Specific reaction rate of acetylcholine receptor-controlled ion translocation: A comparison of measurements with membrane vesicles and with muscle cells

(*Electrophorus electricus*/flow-quench technique/carbamoylcholine)

GEORGE P. HESS, HITOSHI AOSHIMA*, DEREK J. CASH, AND BERNARD LENCHITZ

Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, 270 Clark Hall, Cornell University, Ithaca, New York 14853

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ABSTRACT The specific reaction rate ($\bar{J}$) of the acetylcholine receptor-controlled ion translocation has been determined. In eel Ringer's solution (pH 7.0) at 1°C, $\bar{J} = 3 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. $\bar{J}$ is an intrinsic constant that is characteristic of the receptor and independent of other properties of a receptor-containing cell that also determine the rates of ion translocation. Membrane vesicles (prepared from the electric organ of *Electrophorus electricus*) and a flow-quench technique that has a millisecond time resolution were used to measure the receptor-controlled ion translocation. Using the value of $\bar{J}$ and the molar concentrations of receptor sites and inorganic ions, we calculated that 6×10^3 ions are translocated per msec per receptor. Analysis of electrical noise in frog muscle cells at temperatures above 8°C [Neher, E. & Stevens, C. F. (1977) *Annu. Rev. Biophys. Bioeng.* 6, 345–381] gave a value of about 1×10^4 ions msec⁻¹ per channel. Thus, each technique gives essentially the same result. It is now possible, therefore, 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, B. & Miledi, R. (1972) *J. Physiol. (London)* 224, 665–699], which allows one to measure elementary steps in the formation of ion channels through the cell membrane.

The initial rate of acetylcholine receptor-controlled movement of ions across the membrane of a particular cell determines the initial change in transmembrane potential (1, 2) and therefore also determines whether or not the electrical signal generated by a nerve cell will be propagated (3) or, in muscle cells, whether a contraction will occur. This initial rate is characterized by the rate coefficient, J_A . The value of J_A is determined not only by the intrinsic properties of the receptor but also by certain properties of the cell, such as the number of receptor sites and the internal volume, and also the internal and external concentrations of freely mobile inorganic ions. We have, therefore, determined the specific reaction rate, $\bar{J}$, which is characteristic of the receptor-controlled ion translocation and independent of these other properties of nerve and muscle cells. $\bar{J}$ can be used to calculate the value of J_A as well as the number of inorganic ions translocated per receptor site per unit time.

Estimates (4, 5) of the number of ions translocated per receptor-formed channel per unit time in membrane vesicles, made without knowledge of $\bar{J}$, are many orders of magnitude lower than the number determined by noise analysis with cells (6, 7) and raise the possibility that the receptors in the vesicles were impaired. The determination of $\bar{J}$, and therefore of the number of ions translocated per receptor-formed channel per unit time in membrane vesicles, is now possible because of the

following recent advances. (i) Techniques for studying transmembrane processes using membrane vesicles (8, 9) in conditions that are not limited by the metabolic restrictions present in intact cells have been developed. (ii) Kinetic techniques for the resolution of the receptor-controlled ion flux from other processes in the membrane preparations have been developed (10). (iii) Methods for separating the vesicles that contain functional receptors from those that do not are available (11). (iv) Flow-quench techniques have been adapted so that ion fluxes across vesicle membranes can be measured in the millisecond time region (12). (v) The rate of receptor-controlled ion translocation over a wide range of both carbamoylcholine and acetylcholine concentrations can be measured (12–15). The interpretation of all these measurements has led to the formulation of a minimum mechanism (Fig. 1) that relates the formation of ligand–receptor complexes to ion translocation (13, 14). The integrated rate equation (Eq. 1) based on this mechanism predicts the rate of receptor-controlled flux over the concentration ranges of carbamoylcholine (13, 14) and acetylcholine that we have investigated (15).

$$\frac{M_t}{M_\infty}$$

$$= 1 - \exp - J_m \{ ([\bar{A}L_2]_0 - [\bar{A}L_2]_\infty) \frac{1 - e^{-\alpha t}}{\alpha} + [\bar{A}L_2]_\infty t \} \quad [1]$$

* J_A is the observed rate coefficient associated with the initial fast influx before the onset of inactivation (desensitization) of the receptor. J_I is the observed rate coefficient associated with the slow phase that occurs after the inactivation process has gone to completion, and α is the rate coefficient for the inactivation process: $J_A = J_m ([\bar{A}L_2]_0)$; $J_I = J_m [\bar{A}L_2]_\infty$; $K_1 = 2[L][A]/[AL] = [L][AL]/2[AL_2]$; $K_2 = [L][IL]/2[IL_2]$; $\Phi = [AL_2]/[\bar{A}L_2]$. J_m is the rate coefficient for the ion flux associated with the open-channel form of the receptor. $\bar{A}L_2$ and AL_2 represent open-channel and closed-channel species, respectively. The symbol M refers to the ⁸⁶Rb⁺ content of the vesicles. The subscripts 0 and ∞ indicate the time at which the measurement was made. The value of the constants when carbamoylcholine is the ligand are known (14). Φ is the equilibrium constant between the closed- and open-channel forms of the receptor.

$$\left(\frac{J_m}{J_A} - 1 \right)^{1/2} = \Phi^{1/2} + [L]^{-1} K_1 \Phi^{1/2}, \quad [2]$$

where $(1 + \Phi)^{-1}$ gives the fraction of receptors in the open-channel form.

* On leave from Yamaguchi University, Yamaguchi, Japan.

FIG. 1. Acetylcholine receptor-controlled ion translocation. Minimum mechanism to account for the receptor-controlled flux of inorganic ions over a 200-fold concentration range of carbamoylcholine and a 160-fold concentration range of acetylcholine. Three states of the receptor are shown—the active form, $\textcircled{\text{A}}$, which gives rise to the open-channel form, $\textcircled{\text{A}}$, and the inactive form, $\textcircled{\text{I}}$. For artistic reasons the unbound form of the ligand has been omitted.

$$v_i = -d \frac{[M']}{dt} = \bar{J} [M'] [R'_0] (1 + \Phi)^{-1}, \quad [3]$$

where v_i is the initial velocity of efflux, M' is the concentration of inorganic ions inside the vesicles, and R'_0 is the moles of receptor per liter of internal vesicle volume.

$$J_A = \bar{J} [R'_0] (1 + \Phi)^{-1} = J_m (1 + \Phi)^{-1}, \quad [4]$$

where R'_0 is characteristic of a cell or a given vesicle population and Φ is characteristic of the activating ligand.

The measurements of the receptor-controlled ion flux discussed in this paper were made by using a flow-quench technique (12) and receptor-rich membrane vesicles prepared from *Electrophorus electricus* electroplax (4, 16). The receptor from this source has antigenic properties in common with the receptor found in muscle cells (17).

The following measurements were made to determine $\bar{J}$: (i) the effect of carbamoylcholine, over a 200-fold concentration range, on the rates of ion translocation—this has been reported previously (13, 14); (ii) the effect of acetylcholine, over a 160-fold concentration range, on the rates of ion translocation; (iii) the effect of inorganic ion concentration on the rate coefficients of the receptor-controlled flux; and (iv) the internal volume (18) and receptor-site density of those vesicles that contain functional receptors.

We found that the results of the two types of measurement of receptor function—chemical kinetic measurements using membrane vesicles and analysis of acetylcholine-induced noise in muscle cells (6, 7)—are in good agreement. Thus, we can now correlate the results of these two complementary kinetic techniques, one that relates the ligand binding and ion translocation processes in membrane vesicles and the other that measures the elementary steps in the formation of receptor channels in muscle cells.

RESULTS

Effect of Carbamoylcholine and Acetylcholine Concentrations on Initial Rate of Ion Translocation Process. The measurements and techniques used to evaluate the rate coefficients in Eq. 1 in the carbamoylcholine-induced flux of $^{86}\text{Rb}^+$ into electroplax membrane vesicles have been described (12–14). The value of J_m , the rate coefficient for ion flux associated with the open channel form of the receptor, can be obtained from the dependence of J_A on the concentration of activating ligand, L , by using Eq. 2 (14). Also, as we have reported previously, Eq. 2 is obeyed over a 200-fold range of carbamoylcholine concentration (Fig. 2A; ref. 14). Now, it can be seen that Eq. 2 is also obeyed over a 160-fold range of acetylcholine concentration. The value of J_m that gives the best fit of the data to Eq. 2 was obtained by iteration, and the coordinates of the lines were computed by using a least-squares computer program.

FIG. 2. Acetylcholine receptor-mediated influx of $^{86}\text{Rb}^+$ into membrane vesicles at pH 7.0 and 1°C . (A) Influx measurements in presence of carbamoylcholine ($\square$) or acetylcholine ($\circ$). Experiments were carried out in eel Ringer's solution (19) (169 mM NaCl/5 mM KCl/3 mM CaCl_2 /1.5 mM MgCl_2 /1.5 mM phosphate buffer, pH 7.0). All measurements of acetylcholine-induced influx were conducted in the presence of 0.05 mM Tetram (*O,O*-diethyl 5-diethylaminoethyl phosphorothiolate) to inhibit the acetylcholinesterase present in the preparations. Influx measurements with carbamoylcholine in presence and absence of Tetram gave results which were indistinguishable. The coordinates of the lines and the following constants were evaluated by using a least-squares computer program. For carbamoylcholine, $K_1 = 1.9$ mM, $\Phi = 2.8$, $J_A = 9.7$ sec $^{-1}$, $J_m = 37$ sec $^{-1}$, and $\chi^2 = 0.5$. For acetylcholine, $K_1 = 80$ μM , $\Phi = 1.5$, $J_A = 14.8$ sec $^{-1}$, $J_m = 37$ sec $^{-1}$, and $\chi^2 = 0.8$. The values of J_A given are for the saturating concentrations of the respective ligands. (B) Influx in presence of 2 mM carbamoylcholine and various concentrations of inorganic salts. Concentrations of CaCl_2 , MgCl_2 , and phosphate buffer were the same as in eel Ringer's solution, and concentrations of other inorganic ions were as indicated: $\blacktriangle$, 24 mM NaCl, 145 mM glucosamine; $\circ$, the ratio of NaCl to KCl was the same as in eel Ringer's solution, but their concentrations were decreased to give a total salt concentration of 93 mM; $\square$, the ratio of NaCl to KCl was the same as in eel Ringer's solution, but the concentrations were increased to give a concentration of 360 mM. The flow-quench apparatus, the method of measurement, the evaluation of the rate coefficients, and the chemicals used have been described (12–14). The method for making the vesicles (4, 16) has been modified to obtain larger quantities of a kinetically uniform vesicle preparation and somewhat lower rates of receptor-controlled ion translocation ($J_A = 1.7 \pm 0.4$ sec $^{-1}$ in presence of 1 mM carbamoylcholine) than previously reported (14). [^3H]glucosamine (62 $\mu\text{Ci}/\text{ml}$; 1 Ci = 3.7×10^{10} becquerels) was used to determine that, within the time of the measurements (2 min), the movement of this amino sugar through the membrane was negligible both in the absence and the presence of 2 mM carbamoylcholine. D-(+)-glucosamine and [^3H]glucosamine were obtained from Eastman and Amersham, respectively. Gramicidin D (G-5002) was supplied by Sigma.

The same value of J_m (37 sec $^{-1}$) gives a satisfactory fit for both the acetylcholine and the carbamoylcholine data [$\chi^2 < 0.8$ (20)]. The abscissa intercept provides the value of K_1 , and the ordinate

intercept provides the value of $\Phi^{1/2}$. One prediction of the minimum mechanism, the independence of J_m but the dependence of Φ on the activating ligand (14), is borne out by the data shown in Fig. 2A.

Effect of Inorganic Ion Concentration on the Rate Coefficients. J_A is independent of the internal concentration of sodium and potassium ions in the physiologically interesting region (24–360 mM) (Fig. 2B). The solid line in Fig. 2B is based on the mean value of all the $^{86}\text{Rb}^+$ influx measurements made in the presence of 2 mM carbamoylcholine. The line was computed by using Eq. 1, a nonlinear least-squares computer program that gives the coordinates of the line, and the following values: $J_A = 4.1 \text{ sec}^{-1}$, $J_I = 0.02 \text{ sec}^{-1}$, $\alpha = 3.0 \text{ sec}^{-1}$, and $\chi^2 = 0.5$ (20). The respective values obtained are the same, within experimental error, in presence of low and high concentrations of inorganic ions. In addition, the data indicate that the rate coefficients are independent of ionic strength. Glucosamine, which was used to adjust the ionic strength, does not detectably cross the membrane during the measurements.

Isolation and Characterization of Vesicles that Contain Functional Receptors. A method has been devised to separate vesicles that contain functional receptors from those that do not (11). The principle of the method is that one can equilibrate a heterogeneous vesicle population with CsCl and then exchange the Cs^+ for Na^+ in only those vesicles that contain functional receptors (11). The vesicles can then be separated on the basis of their density by using a CsCl/sucrose or a CsCl/Percoll density-gradient centrifugation (21). The results of a typical experiment are shown in Fig. 3. The extent to which the vesicles were loaded with tracer ions was determined in two ways. One sample was exposed to $^{86}\text{RbCl}$ and 2 mM carbamoylcholine for 30 sec, and then the $^{86}\text{Rb}^+$ content was determined by using a Millipore filter assay (4). The second sample was allowed to equilibrate with $^{86}\text{RbCl}$ of the same specific activity for 20 hr; the inorganic ions in the vesicles, whether they contain func-

FIG. 3. Purification of vesicles containing functional receptors by using a 190 mM CsCl/Percoll density-gradient centrifugation. Fraction numbers start from the bottom of the centrifuge tube, and each fraction contains ≈ 1 ml. The procedure for the purification of vesicles containing functional receptors differs from the one previously reported (11) as follows: Instead of a continuous sucrose/190 mM CsCl density-gradient, a 15% Percoll/190 mM CsCl density-gradient was used (21). Three milliliters of membrane solution (10 mg of protein/ml) were mixed with 35 ml of the Percoll/CsCl solution. The solutions were centrifuged for 30 min at 15,500 rpm in a Sorvall centrifuge with an SS-34 rotor. Each 1-ml fraction was collected separately. Influx was allowed to proceed for 30 sec in the presence of 2 mM carbamoylcholine. The details of the influx assays and the determination of the tracer content of the vesicles by using gramicidin (18) and of the determination of the protein concentration of the membrane vesicles (10, 21) have been described. The purification and characterization of α -bungarotoxin and the method used to determine the concentration of toxin-binding sites have been described (22, 23). Percoll was obtained from Pharmacia.

tional receptors or not, equilibrate with those in the external solution within this time (10). The $^{86}\text{Rb}^+$ content of the vesicles was then determined by measuring the efflux of tracer in the presence of gramicidin (25 $\mu\text{g}/\text{ml}$) (18), which releases $^{86}\text{Rb}^+$ from all the vesicles present. When these experiments were done with the most active vesicle fraction (fraction 35, see Fig. 3) the $^{86}\text{Rb}^+$ content of the vesicles was found to be the same in both experiments. This indicates that within the error of the measurements, the fraction contained only vesicles that responded to carbamoylcholine.

From the $^{86}\text{Rb}^+$ content of the vesicles and the specific activity of the solution in which the vesicles were incubated, one can calculate their internal volume. The calculations are based on reports that $^{86}\text{Rb}^+$ is an effective replacement for K^+ (24) and that receptor channels do not discriminate between K^+ and Na^+ (25). The internal volume (18), determined in 10 experiments, each with a different membrane preparation, each from a different eel, was $2.0 \pm 0.3 \mu\text{l}/\text{mg}$ of membrane protein. The concentration of α -bungarotoxin-binding sites in the most active fraction, determined in seven different membrane preparations, each from a different eel, was $4.8 \pm 0.7 \text{ pM}/\text{mg}$ of membrane protein. We have shown previously that the stoichiometry of α -bungarotoxin sites and receptor-ligand binding sites is 1:1 in electroplax membranes (16, 23) and that the binding of two ligand molecules is required to initiate ion translocation (14). From the concentration of toxin-binding sites and the internal volume of the vesicles, the receptor concentration, $[R_0]$, is calculated to be $1.2 \mu\text{M}$, where the prime indicates that we are considering moles per liter of internal volume of the vesicles.

DISCUSSION

When the rate coefficients for the receptor-controlled flux are independent of inorganic ion concentration (see Fig. 2B) and proportional to the number of receptor-formed ion channels through the membrane, the rate expression for the ion translocation process is given by Eq. 3. As $J_m = \bar{J} [R_0]$ (Eqs. 3 and 4), the value of $\bar{J}$ depends on two experimentally determined quantities only: J_m , which is based on the ligand concentration dependence of the influx over a wide range of both carbamoylcholine and acetylcholine concentrations (see Fig. 2A), and R_0 , which has been determined here and previously (14). The value for $\bar{J}$ ($3 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ at pH 7 and 1°C) falls within the range of bimolecular rate constants typically observed in small molecule-protein reactions (26). $\bar{J}$ may, however, contain the rate coefficients for several different elementary steps; for example, the diffusion of inorganic ions past an energy barrier in the channel.

Having obtained the value of $\bar{J}$, we can now relate the analysis of electrical noise, induced by acetylcholine in cells, to chemical kinetic measurements using membrane vesicles, because both techniques allow us to determine the number of ions translocated per receptor site per unit time. Originally, it was estimated that in vesicles prepared from the electroplax of *E. electricus*, less than 1 ion was transferred per receptor site per msec (4). In investigations using vesicles prepared from *Torpedo* electroplax (5), the transfer of 3.5 ions per α -bungarotoxin-binding site per msec was reported. These values are lower, by several orders of magnitude, than those determined by electrophysiological measurements with cells (6, 7) and in the flow-quench measurements with *E. electricus* vesicles reported here. We found that 6×10^3 ions are translocated per receptor-formed channel per msec. This number, which is obtained by multiplying $\bar{J}$ by $[M']$ (200 mM), is in excellent agreement with previous measurements (11) in which the functional vesicles were

isolated on a CsCl/sucrose density gradient. In those experiments, using $^{22}\text{Na}^+$, it was determined that the vesicles contained 10^5 inorganic ions per toxin site per mg of vesicle protein. Using the value of J_m determined here, we calculate that 7×10^3 ions are transferred per receptor site per msec (2×10^5 ions per receptor site $\times 0.037 \text{ msec}^{-1}$). These values, determined at pH 7.0 and 1°C , can be compared with electrical noise analysis in frog muscle cells, which give a value of $\approx 1 \times 10^4$ ions translocated per receptor-formed channel per msec at 8°C (7). This value depends on temperature (6), transmembrane voltage (7), and, as we have shown here (see Fig. 2B), on the internal concentrations of NaCl and KCl.

Many of the factors that contributed to the very low estimates by Kasai and Changeux (4) of channel efficiency have been discovered. Only a small fraction of the vesicles in the heterogeneous preparation (4) contained functional receptors, and ion fluxes from vesicles that did not contain functional receptors dominated such measurements (10). The high concentrations of KCl used (4) inactivate the receptor in eel vesicles (27).

The determination of $\bar{J}$ enables one to calculate the initial rate, J_A , of the receptor-controlled translocation of ions across the membrane of a particular cell by using Eq. 4. We can now also appreciate the short-comings of chemical kinetic measurements of receptor-controlled flux in cells. Ion flux measurements made by using cultured muscle cells led to a low estimate of receptor-channel efficiency (28). From toxin binding data (28) and the internal volume of muscle cells ($5 \mu\text{l}/\text{mg}$ of cell protein; unpublished results), we can calculate that $[R_0] = 20 \text{ nM}$, which corresponds to a J_A value of 0.3 sec^{-1} at saturating carbamoylcholine concentrations. If we assume that the maximal rate for receptor inactivation ($\alpha_{\text{max}} = 7 \text{ sec}^{-1}$) is similar in eel vesicles and in cultured muscle cells, we can calculate, by using Eq. 1, that less than 4% of the total influx occurs during the initial fast phase, which terminates before the first measurement made in the muscle cell experiments (28). The observed influx was, therefore, due to a mixture of receptor forms (A and I; see Fig. 1) in which the active channel-forming species ($\bar{A}L_2$) constituted only a small percentage of the total. However, the efficiency of the ion translocation was calculated (28) by assuming that all the receptors present could give rise to open channels.

Φ (the channel-opening equilibrium constant) is smaller for acetylcholine than it is for carbamoylcholine (Fig. 2A). This means that, at saturating concentrations of ligand, the concentration of receptor-formed channels is greater in the presence of acetylcholine than it is in the presence of carbamoylcholine. Analysis of acetylcholine-induced noise in cells indicates that the mean time the receptor-formed channels are open depends on the activating ligand (7) and that it is longer in the presence of acetylcholine than in the presence of carbamoylcholine. Unlike Φ , the mean time the channel is open determines neither the initial rate of ion translocation nor the initial change in the transmembrane potential of a cell, which are controlled by both the rates of opening and closing. An activating ligand characterized by a large mean open time of the channel may conceivably be associated with a small channel-opening equilibrium constant (Φ) and a low value of J_A .

In principle, the statistical method (noise analysis) and the measurements of the rate of macroscopic changes in concentration used in our studies are capable of producing equivalent and complementary results. In practice, each method has its advantages. The techniques for obtaining important information about elementary steps in the channel-forming process, first developed by Katz and Miledi (6), have been elegantly advanced by Neher and Sakmann (29). These techniques have been particularly useful at low concentrations of ligand (6, 7,

29). To obtain information about the relationship between ligand binding mechanisms and ion translocation, the flow-quench method for measuring fast fluxes in vesicles (12) has a number of advantages. The solution compositions inside and outside the vesicles can be varied and known accurately. The number of receptors exposed to a definite concentration of ligand can be determined. Measurements can be made before inactivation (desensitization) of the receptor by the receptor ligand used. A wide range of ligand concentrations can, therefore, be used in the measurements, which have a precision comparable with that obtained with regulatory enzymes in solution (13, 14). In addition, the dependence on ligand concentration of four different properties—ion translocation, receptor inactivation, recovery from inactivation, and the rate of ion translocation mediated by equilibrium mixtures of active and inactive receptor forms—of the receptor-controlled translocation of ions can be investigated (13). Each phase exhibits simple kinetics that allow the use of simple analytical expressions for the concentration dependence of each process (13, 14). Chemical kinetic techniques will, however, fail both when $[R_0]$ is too low, as in cultured muscle cells, and when it is too high. In the latter case, the time resolution of the flow-quench technique will not be sufficient.

Both the determination of the specific reaction rate for the receptor-controlled translocation of ions and the results of noise analysis enable one to calculate the number of ions translocated per receptor-formed channel in a given cell. Therefore, it is now possible to integrate the results obtained in the two different types of measurement of receptor function, chemical kinetics and noise analysis, which relate the ligand binding and ion translocation processes in membrane vesicles, and to measure the elementary steps in the formation of receptor-formed ion channels in muscle cells.

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