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ACETYLCHOLINE RECEPTOR-CONTROLLED ION FLUX IN ELECTROPLAX MEMBRANE VESICLES: A MINIMAL MECHANISM BASED ON RATE MEASUREMENTS IN THE MILLISECOND TO MINUTE TIME REGION

H.Aoshima, D.J.Cash and G.P.Hess*

Section of Biochemistry
270 Clark Hall
Cornell University, Ithaca, N.Y. 14853

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Summary The dependence of acetylcholine receptor-controlled transmembrane ion flux on carbamylcholine concentration was measured in the msec time region, using membrane vesicles and a quench flow technique. 4 Measurements were made: (1) transmembrane ion influx, (2) rate of inactivation of the receptor by carbamylcholine, (3) rate of recovery, and (4) ion influx mediated by "inactivated" receptor. The minimal model, based on the measurements, accounts for the time dependence of receptor-controlled ion flux over a 200-fold carbamylcholine concentration range. The maximum flux rate of 84 sec^{-1} indicates that we have succeeded in measuring the receptor-controlled processes which give rise to electrical signals in cells.

The amplitude and sign of receptor-controlled electrical signals in nerve or muscle cells determines whether the signals are transmitted to another cell or whether the muscle contracts. These electrical signals depend on the concentration of acetylcholine which binds to the receptor (1) and have been investigated extensively during the last decade (for a review see (2)). The underlying molecular processes which give rise to these signals are the receptor controlled fluxes of inorganic ions through the cell membrane. The relationship between the receptor-mediated flux rates and the concentration of receptor ligands is not understood and has been investigated intensively (3-12).

Here we report that we have succeeded in measuring the dependence of the acetylcholine receptor-controlled ion flux on the concentration of carbamylcholine, a stable analog of acetylcholine, in the msec to min time region. Measurements were made with membrane vesicles prepared from the electric organ of the electric eel, *Electrophorus electricus*. The vesicles contain functional acetylcholine receptors (3). $^{86}Rb^{+}$, an effective replacement for $^{42}K^{+}$ (20), was

*To whom reprint requests and correspondence should be addressed.

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used in the experiments. The investigations differ from electrophysiological measurements and previous flux measurements with cells or vesicles in several important ways. (1) The techniques which we have developed allow the measurement of flux rates of a particular inorganic ion across the membrane in the msec to min time region (14), without interference by processes not related to the receptor (7). (2) The internal and external ion concentration can be varied and determined. (3) The number of receptor sites associated with a definite volume of solution and exposed to a definite concentration of ligands can be measured.

Four different kinetic measurements have been made to establish the relationship between receptor-controlled flux and ligand concentration. (1) Measurements of the flux of $^{86}Rb^{+}$ into the vesicles in the 25 msec to 99 sec time region, examples of which are shown in Fig. 1a. The influx induced by 5 mM carbamylcholine has an apparent $t_{1/2}$ value of 40 msec. The influx induced by 0.25 mM carbamylcholine is biphasic. An initial relatively rapid phase is followed by a second, slower phase. (2) The inactivation of the receptor by exposure to acetylcholine and its analogs has been observed in electrophysiological measurements (15). In the experiment in Fig. 1b (solid symbols) the receptor was mixed with 7.5 mM carbamylcholine for the time indicated on the abscissa and $^{86}Rb^{+}$ influx was then allowed to proceed for 1.2 sec. In the control experiment carbamylcholine was not present in the preincubation. Comparison between these two measurements gives the extent of inactivation of the receptor. A graph of the percent of inactivation, on a logarithmic scale, *versus* time, is shown in Fig. 1b. The inactivation follows a first order rate law with a $t_{1/2}$ value of 250 msec. (3) The rate with which the inactivated receptor is converted to the active states can be measured (Fig. 1b, open symbols). In the experiment the vesicles were pre-incubated with 70 μM carbamylcholine until an equilibrium between active and inactive forms of the receptor was reached, and influx was then measured (see Fig. legend). In the control experiments the vesicles were not preincubated with carbamylcholine. A comparison between these two measurements gives the extent of reactivation. The percent of reactivation on a logarithmic scale is plotted

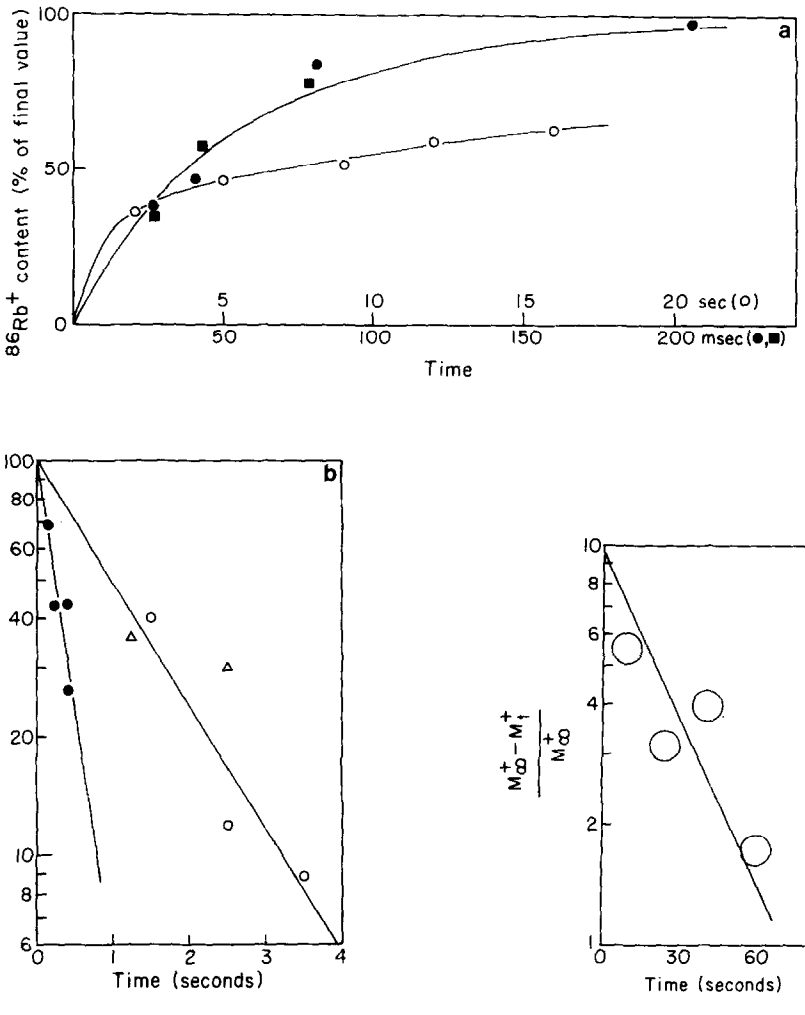

Fig. 1 The effect of carbamylcholine on receptor-mediated influx of $^{86}Rb^+$, pH 7.0,1°C. (a) $^{86}Rb^+$ influx in presence of 5 mM carbamylcholine (●,■) and 0.25 mM carbamylcholine (○). Each point is the mean of 3 determinations. The lines are computed using eq.(1) and the constants evaluated from the various experiments (see the text). (b) Inactivation of the receptor (●). The vesicles were pre-incubated with 7.5 mM carbamylcholine for the times indicated on the abscissa and then for 1.2 sec with 5 mM carbamylcholine and $^{86}RbCl$. In control experiments carbamylcholine was omitted from the preincubation. Reactivation of the receptor (○,△). The vesicles were pre-incubated with 70 uM carbamylcholine for 2 h and then diluted 7-fold. After the time indicated on the abscissa, $^{86}Rb^+$ influx was allowed to proceed for 1.2 sec in presence of 5 mM carbamylcholine. In the control experiments carbamylcholine was omitted from the preincubation. Each point is the mean of 2 determinations. Different *symbols represent different membrane preparations.* (c) Influx mediated by the equilibrium mixture of active and inactive receptor conformations. The vesicles were preincubated with 2 mM carbamylcholine for 30 min and $^{86}Rb^+$ influx was then allowed to proceed for the times indicated on the abscissa in 2 mM carbamylcholine. The symbol M^+ refers to the $^{86}Rb^+$ content of the vesicles and the subscript to the time of measurement. Each point is the mean of 2 determinations

E.electricus were obtained from World Wide Scientific Animals. The vesicles were prepared as described by Fu *et al.* (25). Measurements were made in eel Ringer solution (26) using a pulsed quench flow apparatus (27). Protein concentrations were measured by the method of Lowry *et al* (28). Carbamylcholine chloride and *d*-tubo-

versus time in Fig. 1b. The reactivation follows a first order rate law with a $t_{1/2}$ value of about 1 sec in 10 μM carbamylcholine. (4) The rate of influx regulated by an equilibrium mixture of active and inactive receptor can be measured as a function of ligand concentration. In the experiment in Fig. 1c prior to addition of $^{86}Rb^{+}$ the membrane vesicles were pre-incubated with 2 mM carbamylcholine for 30 min to obtain an equilibrium mixture of active and inactive receptor states. Experiments similar to those illustrated in Fig. 1b show that this equilibrium is reached within seconds at the carbamylcholine concentration used and that the inactivation process is reversible. The experiment (Fig. 1c) indicates that influx follows a first order rate law with a $t_{1/2}$ value of 21 sec in 2 mM carbamylcholine.

On the basis of electrophysiological measurements with muscle cells, Katz and Thesleff (15) suggested that the receptor exists in an active and in an inactive state. The model in Fig. 2 accounts for the underlying processes which give rise to these electrophysiological measurements, the receptor-controlled flux of inorganic ions through the membrane. The minimum number of intermediates and constants, which can account for the experimental results, are: an active state (A), an inactive state (I), and the open channel form ($\bar{A}$). The binding of two ligand molecules to the A state is required for channel opening and ion flux while inactivation proceeds with one or two ligand molecules bound. One ligand dissociation constant, K_1, an equilibrium constant for channel opening, Φ^{-1}, one ion flux rate constant, J_m, one constant for the conformational equilibrium between the A and I states, K_{c2}, and one ligand dissociation constant pertaining

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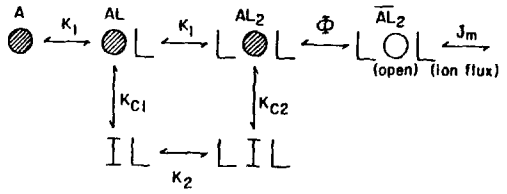
curarine chloride were obtained from Sigma Chemical Co. $^{86}RbCl$ was obtained from New England Nuclear. All other chemicals were reagent grade.

In the quench flow apparatus the vesicle suspension (∿800 μg protein per ml, 0.23 ml) was mixed with an equal volume of the carbamylcholine solution, or with 6 volumes of buffer in the reactivation experiments, or with a solution containing both carbamylcholine and $^{86}RbCl$ (100 μCi/ml). After a desired time interval the reaction mixture was quenched by mixing with a solution of *d*-tubocurarine (30 mM, 0.23 ml). The vesicles were then removed from the reaction mixture by a Millipore filter (HAWP 025) and their $^{86}Rb^{+}$ content was determined by scintillation counting. Complete quenching of the flux by *d*-tubocurarine was demonstrated by the absence of receptor-controlled flux with very short reaction times or when *d*-tubocurarine was added simultaneously with carbamylcholine. For each measurement with carbamylcholine a control experiment in absence of carbamylcholine was performed.



$$-\ln \frac{M_\infty - M_t}{M_\infty} = \{J_A(obs) - J_I(obs)\} \frac{1 - e^{-\alpha t}}{\alpha} + J_I(obs)t \quad (1)$$

$$J_A(obs) = J_m \left\{ \frac{L_2}{L^2 (1 + \phi) + 2K_1 L\phi + K_1^2 \phi} \right\} \quad (2)$$

$$J_I(obs) = J_m \frac{K_{c2} L^2}{K_{c2}(L^2(1 + \phi) + 2K_1 L\phi + K_1^2\phi) + \phi L(L + 2K_2)} \quad (3.i)$$

$$J_I(max) = J_m \frac{K_{c2}}{\phi} \quad (3.ii)$$

$$K_{c1} = \frac{k_{21}}{k_{12}} = \frac{AL}{IL} \qquad K_{c2} = \frac{k_{43}}{k_{34}} = \frac{AL_2}{IL_2} \quad (4)$$

$$\alpha = \frac{k_{43} L + 2k_{21} K_2}{(L + 2K_2)\phi} + \frac{k_{34} L^2 + 2k_{12} K_1 L}{L^2(1 + \phi) + 2K_1 L\phi + K_1^2 \phi} \quad (5)$$

$$\ln \frac{M_\infty^+ + M_t^+}{M_\infty^+} = -J_I(obs)\, t \quad (6)$$

$$\left\{ \frac{J_m}{J_A(obs)} - 1 \right\}^{1/2} = \phi^{1/2} + \frac{K_1}{L} \phi^{1/2} \quad (7)$$

$J_m = 84\ sec^{-1}$ $K_1 = 1.7\ mM$ $K_{c1} = 4 \times 10^{-2}$

$J_I(max) = 0.05\ sec^{-1}$ $K_2 = 70\ \mu M$ $K_{c2} = 2 \times 10^{-3}$

$\phi = 3.4$

Fig 2 Minimum mechanism to account for the acetylcholine receptor-controlled flux of inorganic ions, and the integrated rate equations pertaining to this scheme, with the values for the parameters which fit the data.

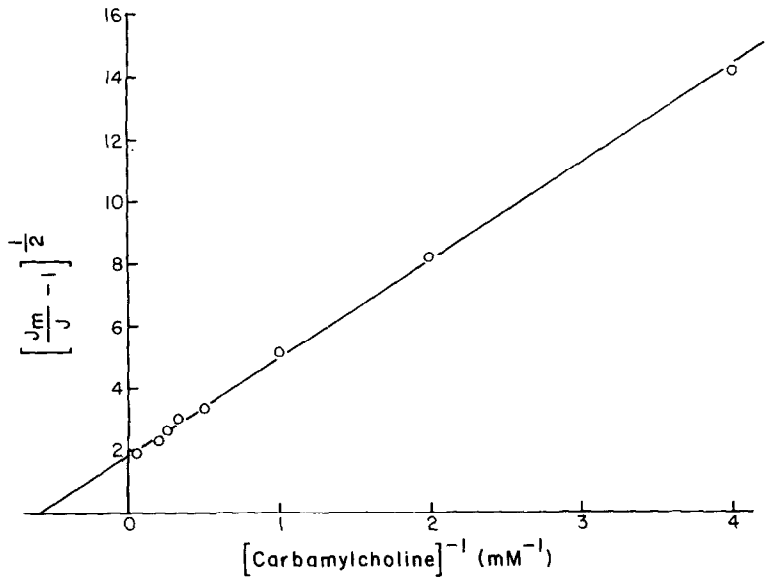


Fig 2 Inset A plot of the data according to eq. (7). The line is drawn through the points which are calculated using J_m=84 sec^{-1}.

to the I state, K_2, are required. With the assumption that all ligand binding steps and the equilibration between open and closed channels are fast relative to the interconversion between the A and I states, the integrated rate equation for the receptor-controlled flux is given by equation (1) in the legend to Fig. 2. All terms in the equation and the values for the constants are given in the legend. The rate constants for the isomerization of receptor forms are contained in α. At each ligand concentration α is obtained from measurements illustrated in Fig. 1b. The integrated rate equation for the ion flux characteristic of the equilibrium mixture of active and inactive receptor states is given by equation (6) and the value of J_I(obs) at each ligand concentration can be obtained from the measurements illustrated in Fig. 1c. The ion flux associated with the receptor prior to inactivation, J_A(obs), is now the only unknown term in equation(1). The term was evaluated at each ligand concentration from experiments illustrated in Fig. 1a with a curve fitting procedure and use of a computer program. The solid lines in Fig. 1a are calculated using the values obtained in the different measurements. The dependence of J_A(obs) on carbamylcholine concentration indicates that ligand binding to the active receptor state is cooperative. The minimal model

requires the binding of two ligand molecules to the active receptor form, and two constants, K_I and Φ^{-1}. A plot of equation (7), which is based on this model, is shown in the inset to Fig. 2. This equation is obeyed over the whole concentration range of carbamylcholine used. The values of K_1, Φ^{-1}, and J_m obtained from this graph are listed (Fig. 2 legend). The maximum rate of inactivation of the receptor (Fig. 1b) is reached at a ligand concentration at which the flux rate has not reached its saturation value. This is accounted for by the model, in which the binding of only one ligand molecule is required to induce interconversion of the active to an inactive state which binds ligand with higher affinity, i.e. $K_1 >> K_2$. This inequality of binding constants is supported by the influx controlled by the equilibrium mixture of active and inactive states. When the maximum flux rate of 0.03 sec^{-1} associated with the equilibrium mixture is attained (Fig. 1c), K_{c2} can be evaluated (eq.3.ii). The value of K_2 is obtained from the effect of carbamylcholine concentration on J_I(obs) (eq.3.i). The value for K_{c2} of 2×10^{-3} requires that $k_{34} >> k_{43}$. As can be seen in Fig. 1b, the rates of inactivation and reactivation differ by only a factor of 4. This observation is accounted for by recovery of the receptor occurring from a species different from IL_2 and justifies the inclusion of IL_1 in the model. The existence of an inactive state of the receptor in the absence of ligand is neither required nor excluded by our data that channel opening represents a rapid equilibrium between open and closed receptor forms; this process alone is sufficient to account for the observed cooperativity. This interpretation leads to the conclusion that, when the carbamylcholine is the ligand, only one quarter of the AL_2 species gives rise to open channels.

A number of electrophysiological experiments have indicated a requirement for the binding of more than one molecule for receptor-mediated conductance changes (15-17). Previously, with the same preparation, two ligand binding sites on the receptor, and a minimum reaction scheme essentially identical to that in Fig. 2, were required to account for the reaction of α-bungarotoxin with the receptor (18, 19). The biphasic nature of the receptor-controlled flux shown in Fig. 1a (open circles) was first detected in efflux experiments (20). While the

initial phase of this flux was too fast to be measured by the techniques then available, a dissociation constant for the carbamylcholine induced flux associated with the slow phase of 70 μM was determined (7), in good agreement with the value obtained here.

Using purified membrane vesicles and flux rates associated with the slow phase of the receptor-controlled flux, we estimated the initial velocity, v_i, of the receptor-controlled ion translocation process to be 3×10^4 ions per toxin site per min (21). Based on the value of J_m determined in this study, v_i is 1.7×10^4 times greater than this, prior to inactivation of the receptor, and is well within the range of values obtained by electrophysiological techniques with muscle cells (22, 23). Thus it appears that with the techniques we have developed (14,24) we have succeeded, using variable but well-defined conditions, in measuring the receptor-controlled processes which give rise to electrical signals in cells, and which determine the transfer of information between these cells.

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