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ACETYLCHOLINE-INDUCED RECEPTOR-CONTROLLED ION FLUX INVESTIGATED BY FLOW QUENCH TECHNIQUES

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Summary: Using a quench flow technique with membrane vesicles, the acetylcholine receptor-controlled transmembrane ion flux and the inactivation of the receptor with acetylcholine were measured in the msec time region. The ion flux was followed by influx of radioactive tracer ion and the inactivation was followed by an ion flux assay of receptor pre-incubated with ligand. The measurements covered a concentration range to complete saturation of the active state of the receptor with ligand, and were consistent with a minimal model previously proposed on the basis of experiments with carbamylcholine. The ion translocation rate at saturation with acetylcholine is about twice that at saturation with carbamylcholine and this reflects a more favored channel opening equilibrium for acetylcholine.

The electric organ of the electric eel, Electrophorus electricus (1) has been a convenient source of material for the study of the acetylcholine receptor. The presence of an acetylcholine receptor with antigenic determinants in common with those of a muscle cell (2) in a membrane which is excitable (3) provides a model for the neuromuscular junction. The generous size of the organ provides sufficient quantities for the characterization of physical and chemical properties and the large size of the electroplax cells has been convenient for electrophysiological studies (4, 5). The preparation from electroplax of membrane vesicles containing the acetylcholine receptor (6) allowed the study of the receptor-controlled transmembrane ion translocation, which determines the magnitude of the receptor controlled change in the electrical membrane potential, in a model system free of whole cells. This system enables the time course of transmembrane ion flux to be followed with a known uniform constant concentration of ligand with vesicles of which the volume and number of receptors can be characterized. The results of such measurements, complementary to electrophysiological studies, are expected to be in agreement with them.

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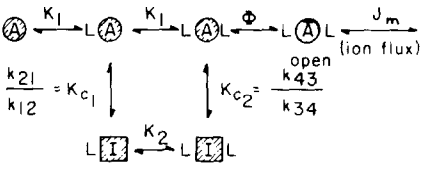
Early measurements (6) of the acetylcholine receptor-mediated ion flux in electroplax membrane vesicles in which radioactive tracer ions were used gave rates per receptor which were much smaller than those indicated by electrophysiological measurements (7). The important sources of this discrepancy were firstly the inclusion in the measurements of the total non-specific ion flux in addition to the receptor-mediated ion flux exhibited by a small portion of the total vesicle population (8). Secondly in the conditions used, with ligand at concentrations lower than required to saturate the active state of the receptor, inactivation is relatively faster than ion flux (11) and the ion flux observed proceeds at the rate of the inactivated receptor (11, 12).

We have recently reported a study of the ligand dependence of ion flux with acetylcholine receptor-containing vesicles with carbamylcholine over a 200-fold concentration range covering saturation of the receptor in its active state (11). The results were consistent with the minimum mechanism shown in Fig. 1. Ion flux required two bound ligand molecules while inactivation of receptor proceeded with only one bound ligand molecule. Thus inactivation could occur without channel opening. We are now reporting that with acetylcholine as the ligand, also over a concentration range covering saturation of the active state of the receptor, the ion flux and inactivation rates again follow the integrated rate equation (1) derived from the reaction scheme in Fig. 1.

Ion translocation was followed with the quench flow technique as in our previous reports (11-13). The radioactive isotope tracer ion was rubidium-86, an analog of potassium (15). Influx of tracer was initiated by mixing the membrane vesicles with the acetylcholine solution containing ^{86}Rb. The ion flux was terminated by admixture with curare after a predetermined reaction time, and the radioactive content of the vesicles was measured with a millipore filter assay (11). The membrane vesicle preparation at 800 µg protein per ml contained Tetram (0,0-diethyl-S-diethylaminoethyl thiophosphate) to inhibit acetylcholinesterase.

Minimum Mechanism



$$\frac{M_t}{M_\infty} = 1 - \exp - J_m\left\{\left(\left[\bar{A}L_2\right]_o - \left[\bar{A}L_2\right]_\infty\right)\frac{1-e^{-\alpha t}}{\alpha} + \left[\bar{A}L_2\right]_\infty t\right\} \qquad (1)$$

Fig. 1: The minimum mechanism (11) which accounts for the observed rates of acetylcholine receptor-mediated ion translocation and receptor inactivation and their dependence on the concentrations of acetylcholine and carbamylcholine. The active forms of the receptor, A, and the inactive forms, I, bind ligand, L, with different affinities. Active receptor with two bound ligands, AL_2 is in equilibrium with the open channel form, $\bar{A}\bar{L}_2$, where $1/\Phi$ is the equilibrium constant for opening the channel. $\bar{A}\bar{L}_2$ permits ion flux with a first order rate constant J_m. Ligand binding and channel opening processes are assumed to be rapid compared with all other processes. Conversions between the active and inactive forms occur with the first order rate constants, k which are measurable with our techniques.

Equation 1 is the integrated rate equation pertaining to this scheme where

$$J_A = J_m\left((\bar{A}\bar{L}_2)_o - (\bar{A}\bar{L}_2)_\infty\right)$$

$$J_I = J_m\,(\bar{A}\bar{L}_2)_\infty$$

$$K_1 = \frac{2(L)(A)}{(AL)} = \frac{(L)(AL)}{2(AL_2)}$$

$$K_2 = \frac{(L)(IL)}{2(IL_2)} \qquad \Phi = \frac{(AL_2)}{(\bar{A}\bar{L}_2)}$$

J_A is the observed rate constant of the initial fast influx prior to inactivation of the receptor. J_I is the observed rate constant of the slow phase which occurs after inactivation. α is the rate constant for inactivation. The subscripts o and ∞ denote concentrations immediately and at equilibrium after ligand has been added. M represents the radioactive tracer concentration inside the vesicles and so M_t/M_∞ is the fraction of total equilibration which has occurred at time, t.

A typical influx curve with a saturating concentration of acetylcholine shown in Fig. 2a illustrates that our time resolution is adequate to follow the whole course of ion flux with the active receptor. Saturating ligand concentration was established by showing that the same influx kinetics is observed over a wide range of acetylcholine concentration at saturation with ligand. The first point is at 7 msec and the equilibration is essentially complete at

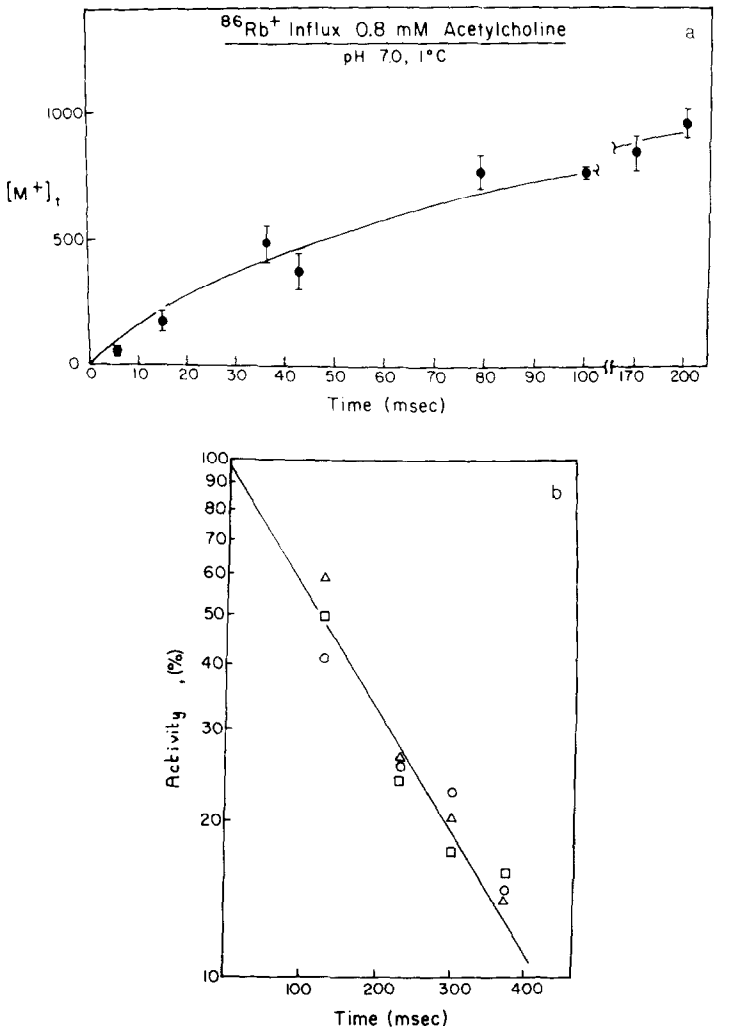


Fig. 2: Acetylcholine induced ion flux in membrane vesicles, pH 7.0, 1°C. (a) Ion translocation with a saturating concentration (0.8 mM) of acetylcholine. The membrane vesicles equilibrated with eel Ringer's solution (168 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.5 mM sodium phosphate, pH 7.0) treated with 0.05 mM Tetram (0,0-diethyl-S-diethylaminoethyl thiophosphate), an acetylcholine esterase inhibitor, were mixed with an equal volume of acetylcholine with ^{86}Rb (100 μCi/ml) in eel Ringer. After the time on the abscissa, the mixture was mixed with the same volume of 30 mM d-tubocurarine and the vesicles were separated and washed with the Millipore filter assay (11). The ^{86}Rb content of the vesicles was measured by scintillation counting. The same procedure was followed in absence of ligand, and the difference between these two measurements at each time is plotted against reaction time. The points are experimental, and the line is calculated from equation 1 with $J_A = 14.5\ sec^{-1}$, $J_T = 0.01\ sec^{-1}$ and $\alpha = 5.6\ sec^{-1}$. (b) Inactivation of the acetylcholine receptor by 100 μM acetylcholine. After incubation for the times on the abscissa the activity was assayed in a second incubation of 1.2 sec with 5 mM carbamylcholine and $^{86}RbCl$. In the control experiment, ligand was omitted from the first incubation. The remaining activity is plotted as a function of incubation time with 100 μM acetylcholine (12).

All other details of the experimental procedure, the evaluation of the rate coefficients, and the source of the chemicals used has been given previously (11).

200 ms. With lower acetylcholine concentrations inactivation becomes important during the course of the ion translocation and the deviation from first order kinetics due to a continually decreasing number of active receptors during ion flux becomes apparent. The ion flux curves follow equation 1 (Fig. 1). where $\frac{M_t}{M_\infty}$ is the fraction of the ion influx relative to the equilibration of tracer ion. J_A, α and J_I are the first order rate constants for ion flux with receptor in the active state, for inactivation of receptor and for ion flux with the equilibrium mixture of inactive and active receptor at the particular ligand concentration respectively. After inactivation is completed the ion flux continues again with first order kinetics (8, 10) at the rate given by J_I (11, 12).

With intermediate concentrations of ligand, α as well as J_A can be determined by analysis of the ion flux curves (equation 1) at short reaction times (up to a few seconds). With high ligand concentrations (ion flux is complete before much inactivation) or low ligand concentrations (ion flux is very small when inactivation is completed) the determination of α from influx measurements is not accurate. Therefore we have measured the rate of inactivation independently by a different method which we previously described using carbamylcholine (12) and which we now report using acetylcholine. Using the quench flow technique with two incubations, the membrane vesicles are first mixed with acetylcholine and after a predetermined incubation time the mixture is mixed with 5 mM carbamylcholine and radioactive tracer ion to determine the activity. These determinations of α agreed with the values from analysis of the ion flux curves at intermediate concentrations of ligand. A typical experiment is shown in Fig. 2b.

The ligand dependence of the ion flux via active receptor, J_A and of receptor inactivation, α is given for acetylcholine in Fig. 3. While J_A follows a sigmoid dependence on ligand concentration, α does not. The saturating value for ion flux is about twice as high for acetylcholine as for carbamylcholine (11)

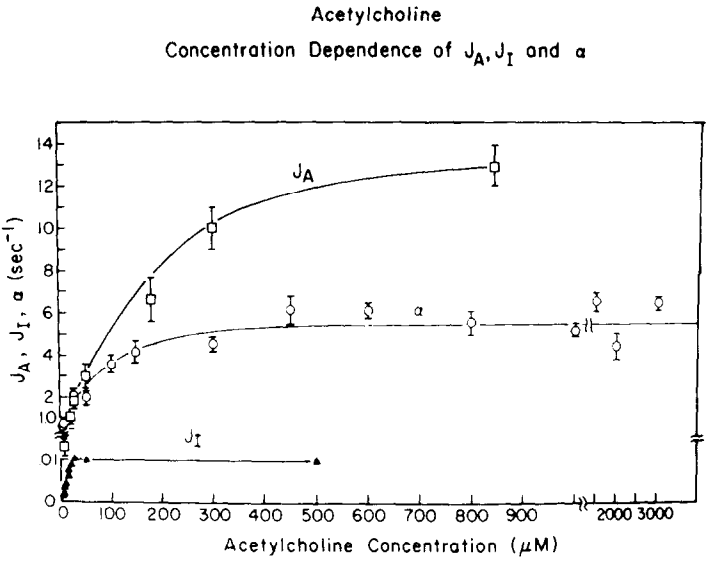


Fig. 3: The rate constants for initial flux, J_A, flux after inactivation, J_I, and inactivation of receptor, α as a function of acetylcholine concentration. J_A was determined in the ion flux measurements (e.g. Fig. 2a), α was determined by ion flux after preincubation with ligand (e.g. Fig. 2b) and also from ion flux measurements. J_I was determined by a method of sampling a single incubation described elsewhere (14).

These ion flux rates are somewhat lower than those previously reported (11) after slight modification of the preparation (16) to give larger quantities of a kinetically homogeneous membrane vesicle population. This variation of J_A for the two ligands results from different values for the channel opening equilibrium constant, $1/\Phi$ and is consistent with a single common value for ion flux through the open form of the channel (17). The saturating rate constants for ion flux with the equilibrium concentration of active and inactive receptor, J_I are slower than J_A by a factor of 1300 for acetylcholine and 500 for carbamyl choline (11).

In conclusion, the measurements of ion flux and inactivation of receptor with acetylcholine are consistent with the minimal reaction scheme previously proposed, on the basis of experiments with carbamylcholine. With acetylcholine the channel opening equilibrium for receptor with two bound ligands is more favored than with carbamylcholine, giving rise to a larger ion flux at saturation with acetylcholine.

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