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Acetylcholine Receptor: Evidence for a Voltage-Dependent Regulatory Site for Acetylcholine. Chemical Kinetic Measurements in Membrane Vesicles Using a Voltage Clamp†

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ABSTRACT: Acetylcholine receptor mediated ion translocation in membrane vesicles prepared from the *Electrophorus electricus* electroplax was investigated under voltage clamp conditions by using a quench-flow technique that allows the translocation to be measured in the millisecond to second time region. Two rate coefficients were measured over a 500-fold concentration range of acetylcholine, at a transmembrane voltage, V_m, of −45 mV, at pH 7.0, 1 °C. J_A is the rate coefficient for ion translocation by the active state of the receptor in the absence of inactivation (desensitization), and α is the rate coefficient for the inactivation of the receptor by acetylcholine. (1) The values of J_A and α increase with increasing acetylcholine concentration up to 300 μM. At higher concentrations, a concentration-dependent decrease in the ion flux rate was observed without a concomitant change in the inactivation rate. This inhibitory effect has not been reported previously and was not observed with acetylcholine or carbamoylcholine in the absence of a transmembrane voltage. (2) The value of the maximum influx rate coefficient, 26 s^{-1}, is approximately twice that observed at 0 mV [$J_A(max)$ = 15 s^{-1}]. This is consistent with previous interpretations that related $J_A(max)$ values to the channel-opening equilibrium constant, $1/\Phi$, and with the relation of $1/\Phi$ to the mean lifetime of the open receptor channel in muscle cells, which is dependent on V_m. (3) The maximum observed inactivation rate coefficient [$\alpha(max)$ = 8.5 s^{-1}] is somewhat larger than that observed at 0 mV [$\alpha(max)$ = 5 s^{-1}]. The consequence of the newly discovered voltage-dependent regulatory site, characterized by a dissociation constant K_R = 800 μM (V_m = −45 mV), is that the ability of the receptor to translocate ions, and therefore to initiate the transfer of signals between cells, depends significantly and unsuspectedly on the resting transmembrane voltage of the cell. The regulatory site for acetylcholine can be fitted into a general model, proposed in this paper, which also explains activation, inactivation, and voltage-dependent modulation of the receptor function.

Chemical kinetic investigations of acetylcholine receptor controlled ion flux in *Electrophorus electricus* membrane vesicles, using carbamoylcholine or acetylcholine as the activating ligand, led to the proposal of a minimum mechanism that relates ligand binding to ion fluxes (Cash & Hess, 1980; Aoshima et al., 1981; Cash et al., 1981) [see left-hand side of Figure 1 in the preceding paper (Pasquale et al., 1983)]. More recently, the mechanism was extended [see Figure 1 in the preceding paper (Pasquale et al., 1983)] on the basis of studies with suberyldicholine, which indicated the existence of a regulatory site that exerts its inhibitory action at high (>50 μM) suberyldicholine concentrations (Pasquale et al., 1983). A description of the model and the definitions of the constants used appear in Figure 1 of the preceding paper (Pasquale et al., 1983) and in its legend. The equations pertaining to the model appear in Table I of the preceding paper (Pasquale et al., 1983). The existence of an inhibitory binding site has been proposed previously in order to explain the effect of local anesthetics (Adams, 1977; Koblin & Lester, 1978; Heidman & Changeux, 1978; Neher & Steinbach, 1978). The interaction of positively charged local anesthetics with the receptor is voltage dependent and increases as the transmembrane voltage becomes more negative (Neher & Steinbach, 1978; Colquhoun, 1981).

All the available evidence indicates that local anesthetics and other noncompetitive inhibitors act primarily at sites other than the acetylcholine-binding site (Steinbach, 1968; Cohen

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et al., 1974). Here, we present evidence that a regulatory voltage-dependent binding site for acetylcholine exists on the acetylcholine receptor, a site that is distinct from the sites that are involved in the opening of receptor channels and in the inactivation of the molecule. The biological significance of this property of the receptor may be that the receptor-controlled transmission of signals between cells may be variable and adjustable, depending on the transmembrane potential of the cells.

Experimental Procedures

Materials. Membrane vesicles were prepared from the electric organ of *E. electricus* (World Wide Scientific Animals, Apopka, FL) according to the method of Kasai & Changeux (1971) with a number of modifications (Fu et al., 1976; Sachs et al., 1982). The vesicles finally obtained were suspended in buffer A (90 mM NaCl, 1 mM $CaCl_2$, 1.5 mM sodium phosphate buffer, pH 7.0) and stored in liquid N_2 until used. In some experiments, the vesicles were suspended in buffer B (90 mM KCl, 1 mM $CaCl_2$, 1.5 mM sodium phosphate buffer, pH 7.0) or buffer C (400 mM NaCl, 1 mM $CaCl_2$, 1.5 mM sodium phosphate buffer, pH 7.0). The influx rate and its inactivation rate measured with the vesicles in these buffers are the same as in *E. electricus* Ringer's solution (169 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.5 mM sodium phosphate buffer, pH 7.0) [also see Hess et al. (1981)]. To inhibit the activity of acetylcholinesterase, 50 μM Tetram [*O,O*-diethyl *S*-[2-(diethylamino)ethyl] thiophosphate (a gift from Dr. R. D. O'Brien)], was added to the vesicle suspension. $^{86}RbCl$ (in 0.5 M HCl) obtained from New England Nuclear was neutralized with 0.5 M NaOH and diluted with H_2O to give a stock solution, 10 mCi/mL in 0.2 M NaCl. Acetylcholine bromide and *d*-tubocurarine were obtained from Eastman Kodak and Calbiochem-Behring Corp., respectively. All other chemicals were reagent grade.

Establishment of a Voltage Difference across the Vesicle Membranes. We have shown previously that Na^+, K^+, and Cl^- in *E. electricus* vesicles exchange very slowly ($t_{1/2} > 0.5$ h) with ions in the external medium (Hess & Andrews, 1977) when no activating ligand is present. Therefore, if an asymmetric distribution of monovalent cations exists across the membrane and the osmotic balance is maintained in the solutions by use of an impermeant cation, then making the vesicle membrane selectively permeable only to the asymmetrically distributed cation should result in the formation of a Nernst voltage across the membrane. In the experiment illustrated in Figure 1a, different asymmetric distributions of K^+ ($[K^+]_{inside} > [K^+]_{outside}$) existed across the vesicle membrane. The distribution of a tracer cation, $^{86}Rb^+$, across the vesicle membrane, which had been made permeable to both K^+ and $^{86}Rb^+$ by the addition of the ionophore valinomycin, was used as an indicator of the transmembrane voltage, and we see that the Nernst equation is obeyed (Figure 1a).

When the concentration of $^{86}Rb^+$ is the same inside the vesicles as in the external solution, it follows that the ratio of the $^{86}Rb^+$ content of the vesicles in the presence of a transmembrane voltage, V_m, to that in the absence of a transmembrane voltage (V_m = 0 mV) will be given by eq 1 (see Equations).

We used arginine chloride in the external solutions to maintain the osmotic balance and constant ionic strength in the solutions on either side of the membrane. We ascertained that in the time period required for our measurements, at most 3 s, the exposure of the vesicles to arginine does not affect the values of J_A and α when $^{86}Rb^+$ influx was measured in the presence of 3 mM acetylcholine (Figure 1b) and 600 μM acetylcholine, respectively (data not shown). We also ascertained that, in the presence of 1 mM acetylcholine, there is no detectable transmembrane flux of [^{3}H]arginine in the second to minute time region when the concentrations of arginine are those used in our experiments. In the presence of activating ligands, both monovalent cations (Hess et al., 1979) and Ca^{2+} (E. B. Pasquale, K. Takeyasu, and G. P. Hess, unpublished results) exchange in the millisecond time region. Opening of the channels in the presence of an asymmetric distribution of Na^+ and Ca^{2+} allows rapid exchange of cations, which produces a transmembrane voltage that is maintained (clamped) by the relatively immobile chloride ions (Kim & Hess, 1981). The voltage across a membrane permeable to both Na^+ and Ca^{2+} is given by eq 2 (see Equations), which is obtained from the Nernst–Planck equation.

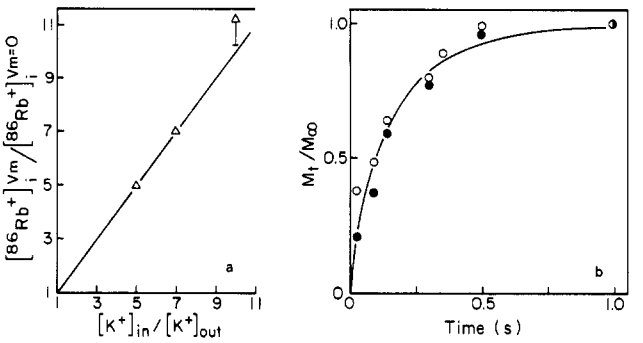

FIGURE 1: (a) Distribution of $^{86}Rb^+$ according to a transmembrane voltage that was produced by the asymmetrical distribution of K^+ inside and outside the vesicles in the presence of valinomycin. The membrane vesicles (100 μg of protein/mL) equilibrated with buffer B (see Experimental Procedures) were mixed with an isotonic arginine solution (90 mM arginine chloride, 1 mM $CaCl_2$, 1.5 mM sodium phosphate buffer, pH 7.0) to give a ratio of K^+ concentration outside to inside the vesicles as indicated on the abscissa. $^{86}Rb^+$ (50 μCi/mL) and valinomycin (5 μM) were added to the vesicle suspension for 12 s, and the reaction mixture was then applied to a Millipore filter and washed with 10 mL of buffer A (see Experimental Procedures). In control experiments (V_m = 0 mV), the vesicles were diluted with buffer B. The distribution of $^{86}Rb^+$ was expressed as the ratio of $^{86}Rb^+$ content of the vesicles in the presence of a transmembrane voltage to that in the absence of the voltage. The points were obtained experimentally, and the line was calculated by using the Nernst equation and eq 1 (see Experimental Procedures). (b) Arginine does not affect the function of the acetylcholine receptor. By use of a quench-flow apparatus, the flux of $^{86}Rb^+$ into the membrane vesicles was measured in the presence (●) and absence (○) of 80 mM arginine chloride. One volume of the membrane vesicles (1.6 mg of protein/mL) equilibrated with buffer C was diluted with 9.5 volumes of either buffer C or a buffer containing 311.4 mM NaCl, 88.6 mM arginine chloride, 1 mM $CaCl_2$, and 1.5 mM sodium phosphate buffer, pH 7.0, in the presence of $^{86}Rb^+$ and 3 mM acetylcholine. The final concentrations of Na^+ inside and outside the vesicles were 403 and 324 mM (containing 1 mM Na^+ from the $^{86}Rb^+$ stock solution), respectively, in the presence of 80 mM arginine chloride. This produces a negligible transmembrane voltage (eq 2, Experimental Procedures). The line was drawn by using the values J_A = 11 s^{-1} and α = 5 s^{-1} calculated from eq 3 (Table I in the preceding paper).

Measurement of $^{86}Rb^+$ Influx and Receptor Inactivation in the Presence of a Transmembrane Voltage. The ligand incubation and ion flux measurements were performed at a transmembrane voltage V_m of −45 mV at 1°C. The quench-flow technique used in measurements of ion flux with these vesicles (Hess et al., 1979) has been described in detail (Cash & Hess, 1981). One volume of the vesicles (1.6 mg of protein/mL) equilibrated with buffer A was mixed with 9.5 volumes of isotonic arginine solution (90 mM arginine chloride, 1 mM $CaCl_2$, 1.5 mM sodium phosphate buffer, pH 7.0) in the quench-flow apparatus itself. Hence, during both types of measurements (see legend to Figure 2 for more details), the concentrations of Na^+ inside and outside the vesicles were 93

and 12.5 mM, respectively, while the concentration of Ca^{2+} was the same (1 mM) on either side of the membrane. According to eq 2, this distribution of ions across the membrane results in a transmembrane voltage of −45 mV. Linear and nonlinear least-squares programs were used to obtain an optimal fit of the data to theory (Bevington, 1969).

Equations. Relationship between Transmembrane Voltage and Value of J_A and Concentration of Inorganic Ions.

$$[^{86}Rb^+]_i^{V_m}/[^{86}Rb^+]_i^{V_m=0} = e^{-V_mF/(RT)} \quad (1)$$

where $[^{86}Rb^+]_i^{V_m}$ is the $^{86}Rb^+$ content of vesicles allowed to equilibrate with external $^{86}Rb^+$ in the presence of a transmembrane voltage, V_m, $[^{86}Rb^+]_i^{V_m=0}$ is the $^{86}Rb^+$ content of vesicles allowed to equilibrate with external $^{86}Rb^+$ when V_m = 0 mV, and F, R, and T represent the Faraday constant, the gas constant, and absolute temperature, respectively.

$$\frac{d([Na^+]_i + [Ca^{2+}]_i)}{dt} = 0 = f_oJ_A[Na^+]_o + 2f_o'J_A'[Ca^{2+}]_o - f_iJ_A[Na^+]_i - 2f_i'J_A'[Ca^{2+}]_i \quad (2)$$

where J_A and J_A' are the rate coefficients for ion translocation in absence of inactivation and transmembrane voltage for Na^+ and Ca^{2+}, respectively, and the ratio of J_A'/J_A = 0.5 (E. B. Pasquale, K. Takeyasu, and G. P. Hess, unpublished results). The subscripts i and o refer to the inside and outside of the vesicles. f and f' are the Planck factors for monovalent and divalent ions, respectively, which take into account the acceleration and retardation of the ions in the electric field (Planck, 1890) where f_i and $f_i' = [ZV_mF/(RT)](1 - \exp[-ZV_mF/(RT)])^{-1}$, f_o and $f_o' = [ZV_mF/(RT)](\exp[ZV_mF/(RT)] - 1)^{-1}$, $f_o/f_i = \exp[-ZV_mF/(RT)]$, and Z represents the valency of the ion.

Results

In Figure 2a is shown the $^{86}Rb^+$ content of vesicles at different times after the addition of acetylcholine (60 μM) in the presence and absence of a transmembrane voltage of −45 mV. The determination of J_A, the influx rate coefficient, by use of eq 3 of Table I of the preceding paper (Pasquale et al., 1983), and the derivation of the equation have been described in detail (Hess et al., 1983a). The determination of α in the presence and absence of transmembrane voltage is shown in Figure 2b. The evaluation of α has been described in detail (Aoshima et al., 1981). The value of M_∞ (which is the $^{86}Rb^+$ content of vesicles when they are equilibrated with external $^{86}Rb^+$) to be used in eq 3 of Table I of the preceding paper (Pasquale et al., 1983) in the presence of a transmembrane voltage is determined by the use of eq 1 (Experimental Procedures): the M_∞ value in the absence of voltage is multiplied by $e^{-V_mF/(RT)}$. This exponential factor has a value of 6.8 when V_m = −45 mV. It is to be noticed from the slopes of the lines, which are proportional to the rate coefficient for receptor inactivation, α (Aoshima et al., 1981), that α has a greater value when V_m = −45 mV than when V_m = 0 mV. Under conditions where the external volume is much larger than the internal volume, the receptor-controlled flux rate is determined by the efflux rate coefficient, which, in the presence of a membrane potential, is given by eq 2. It is further assumed that any barriers to ions moving through receptor-formed channels are independent of the transmembrane voltage. The experimental evidence is that the conductance of receptor channels, which is directly related to the values of J_A (Hess et al., 1983b), is independent of the transmembrane voltage (Fatt & Katz, 1951; Anderson & Stevens, 1973). The values

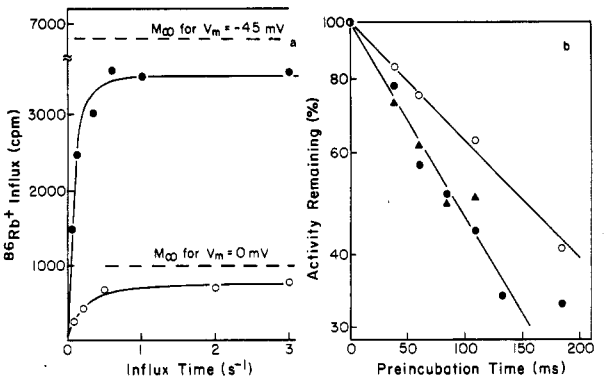

FIGURE 2: Activation and inactivation kinetics of acetylcholine receptor controlled flux of $^{86}Rb^+$ into the membrane vesicles prepared from *E. electricus* electroplax, in the presence of a transmembrane voltage, pH 7.0, 1 °C. (a) Acetylcholine- (60 μM) induced $^{86}Rb^+$ influx in the presence (●) and absence (○) of a transmembrane voltage (V_m = −45 mV). The lines were drawn by using the values of J_A and α calculated from eq 3 of Table I in the preceding paper (Experimental Procedures): for V_m = −45 mV, J_Af_i = 5.1 s^{-1} and α = 7.0 s^{-1}; for V_m = 0 mV, J_A = 3.3 s^{-1} and α = 2.3 s^{-1}. (b) Inactivation of acetylcholine-induced $^{86}Rb^+$ influx in the presence (●, ▲) and absence (○) of a transmembrane voltage (V_m = −45 mV): (○) preincubated with 1 mM acetylcholine, V_m = 0 mV (α = 5.5); (▲ and ●) preincubated with 1 and 5 mM acetylcholine, respectively, V_m = −45 mV (α = 8 s^{-1}). It should be noted that 1 and 5 mM acetylcholine gave the same inactivation rate coefficient in the presence of a transmembrane voltage. One volume of the membrane vesicles (1.6 mg of protein/mL) equilibrated with buffer A was mixed with 9.5 volumes of either buffer A or isotonic arginine solution containing various concentrations of acetylcholine. After various lengths of time, the activity was assayed in the second incubation of 1.5 s with $^{86}Rb^+$ (40 μCi/mL) and 1 or 5 mM acetylcholine. The value of α was calculated from $\ln([M_\infty - (M_t)_T]/M_\infty)/\ln([M_\infty - (M_t)_{T=0}]/M_\infty) = e^{-\alpha T}$. The activity remaining is indicated on the left side of the equation (Aoshima et al., 1981). $(M_t)_T$ represents the radioactivity inside the vesicles when influx was measured for time t (1.5 s) after various preincubation times (T). $(M_t)_{T=0}$ and M_∞ are the radioactivity inside the vesicles at time t (1.5 s) and at equilibrium, respectively.

of J_A in the presence and absence of a transmembrane voltage (Figure 3a) are normalized to each other by dividing the efflux rate coefficient values obtained at −45 mV (which are given by J_Af_i) by f_i (which has a value of 0.33 at V_m = −45 mV) (see eq 2, Experimental Procedures). It is to be noticed from the data in Figure 3a that the J_A values obtained at V_m = 0 mV increase and then reach a plateau value as the acetylcholine concentration is increased. In the same concentration region, the J_A values obtained at V_m = −45 mV first increase and then decrease. The minimum model [Figure 1 of the preceding paper (Pasquale et al., 1983)] accounts for this decrease, which is given by eq 1-II in Table I of the preceding paper (Pasquale et al., 1983). In contrast, the values of the inactivation rate coefficient, α, remain constant in the concentration region in which J_A decreases (Figure 3b). The minimum model and eq 2-II [Figure 1 and Table I of the preceding paper (Pasquale et al., 1983)] account for this. At acetylcholine concentrations <300 μM, the term $K_R(K_R + L)^{-1}$ in eq 1-II [in Table I of the preceding paper (Pasquale et al., 1983)] can be neglected, and the J_A values shown in Figure 3a can be plotted according to eq 1b-I in Table I of the preceding paper (Pasquale et al., 1983).

The equilibrium constant for channel opening, $1/\Phi$, and the dissociation constant, K_1 [see the model in Figure 1 and the legend in the preceding paper by Pasquale et al. (1983)], are obtained from the ordinate and intercept of the lines, respectively. While the dissociation constant for acetylcholine and the active conformation of the receptor, K_1, changes only slightly with transmembrane voltage, the channel-opening equilibrium constant ($1/\Phi$) increases from a value of 0.7 to

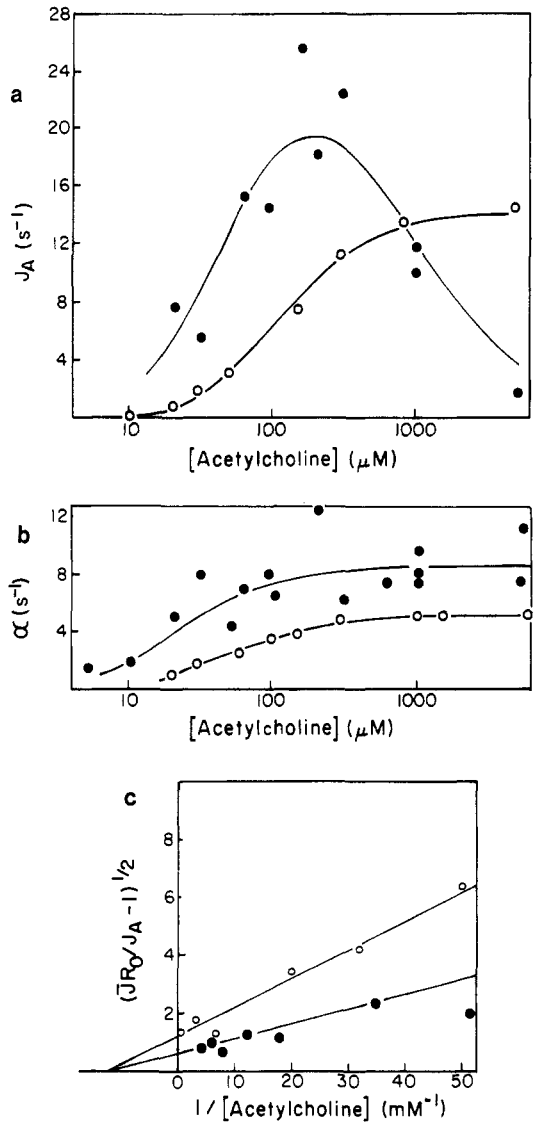

FIGURE 3: Effects of a transmembrane voltage on functioning of acetylcholine receptor from *E. electricus* electroplax. (a) Effect of a transmembrane voltage (−45 mV) on the dependence of the influx rate coefficient, J_A, on acetylcholine concentration. One volume of the membrane vesicles equilibrated with buffer A (see Experimental Procdures) was mixed with 9.5 volumes of isotonic arginine solution containing $^{86}Rb^+$ (40 μCi/mL) and various concentrations of acetylcholine. After various lengths of incubation time, the reaction was quenched by adding 5 volumes of *d*-tubocurarine (20 mM) in a buffer solution (11.1 mM NaCl, 81 mM arginine chloride, 1 mM $CaCl_2$, 1.5 mM sodium phosphate, pH 7.0). An example of the measurement of the acetylcholine-induced $^{86}Rb^+$ influx is shown in Figure 2a. For control experiments (V_m = 0 mV), the vesicles were diluted with buffer A containing $^{86}Rb^+$ and acetylcholine. The values of J_A in the presence (●) and absence (○) of the transmembrane voltage were calculated from the integrated rate equation (eq 3, Table I of the preceding paper) and normalized to each other by using a factor, f_i, of 0.33 at −45 mV, pH 7.0, 1 °C (eq 2). The points were obtained experimentally, and the lines in the absence and presence of the transmembrane voltage were calculated from eq 1a-I and 1-II, respectively, of Table I in the preceding paper by using the equilibrium constants listed in (c) and K_R = 800 μM. (b) Effect of a transmembrane voltage (V_m = −45 mV) on the dependence of the inactivation rate coefficient, α, on acetylcholine concentration: (○) V_m = 0 mV; (●) V_m = −45 mV. (c) A linear plot of the relationship between the influx rate coefficient, J_A, and acetylcholine concentration, L: (○) V_m = 0 mV; (●) V_m = −45 mV. Data from (a) were replotted by using eq 1b-I (Table I of preceding paper), neglecting the effect of inhibition at low acetylcholine concentrations (20–300 μM). This procedure would give the maximum possible value of Φ in the presence of a voltage. When V_m = 0 mV, K_1 = 80 ± 2 μM, $1/\Phi$ = 0.7, J_A(max) = 15 s^{-1}, and $\bar{J}$ = 3 × 10^7 M^{-1} s^{-1}. When V_m = −45 mV, K_1 = 75 ± 15 μM, $1/\Phi$ = 2.5, J_A(max) = 26 s^{-1}, and $\bar{J}$ = 3 × 10^7 M^{-1} s^{-1}.

a value of 2.5 as the transmembrane voltage is decreased. The value of the specific reaction rate for the receptor-controlled ion translocation, $\bar{J}$ = 3 × 10^7 M^{-1} s^{-1}, was determined previously at zero transmembrane voltage with acetylcholine, carbamoylcholine (Hess et al., 1981), and suberyldicholine (Hess et al., 1982) as the activating ligand and was found to give a satisfactory fit to the data in Figure 3c. K_R was evaluated by fitting the J_A values to eq 1-II in Table I of the preceding paper. A K_R of 800 μM was obtained.

Discussion

The minimum mechanism in Figure 1 of the preceding paper accounts for the effect of suberyldicholine concentration over a 10 000-fold range on the receptor-controlled flux and for the inhibition of the influx at high suberyldicholine concentrations without a concomitant decrease in the inactivation rate (Pasquale et al., 1983). The same mechanism accounts for the newly discovered voltage-dependent inhibition of receptor-controlled ion translocation by acetylcholine that we have described here. The binding of acetylcholine to a single regulatory site present on all forms of the receptor and characterized by a single dissociation constant, K_R (Figure 1, eq 1-II in the preceding paper), is required to fit all the data presented here.

The model is consistent with previous chemical kinetic measurements that led to the conclusion that ion flux in the millisecond time region requires the binding of two acetylcholine molecules to the receptor before the channel opens, activating sites that are characterized by the intrinsic dissociation constant K_1 (Figure 1, eq 1-II and 2-II in the preceding paper). The data in Figure 3c are in agreement with results obtained previously with carbamoylcholine (Cash & Hess, 1980; Aoshima et al., 1981), acetylcholine (Cash et al., 1981), and suberyldicholine [Hess et al., 1982; Pasquale et al., 1983 (preceeding paper)]. The minimum model (Figure 1 in the preceding paper) is consistent with a value of 3 × 10^7 M^{-1} s^{-1} for $\bar{J}$, the specific reaction rate for the ion translocation (Hess et al., 1981), a value that has also been found when acetylcholine, carbamoylcholine (Hess et al., 1981), and suberyldicholine (Hess et al., 1982; Pasquale et al., 1983) were used as the activating ligand. The model is also consistent with electrical measurements of the properties of acetylcholine-receptor channels in cells. The value of $\bar{J}$ is consistent with that obtained when the single-channel current measurement technique (Neher & Sakmann, 1976) is used with *E. electricus* electroplax cells (Hess et al., 1983b) from which the vesicles are prepared. Further correlations between single-channel current recordings with *E. electricus* electroplax cells and chemical kinetic measurements are possible. The rate constant for channel closing, k_{close}, as measured by the mean lifetime of the open channel, τ, in electroplax cells decreases *e*-fold for a decrease in transmembrane voltage of about 60 mV (Sheridan & Lester, 1977), and it has been suggested that the rate constant for channel opening k_{open}, is independent of transmembrane voltage (Neher & Sakmann, 1975; Sheridan & Lester, 1975). The increase in the channel-opening equilibrium constant ($1/\Phi = k_{open}/k_{close}$) by about a factor of ~3 for a change in transmembrane voltage of −45 mV is, therefore, consistent with the effect of transmembrane voltage on the lifetime of the open acetylcholine-receptor channel. Electrophysiological studies indicate qualitatively that membrane potential affects inactivation rates (Magazanik & Vyskocil, 1970; Scubon-Mulieri & Parsons, 1978). Here we demonstrate, using chemical kinetic techniques (Aoshima et al., 1981), that a negative transmembrane voltage of −45 mV causes the rate of inactivation, α, to increase about 2-fold with

respect to the rate at $V_m = 0$ mV, over the entire concentration range studied.

The existence of a voltage-dependent inhibitory binding site for cationic local anesthetics on the acetylcholine receptor has been well documented (Adams, 1977; Neher & Steinbach, 1978; Steinbach, 1980; Colquhoun, 1981). The effect of local anesthetics on single-channel current recordings, a chopping of the lifetime of the open channel into smaller segments of channel-open and -blocked times, called flickering, has been analyzed in detail, and an elegant mechanism for this observation has been proposed (Neher & Steinbach, 1978). These authors showed that an inhibitory site that involves only the open channel is sufficient to explain their results. We have shown (Pasquale et al., 1983) that a model that involves an inhibitory site only in the open-channel form predicts that the inactivation rate coefficient, α, as well as the flux rate coefficient, J_A, will decrease at high ligand concentration, unless additional isomerization steps are included to account for inactivation of the receptor. The chemical kinetic measurements with suberyldicholine (Pasquale et al., 1983) and those presented here (Figures 2 and 3) show that J_A decreases without a concomitant decrease in α. The model in Figure 1 of the preceding paper involves a preexisting regulatory site on the receptor protein and accounts for both the chemical kinetic measurements and for the single-channel currents measured in the presence of local anesthetics. Recently, the flickering has also been observed with acetylcholine and other activating ligands, and several mechanisms that can account for the observation have been proposed (Nelson & Sachs, 1979, 1982; Colquhoun & Sakmann, 1981; Auerbach & Sachs, 1983; Jackson et al., 1983). The voltage independence of the ligand-binding sites characterized by K_1 is consistent with structural information that places these binding sites above the plane of the membrane (Kistler et al., 1982).

It is to be remembered that cocaine also affects the flux rate without affecting the rate of receptor inactivation (Karpen et al., 1982). Although it is too soon to speculate whether the regulatory site for acetylcholine is the same as the inhibitory site for local anesthetics, it is significant that even in the absence of a local anesthetic, noise analysis of iontophoretically applied agonist yields a two-component power spectum at high doses of agonist, which might be explained by a local anesthetic effect of the agonist itself (Neher & Steinbach, 1978). Knowledge of the existence of a voltage-dependent regulatory site for acetylcholine is expected to be helpful in interpreting the single-channel current recordings.

The data in Figure 3a indicate that when $V_m = -45$ mV, a concentration of acetylcholine of 300 μM results in a higher efflux rate and, therefore, a more efficient propagation of the receptor-controlled signal between cells than higher concentrations. The inhibition of the receptor by acetylcholine, as by local anesthetics (Neher & Steinbach, 1978), is expected to involve a simple bimolecular process, to occur in the microsecond time region, and to take place before channel opening occurs, the latter event requiring two consecutive binding steps and a change in protein conformation.

It has been assumed that the release of acetylcholine alone can control the transmission of signals between cells (Kandel & Schwartz, 1982). The experiments presented here suggest that the resting transmembrane voltage of a cell and its influence on the receptor can have a significant effect on the rate of ion translocation (Figure 3a) and, therefore, on the transmission of signals between cells. Depending on the resting transmembrane voltage of the cell and on the amount of acetylcholine released, one can obtain conversion of the receptor to an inhibited state [α is not affected by acetylcholine binding to the regulatory site (Figure 3b in this paper and eq 2-II in Table I of the preceding paper)] without channel opening and signal transmission also occurring. It is also important to notice that the channel-opening equilibrium constant increases significantly with decreasing voltage. An increase in $1/\Phi$ not only increases the influx rate but also increases the cooperativity of ligand binding to the active form of the receptor (Cash & Hess, 1980; Hess et al., 1983a). The biological significance of these properties of the receptor may be that the receptor-controlled transmission of signals between cells may be variable and adjustable, depending on the transmembrane voltage of the cell.

We have now shown that measurements can be made in vesicles at different and constant transmembrane voltages by using the techniques that we have developed (Hess et al., 1979, 1983a) to study the effect of ligand concentration on different molecular processes involved in the ion translocation mediated by the acetylcholine receptor in milliseconds. The general model proposed in this paper can now explain the process of signal reception at the synapse: its *initiation*, *termination*, and *modulation*. We want to emphasize that the model proposed is a minimum model and that more complex models may be required when additional experimental data become available.

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Registry No. Acetylcholine, 51-84-3; carbamoylcholine, 462-58-8; Rb, 7440-17-7; K, 7440-09-7.

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Structural Analysis of Human and Bovine α-Fetoprotein by Electron Microscopy, Image Processing, and Circular Dichroism†

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ABSTRACT: The images of human and bovine α-fetoprotein molecules have been enhanced by combining dark-field electron microscopy with a laser-assisted optical system. This system filters out random background noise while permitting true averaged signal reconstruction of the molecule. A single averaged molecular image was digitized into a matrix, each pixel being assigned a gray scale level to produce a relative mass map for each molecule. These maps were interpreted from the α-helix, β-form, and random coil of the purified proteins as determined by circular dichroism. Results showed that both molecules are "U shaped", apparently monomeric, with outside dimensions of approximately 80 Å. Both molecules have asymmetrical structural features, notably three mass dense regions at both extremities and at the vertex of the molecules. Circular dichroism data suggest a high degree of similar stabilized α-helix and extensive β-form in these regions. Mass map analysis of hAFP correlates with the subdomains organized by disulfide bridges.

Various methods have been used to elucidate the conformation of biological molecules among which are X-ray diffraction analysis (Kendrew, 1963; Blundell, 1976), optical rotary dispersion and circular dichroism (Crabbé, 1972), and electron microscopy (EM)[1] using various staining procedures combined with computer averaging (Frank et al., 1978). The structure of naturally occurring crystalline specimens has also been accomplished by EM (Unwin & Henderson, 1975).

More recently a relatively rapid technique has been developed for the direct visualization of sulfur atoms and small biological molecules in the noncrystalline state such as vasopressin (1056 daltons) and myokinase (21 000 daltons). The procedure combines the features of high contrast dark-field EM, low specimen damage through minimal beam exposure, and image enhancement by means of a laser-assisted optical filter system (Ottensmeyer et al., 1973, 1975, 1977). This technique has been termed "filtering of arrays of images in reciprocal space" (FAIRS) which filters out random background noise while permitting true averaged signal reconstruction of the molecule. EM/FAIRS was first employed on small molecules of known structure, and it was demonstrated that molecular images obtained by this technique were in good agreement with images obtained by X-ray crystallography (Ottensmeyer et al., 1977). Conformations of low molecular weight proteins of unknown structure have also been

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[1] Abbreviations: hAFP, human α-fetoprotein; bAFP, bovine α-fetoprotein; EM, electron microscopy.

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