7 \text{sec}^{-1} (k_{12} \approx k_{34} = 3.7 \text{sec}^{-1},
\]

\[
k_{21} \approx 0.16 \text{sec}^{-1}, k_{43} \approx 0.007 \text{sec}^{-1},
\]

\[
f_m = 84 \text{sec}^{-1},
\]

\[
J = J_m [L(AL)_2]_0.
\]

$J_m = J_m [L(AL)_2]_0$, where $J$ and $J_m$ refer to the ion flux rates before and after inactivation, respectively.

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