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Acetylcholine Receptor: Characterization of the Voltage-Dependent Regulatory (Inhibitory) Site for Acetylcholine in Membrane Vesicles from Torpedo californica Electroplax[†]

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ABSTRACT: Evidence for a voltage-dependent regulatory (inhibitory) site on the nicotinic acetylcholine receptor to which acetylcholine binds was obtained in membrane vesicles prepared from the Torpedo californica electric organ. Two rate coefficients, J_A and α, which pertain to the receptor-controlled ion flux, were measured. A 1000-fold concentration range of acetylcholine was used in a transmembrane voltage (V_m) range from 0 to –48 mV under a voltage-clamped condition at pH 7.4, 1 °C. The following observations were made. (i) At low acetylcholine concentrations, the value of J_A, the rate coefficient for ion translocation by the active (nondesensitized) state of the receptor, increased with increasing concentration. (ii) J_A decreased at high acetylcholine concentrations. (iii) In contrast, α, the rate coefficient for receptor desensitization, did not show such a decrease. (iv) When the transmembrane potential of the vesicle membrane was changed to more negative values, the value of K_R (the dissociation constant for binding of acetylcholine to the regulatory site) decreased by a factor of ~9 for a 25 mV change in V_m, while K_1 (the dissociation constant for binding of acetylcholine to the receptor site that controls channel opening) did not show such a change and has a value of 80 μM. When V_m is –48 mV, K_R has a value of 8 μM. (v) The effect of a transmembrane voltage on the regulatory site was reversible and occurred within the time resolution (5 ms) of the quench-flow technique used in the measurements. These results can be explained by a simple model in which the function of the receptor is regulated by binding of acetylcholine to a voltage-dependent regulatory site, a site that is distinct from the sites responsible for receptor activation and desensitization, and from the inhibitory site for cationic noncompetitive inhibitors such as the local anesthetic procaine. The biological significance of the voltage-dependent regulatory site on the acetylcholine receptor, specific for acetylcholine, is discussed.

The acetylcholine receptor at the vertebrate neuromuscular junction and in the electric organ of electric fish transduces a chemical signal into electrical activity (Fatt & Katz, 1951; Nachmansohn, 1959). Katz and Thesleff (1957) suggested that three fundamental steps are involved in the transduction process: (1) acetylcholine binds to a receptor site that controls the formation of transmembrane channels, (2) the conformation of the receptor changes from a closed-channel state to an open-channel state that allows ions to flow through the receptor channel, and (3) a conformational change of the receptor, from an active to an inactive (desensitized) form, occurs. In the last few years, several models that could account for the signal transduction process mediated by the acetylcholine receptor have been proposed (Cash & Hess, 1980; Dunn & Raftery, 1982; Heidmann & Changeux, 1980; Kistler

et al., 1982; Neubig & Cohen, 1980).

More recently, an additional regulatory step has been found to be involved in the functioning of the acetylcholine receptor in the Electrophorus electricus (Pasquale et al., 1983; Takeyasu et al., 1983) electric organ. Acetylcholine binds to the regulatory site, which is distinct from the sites responsible for activation and inactivation of the receptor; this leads to inhibition of the receptor function in a voltage-dependent manner. Furthermore, this voltage-dependent regulatory site for acetylcholine has been found to be distinct from a voltage-dependent local anesthetic site (Shiono et al., 1984).

In the present study, we characterize the voltage-dependent regulatory site for acetylcholine on the receptor from the Torpedo californica electric organ, further characterize the regulatory site of the receptor from the E. electricus electric organ, and suggest how this regulatory site for acetylcholine can play a decisive role in the transmission of signals between cells.

EXPERIMENTAL PROCEDURES

Materials. Membrane vesicles were prepared from the T. californica electric organ (Pacific Biomarine Lab, Venice, CA) according to the method of Sobel et al. (1977) with slight

modifications. After homogenizing the tissue (400 g) in 2 volumes of a buffer solution (pH 7.4) containing 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenyl-methanesulfonate, and pepstatin (1 μg/mL), the homogenate was centrifuged for 10 min at 6500 rpm in a GSA (Sorvall) rotor. The supernatant obtained was recentrifuged for 2 h at 11 000 rpm in a GSA rotor. The pellet obtained was suspended in 20% sucrose solution (pH 7.4) and centrifuged on a discontinuous sucrose gradient (32%, 36.5%, and 41.5%) for 12 h at 24 000 rpm in an SW27 (Beckman) rotor. All the vesicle bands were combined and diluted 4-fold with buffer A (90 mM NaCl, 1.5 mM sodium phosphate buffer, pH 7.4) and centrifuged for 1 h at 24 000 rpm in an SW27 rotor. The membrane vesicles finally obtained were resuspended in buffer A and stored in liquid N_2 until used. In some experiments, the vesicles were prepared in buffer B (250 mM NaCl, 1.5 mM sodium phosphate buffer, pH 7.4) or buffer C (400 mM NaCl, 1.5 mM sodium phosphate buffer, pH 7.4). The rate of ion flux and the rate at which the flux was inactivated were the same whether measured with vesicles in buffer A, B, or C. The preparation of vesicles from the *E. electricus* electroplax membrane has been described in detail (Fu et al., 1977; Hess et al., 1979; Sachs et al., 1982; Takeyasu et al., 1983).

Suberyldicholine diiodide was a gift from Dr. Ungar, University of Edinburgh. Acetylcholine bromide and *d*-tubocurarine were obtained from Eastman Kodak and Cal-biochem-Behring Corp., respectively. Procaine hydrochloride was from Schwarz/Mann, Inc. ^{86}RbCl (in 0.5 M HCl), obtained from New England Nuclear, was neutralized with 1 M NaOH and diluted with H_2O to give a stock solution that was 10 mCi/mL in 0.2 M NaCl. All the other chemicals were reagent grade.

Establishment of a Voltage Difference across the Vesicle Membrane. The establishment of a transmembrane voltage across the vesicle membrane was achieved by addition of acetylcholine to the vesicle suspension after mixing the vesicles in the quenched-flow apparatus with buffer D (87 mM arginine chloride, 1.5 mM sodium phosphate buffer, pH 7.4) for $V_m = -48$ mV (Takeyasu et al., 1983) or with buffer E (66 mM arginine chloride, 21 mM NaCl, 1.5 mM sodium phosphate buffer, pH 7.4) for $V_m = -25$ mV. If an asymmetric distribution of monovalent cations exists across the membrane and the osmotic balance of the solution is maintained by use of an impermeant cation, then making the vesicle membrane selectively permeable only to the asymmetrically distributed cation results in the formation of a transmembrane voltage across the membrane. In the present study, Na^+ and [arginine]$^+$ were used as the asymmetrically distributed and the impermeant cations, respectively (Takeyasu et al., 1983).

In some experiments done at $V_m = 0$ mV with *E. electricus* vesicles and a concentration of acetylcholine higher than 20 mM, membrane vesicles were mixed with the usual buffer containing 1 mM $CaCl_2$ and 1.5 mM sodium phosphate buffer, pH 7.0, but the NaCl concentration, normally 90 mM, was reduced by the concentration of the added acetylcholine bromide, in order to avoid osmotic effects.

All the measurements where acetylcholine was used as an activating ligand were done in the presence of an acetyl-cholinesterase inhibitor, Tetram (50 μM) [*O,O*-diethyl *S*-[2-(diethylamino)ethyl] thiophosphate (a gift from Dr. R. D. O'Brien)], and were done at 1 °C.

Measurement of J_A and α. The procedure for determination of J_A, the rate coefficient for ion translocation by the active state of the receptor in the absence of inactivation, in *T. californica* (Hess et al., 1982) and *E. electricus* (Cash & Hess,
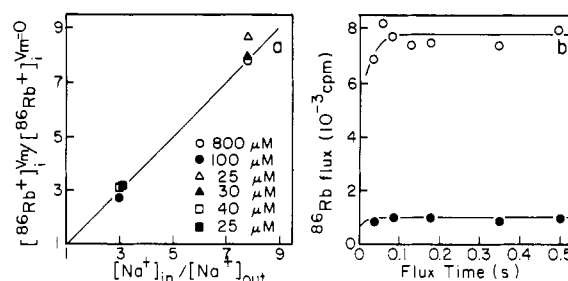

FIGURE 1: Effect of an asymmetric distribution of Na^+ across the vesicle membrane on acetylcholine receptor-controlled ion translocation. (a) Distribution of ^{86}Rb$^+$ according to a transmembrane voltage that was produced by the asymmetric distribution of Na^+ inside and outside the vesicles in the presence of acetylcholine. The *T. californica* vesicles (1.6 mg of protein/mL), after equilibrium with buffer A, were mixed with buffer D and buffer A to give a ratio of the Na^+ concentration inside to outside the vesicles as indicated on the abscissa and a final vesicle concentration of 0.15 mg of protein/mL. ^{86}Rb$^+$ (final concentration 35 μCi/mL) and acetylcholine (final concentration indicated in the graph) were added to the vesicle suspension for 500 ms, and the reaction was quenched by *d*-tubocurarine (final concentration 5 mM) followed by Millipore filtration. 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 the text). (b) Acetyl-choline-induced ^{86}Rb$^+$ flux in the presence (O) and absence (●) of a transmembrane voltage. The *T. californica* vesicles (1.6 mg of protein/mL), after equilibrium with buffer A, were mixed with ^{86}Rb$^+$ (final concentration 35 μCi/mL) and acetylcholine (final concentration 80 μM) in either buffer A or buffer D for the time indicated on the abscissa, and then the reaction was quenched by adding *d*-tubocurarine (final concentration 5 mM). The final vesicle concentration was 0.15 mg of protein/mL. It should be noted that the value of the ratio $[^{86}$Rb$^+]_i^{V_m=-48}/[^{86}$Rb$^+]_i^{V_m=0} = 7.8$, indicating the existence of a transmembrane voltage of -48 mV according to eq 1.

1980) membrane vesicles using a pulsed quenched-flow technique (Fersht & Jakes, 1975) has been described. The method and the underlying theoretical considerations for the measurement of α, the rate coefficient for receptor inactivation (desensitization), have also been described (Aoshima et al., 1981).

RESULTS

When the distribution of a tracer cation, ^{86}Rb$^+$, across the vesicle membrane was measured as an indicator of the transmembrane voltage, it was seen that the Nernst equation was obeyed (Figure 1). Arginine itself did not affect the rate of the ion flux or of its inactivation in the time period required for the measurements (Figure 2).

Figure 1b shows that in the vesicles prepared from the *T. californica* electric organ a transmembrane voltage, produced by an asymmetric distribution of Na^+ across the vesicle membrane in the presence of acetylcholine, can be maintained over the period of time required for quench-flow measurements. The value of M_∞, the concentration of ^{86}Rb$^+$ in the vesicles at equilibrium, obtained experimentally at different transmembrane voltages, was proportional to $\exp[-V_mF/(RT)]$ (Figure 1a), which is given by the ratio of the Na^+ concentration inside to that outside the vesicles:

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

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 $V_m = 0$, and F, R, and T represent the Faraday constant, the gas constant, and absolute temperature, respectively. The value
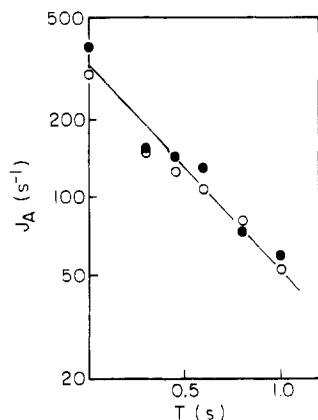
FIGURE 2: Inactivation kinetics of acetylcholine receptor-controlled flux of $^{86}Rb^+$ in the presence (●) and absence (○) of arginine (80 mM). Arginine does not affect the function of the acetylcholine receptor in *T. californica* vesicles in the time region of the experiments (3 s or less). One volume of the vesicles (1.6 mg of protein/mL) equilibrated with buffer C was mixed with 9.5 volumes of either buffer C or a buffer containing 311.4 mM NaCl, 88.6 mM arginine chloride, and 1.5 mM sodium phosphate buffer, pH 7.4, in the presence of acetylcholine (final concentration 1 mM). The final vesicle concentration was 0.15 mg of protein/mL. After various periods of time, indicated on the abscissa of the graph, the J_A value was measured after a second incubation of 5 ms with $^{86}Rb^+$ (final concentration 50 μCi/mL) (Hess et al., 1982). The final concentrations of Na^+ inside and outside the vesicle were 403 and 324 mM, respectively, in the presence of 80 mM arginine chloride outside. This produces a negligible transmembrane voltage. The values of $(J_A)_{T=0}$ and α were calculated by using eq 2 (Appendix). The ordinate intercept of the graph gives the value of the rate coefficient for ion flux in the absence of receptor desensitization $[(J_A)_{T=0}]$. We have determined that arginine does not affect the value of $(J_A)_{T=0}$ within the time region (<3 s) required for our measurements. However, if the vesicles were preincubated with arginine in the minute to hour time region, a progressive inhibition of the receptor by arginine was observed ($t_{1/2}$ > 1.5 min) (data not shown).

of M_∞ obtained experimentally was used for the calculation of J_A and α in the presence of a transmembrane voltage.

In membrane vesicles prepared from the *T. californica* electroplax, the values of J_A and α in the presence and absence of a transmembrane voltage were measured over a 1000-fold range of acetylcholine concentration (Figure 3). At low acetylcholine concentrations, the value of J_A increases with increasing concentration, while at high concentrations, J_A decreases with increasing concentration (Figure 3a). In contrast, the value of α increases with increasing concentration of acetylcholine and reaches a maximum value that is independent of the ligand concentration (Figure 3b).

By use of the value of J_A obtained in the absence of a transmembrane voltage at low acetylcholine concentrations ($L_0 \ll K_R$), K_1, the dissociation constant for acetylcholine binding to the activation site of the receptor before inactivation, and Φ, the equilibrium constant for channel closing, can be calculated by use of eq 5 in the Appendix. K_R in eq 4 represents the dissociation constant for binding of acetylcholine to the regulatory site, and eq 5 is a rewritten form of eq 4 (Appendix) when the term $K_R(K_R + L)^{-1}$ in eq 4 can be neglected.

In eq 5, J is the specific reaction rate of the receptor-controlled ion flux and R_0 the moles of receptors per liter internal vesicle volume (Hess et al., 1981). J is proportional to the conductance of the receptor channel (Hess et al., 1984). A computer program was used to obtain a value for K_1 that accounts for the effect of acetylcholine concentration not only on J_A but also on α. The program was then used to obtain a value for JR_0 that is independent of the transmembrane voltage at which the influx rate coefficient J_A was measured
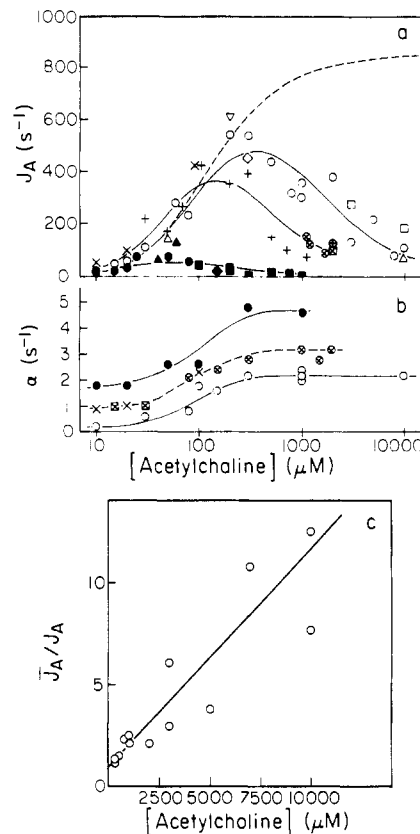

FIGURE 3: Effect of the transmembrane voltage on the ion translocation process mediated by the acetylcholine receptor from *T. californica* electric organ. All measurements were done at pH 7.4, 1 °C. (a) Effect of a transmembrane voltage on the dependence of the flux rate coefficient, J_A, on acetylcholine concentration. The J_A values given are normalized to the J_A values obtained when V_m is 0 mV by multiplying the observed values when V_m is −48 mV by 3.3 and the observed values when V_m is −25 mV by 1.8 (Takeyasu et al., 1983). The points were obtained experimentally, and the lines were drawn according to eq 4 (Appendix) by using the constants listed in Table I. The dashed line represents calculated J_A at 0 mV with the assumption that the receptor has no regulatory site for acetylcholine, i.e., $K_R = \infty$. A total of 0.15 mg of protein/mL and 35 μCi/mL $^{86}Rb^+$ or $^{45}Ca^{2+}$ was used in the influx measurements. M_∞ at 0, −25, and −48 mV was obtained after flux times of 101, 160, and 1000 ms, respectively, with 100 μM acetylcholine. (○) 5-ms Rb^+ flux at 0 mV, (▽) 46-ms Rb^+ flux at 0 mV, (△) 101-ms Rb^+ flux at 0 mV, (□) 59-ms Ca^{2+} flux at 0 mV, (◇) 101-ms Ca^{2+} flux at 0 mV, (⊕) 6-ms Rb^+ flux at −25 mV, (+) 10-ms flux at −25 mV, (▨) 33-ms flux at −25 mV, (×) 47-ms Rb^+ flux at −25 mV, (⊗) 60-ms flux at −25 mV, (▲) 33-ms Rb^+ flux at −48 mV, (●) 500-ms Rb^+ flux at −48 mV, and (■) 1000-ms Rb^+ flux at −48 mV. The J_A value obtained in the $^{45}Ca^{2+}$ influx measurements were multiplied by a factor of 10 to compare them with the J_A values obtained in the $^{86}Rb^+$ influx measurements (the ratio of J_A with $^{86}Rb^+$ to that with $^{45}Ca^{2+}$ had been shown to be 10; Jeng & Cohen, 1982). Four membrane preparations characterized by different J_A values were used. The preparations were normalized to one another by using the J_A values at 1 mM acetylcholine for the $V_m = 0$ mV experiments and at 100 μM acetylcholine for the $V_m = -25$ and −48 mV experiments. (b) Effect of a transmembrane voltage on the dependence of the inactivation rate coefficient, α, on acetylcholine concentration. The experimental conditions were the same as described in (a) and the same symbols as in (a) are used. (c) J_A values obtained at $V_m = 0$ mV [see (a)] for high concentrations of acetylcholine (>300 μM) were plotted vs. acetylcholine concentration according to eq 6 (Appendix). A K_R value of 1 mM at $V_m = 0$ mV was obtained.

and to obtain a best fit of the data to theory (Bevington, 1969). The calculations involved have been described in detail (Hess et al., 1981), and it was shown that the effect of concentration, over a 1000-fold range, of acetylcholine, suberyldicholine, and carbamoylcholine on both J_A and α can be quantitatively accounted for by this approach (Hess et al., 1983). The

Table I: Voltage-Dependent and -Independent Equilibrium Constants Pertaining to Acetylcholine Receptor Function[a]

	for T. californica at V_m (mV) of			for E. electricus at V_m (mV) of	
	0	-25	-48	0	-45
K_1 (μM)[b]	80 ± 11	80[d]	80[d]	80 ± 20 (4.5)	75 ± 15 (4.5[d])
Φ[b]	1.6 ± 0.4	0.9 ± 0.1	0.5[e]	1.5 (1.0)	0.4 (0.4)
K_R (μM)[c]	1000 ± 100	160 ± 20	8 ± 3	50000 (500)	800 (25)

[a] The values were obtained from measurements by using acetylcholine except those in parentheses in which suberyldicholine was the activating ligand. [b] Calculated by using eq 5 (Appendex). [c] Calculated by using eq 6 (Appendix). [d] Assumed from structural information (Kistler et al., 1982) that places the binding site about 50 Å above the plane of the membrane where it is not expected to be perturbed by the electric field across the membrane and from measurements on the E. electricus acetylcholine receptor with acetylcholine (Takeyasu et al., 1983). [e] Obtained with the assumption that there is a linear relationship between the value of ln Φ with V_m (Neher & Sakmann, 1975; Sheridan & Lester, 1975 & 1977; see the text).

calculations, based on the data in Figure 3a,b, give a value for K_1 of 80 μM that is independent of V_m and a channel-opening equilibrium constant ($1/\Phi$) that increases e-fold when V_m is changed from 0 to -48 mV (Table I). The value of K_R, the dissociation constant for acetylcholine binding to the preexisting regulatory site, was evaluated by fitting the J_A values at high acetylcholine concentrations ($L_0 \gg K_R$) to eq 6 (Appendix) by using a least-squares computer program that gives the best fit of the data to theory (Bevington, 1969). A representative plot of the data is shown in Figure 3c. The value of K_R was found to decrease from 1 mM to 8 μM when V_m was changed from 0 to -48 mV (Table I).

The effect of a transmembrane voltage on the inhibition of receptor function is reversible. In Figure 4, the vesicles were preincubated with acetylcholine in the quench-flow machine at a transmembrane voltage of -48 mV, and then they were transferred quickly (within the time resolution of the measurements, 5 ms) to a new medium to give a transmembrane voltage of -25 mV when the value of J_A was measured (solid symbols). Two control experiments were also conducted. (1) The vesicles were preincubated at -25 mV in the presence of acetylcholine, and then the value of J_A was measured at the same transmembrane voltage (open symbols). (2) The vesicles were preincubated in the presence of acetylcholine at -48 mV, and the J_A value was measured at the same V_m value. The results indicate that the effect of a transmembrane voltage can be reversed within the time resolution (5 ms) of the measurements.

Figure 5 also illustrates the effects of a transmembrane voltage on the functioning of the acetylcholine receptor from E. electricus. Here we present the results of experiments with acetylcholine when V_m is 0 mV and, for purposes of comparison, the results obtained when V_m = -45 mV (Takeyasu et al., 1983). The value of K_R for acetylcholine with the E. electricus receptor was calculated to be 50 mM at 0 mV. K_R has a value of 800 μM when V_m is -45 mV (Takeyasu et al., 1983). These K_R values are about 50 times larger than those obtained with the T. californica receptor, but a similar voltage dependence of the value can be seen in the receptors from both sources: the K_R value decreases 50-fold for a change in a transmembrane voltage of -45 mV. This effect of the transmembrane voltage is also observed when suberyldicholine is used as an activating ligand (data not shown). The inactivation rate coefficient, α, remains constant in a concentration region where J_A decreases, both when V_m is 0 mV (Pasquale et al., 1983) and when V_m is -45 mV. The value of K_R decreases from 500 μM when V_m = 0 mV to 25 μM when V_m = -45 mV.

The effects of a transmembrane voltage on the equilibrium constants, K_1, Φ, and K_R, pertaining to acetylcholine receptor function, are summarized in Table I.

Experiments conducted with E. electricus vesicles in the presence and absence of a transmembrane voltage of -45 mV demonstrated that the voltage-dependent inhibitory site for
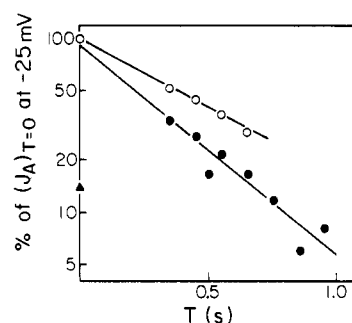

FIGURE 4: Effect of a transmembrane voltage on the T. californica acetylcholine receptor is reversible. (●) Recovery experiment. The experiment was done in a similar manner to the inactivation experiment (Aoshima et al., 1981), but preincubation of the membrane vesicles was done at -48 mV while the influx rate was measured at -25 mV. One volume of the T. californica membrane vesicles suspended in buffer A was mixed with 9.5 volumes of buffer D containing acetylcholine (final concentration 200 μM), which produced a transmembrane voltage of -48 mV. After various lengths of time of preincubation at -48 mV indicated on the abscissa, the J_A value was measured after a second incubation of 71 ms at -25 mV by mixing 2 volumes of the reaction mixture preincubated at -48 mV and 1 volume of a buffer containing 67 mM NaCl, 22 mM arginine chloride, 1.5 mM sodium phosphate buffer (pH 7.4), [86]Rb^{+} (35 μCi/mL), and 200 μM acetylcholine. This buffer reduced the transmembrane voltage to -25 mV. J_A values relative to $(J_A)_{T=0}$ at -25 mV were plotted on the ordinate, where T refers to the preincubation time given on the abscissa of the graph. The final protein concentration was 0.13 mg/mL. The extrapolated J_A value at T = 0 was 91 ± 15% of $(J_A)_{T=0}$ at -25 mV. (O) Control experiment 1. The experiment was done in the same manner as the inactivation experiment (Aoshima et al., 1981). Both preincubation and influx were done at -25 mV. One volume of vesicle suspension in buffer A was mixed with 9.5 volumes of a buffer containing 21 mM NaCl, 66 mM arginine chloride, and 1.5 mM sodium phosphate buffer (pH 7.4) in the presence of 200 μM acetylcholine which produced a transmembrane voltage of -25 mV. After various periods of time, the J_A value was measured after a second incubation of 15 ms with [86]Rb^{+} at -25 mV. (▲) Control experiment 2. The $(J_A)_{T=0}$ at -48 mV was measured without preincubation. One volume of vesicle suspension in buffer A was mixed with 9.5 volumes of buffer D in the presence of Rb^{+} and 200 μM acetylcholine for 101 ms to measure the J_A value at -48 mV. For other details, see Figures 3a and 2.

procaine and the voltage-dependent regulatory site for acetylcholine are different (Shiono et al., 1984). The experiments shown in Figure 6 demonstrate that, in T. californica, the inhibitory site for procaine, characterized by the dissociation constant K_P, and the inhibitory site for acetylcholine are different (measured when V_m is 0 mV). Equations 8 and 9 (Appendix) can be used to distinguish, on the basis of kinetic measurements, between identical or separate inhibitory sites for acetylcholine and the local anesthetic procaine (Shiono et al., 1984). Two different mechanisms can be considered: (I) Acetylcholine at high concentrations ($L_0 \gg K_R$) and procaine occupy the same inhibitory site (eq 9, Appendix). (II) Acetylcholine at high concentrations ($L_0 \gg K_R$) and procaine bind to different inhibitory sites (eq 8, Appendix). When the ratio
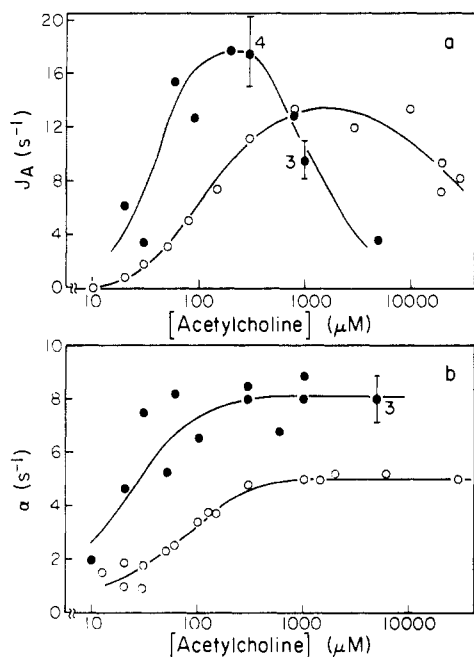
FIGURE 5: Effect of the transmembrane voltage on the ion translocation process mediated by the acetylcholine receptor from *E. electricus* electric organ. All measurements were done at pH 7.0, 1 °C. (a) Effect of a transmembrane voltage on the dependence of the flux rate coefficient, J_A, on acetylcholine concentration. One volume of the vesicles equilibrated with buffer A containing 1 mM $CaCl_2$ was mixed with 9.5 volumes of either buffer A or buffer D containing 1 mM $CaCl_2$, $^{86}Rb^+$ (35 μCi/mL), and various concentrations of acetylcholine. After various periods of incubation, T, the reaction was quenched by adding 5 volumes of d-tubocurarine (25 mM). J_A values were calculated from the influx curve obtained by using eq 7 (Appendix). The values of J_A in the presence of (●) and absence (○) of a transmembrane voltage (−45 mV) were normalized to each other by multiplying the J_A values obtained when $V_m = -45$ mV by 3.3 according to eq 1 Takeyasu et al., 1983). (b) Effect of a transmembrane voltage on the dependence of the inactivation rate coefficient, α, on acetylcholine concentration. One volume of the membrane vesicles (1.6 mg of protein/mL) equilibrated with buffer A containing 1 mM $CaCl_2$ was mixed with 9.5 volumes of either buffer A or buffer D containing 1 mM $CaCl_2$ and various concentrations of acetylcholine. After various lengths of time, T, the activity was assayed in the second incubation of 1.5 s with $^{86}Rb^+$ (final concentration 35 μCi/mL) and 1 mM or higher acetylcholine (Takeyasu et al., 1983). The value of α was calculated from eq 3 (Appendix) (Aoshima et al., 1981). $(M_t)_T$ represents the radioactivity inside the vesicles when flux was measured for 1.5 s after various preincubation times (T). (○) $V_m = 0$ mV; (●) $V_m = -45$ mV.

of the flux coefficients at 0 mV in the absence and presence of procaine, $J_A/J_{A(P)}$, is plotted (Figure 6) vs. the procaine concentration according to eq 8 and 9, the slopes of the lines are inversely proportional to the dissociation constant of procaine, K_P. The same value for K_P, 80 μM, is obtained at low (40 μM, filled circles) and high concentrations of acetylcholine (5 mM, open circles), demonstrating the existence of two independent inhibitory sites. One can calculate from the K_R value for acetylcholine (Table I) using eq 9 that if procaine and acetylcholine occupied the same site, then at high acetylcholine concentrations, the data points would fall along the dashed line in Figure 6, which corresponds to an apparent dissociation constant for procaine that is 5 times larger than the value that is observed.

DISCUSSION

The chemical kinetic measurements presented here on the acetylcholine receptor-controlled ion flux in membrane vesicles prepared from the *T. californica* electroplax display three characteristics that have also been seen with *E. electricus*
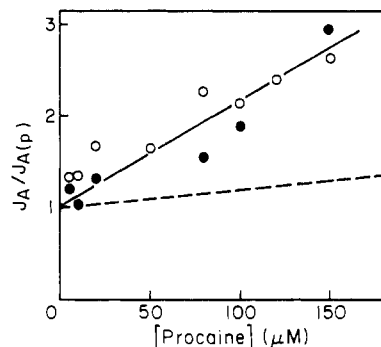

FIGURE 6: Effect of procaine on the rate of ion translocation in *T. californica* vesicles at $V_m = 0$ mV. The ratio of the rate constant for ion flux initiated by a constant concentration of acetylcholine, J_A, to the rate constant obtained in the presence of procaine, $J_{A(P)}$, is plotted vs. procaine concentration according to eq 8 and 9 (Appendix). A total of 0.40 mg of protein/mL and 50 μCi of $^{86}Rb^+$/mL was used in the influx experiments. Acetylcholine: (●) 40 μM; (○) 5 mM (M_t at 17 ms and M_∞ at 200 ms). The coordinates of the solid line were calculated by using all the measurements and a K_P value of 85 ± 5 μM. The dashed line was drawn on the assumption of a common inhibitory site using eq 9 (Appendix), a K_P value of 85 μM, and K_R value of 1 mM. With 5 mM acetylcholine the slope of the dashed line would then give an apparent K_P value of 510 μM.

vesicles (Takeyasu et al., 1983). (1) The values of J_A and α increase with increasing acetylcholine concentrations. At higher concentrations, a concentration- and voltage-dependent decrease in J_A values is observed without a concomitant decrease in α values. This observation suggests that a simple inhibitory blockade of the channel by acetylcholine is unlikely (Pasquale et al., 1983). (2) The channel-opening equilibrium constant, $1/\Phi$, increases when the voltage across the vesicle membrane is changed to a more negative value. (3) The maximum rate coefficient for inactivation (desensitization) increases somewhat as V_m becomes more negative. (4) The value of K_R depends strongly upon the transmembrane voltage: a 50-fold decrease in the K_R value was observed with a change in transmembrane voltage of 45 mV.

These results can be explained by a simple model, where the functioning of the receptor is regulated by binding of acetylcholine to a preexisting regulatory site, a site that is distinct from the site(s) responsible for receptor activation and inactivation (Pasquale et al., 1983; Takeyasu et al., 1983). This model was shown to account quantitatively for the effects of acetylcholine and suberyldicholine, over a 10 000-fold concentration range, on the receptor-controlled ion flux and for the inhibition of the flux at high ligand concentrations without a concomitant decrease in the inactivation rate in *E. electricus* membrane vesicles (Pasquale et al., 1983). Here we show that the same model can quantitatively account for the effect of acetylcholine concentration over a 1000-fold range on J_A and α in the transmembrane voltage range of 0 to −48 mV (Figure 3a,b) on *T. californica* receptor. The coordinates of the solid lines in Figure 3a,b were calculated by using eq 4 and 10 in the Appendix and the values of the constants given in Table I. The K_1 value of 80 μM accounts for the effect of acetylcholine concentration on both J_A and α (Figure 3a,b) and is in agreement with the value of 70 μM obtained in previous studies with *Torpedo* spp. (Neubig et al., 1982) and the value of 80 μM determined in experiments with the *E. electricus* receptors (Table I). The value of K_1 was found to be independent of transmembrane voltage. This is consistent with the structural information that places the pertinent binding sites well above the plane of the membrane (Kistler et al., 1982), where they are not expected to be perturbed by the electric field across the membrane.

The e-fold increase in the channel-opening equilibrium constant when V_m is changed from 0 to −48 mV (Table I) is consistent with electrophysiological measurements of receptor function in cells with the assumption that $\Phi = k_{cl}/k_{op}$ (where k_{op} and k_{cl} represent the rate constant for channel opening and closing, respectively). Electrophysiological measurements indicate that k_{op} is independent of V_m (Neher & Sakmann, 1975; Sheridan & Lester, 1975) and that k_{cl} depends exponentially on V_m (Magleby & Stevens, 1972; Sheridan & Lester, 1975, 1977) with an e-fold change for a 60–80 mV change in V_m.

The strong dependence of K_R on voltage, an e-fold change for a V_m change of 12 mV, with both *T. californica* and *E. electricus* receptor is not without precedent. A large transmembrane voltage-dependent change was observed by Hodgkin and Huxley (1952) in studies of the sodium channel, the conductance of which changes e-fold for a change in V_m of 4 mV. One explanation for such mechanisms is that the voltage-dependent process involves the displacement of more than one electric charge (Hodgkin & Huxley, 1952). This can occur if the process observed involves a conformational change of the protein.

The existence of a voltage-dependent inhibitory site for cationic local anesthetics has been well documented (Adams, 1977; Heidmann & Changeux, 1984; Neher & Steinbach, 1978; Steinbach, 1980; Colquhoun, 1981; Oswald et al., 1983). It was suggested that the binding site for these compounds is inside the transmembrane channel. Similarly, inhibition of receptor function in BC₃H1 cells by high concentrations of acetylcholine, presumably by a channel-blocking mechanism similar to that observed with local anesthetics, has been reported (Sine & Steinbach, 1984a). The effect of transmembrane voltage on the dissociation constant of local anesthetics, K_I (Neher & Steinbach, 1978), is considerably less ($\sim 1/4$) than on K_R and is consistent with a simple binding process involving a monovalent cation. The difference in the effect of transmembrane voltage on K_R and on K_I is consistent with experiments that show that there is a specific regulatory (inhibitory) site for acetylcholine that is different from the inhibitory site for the local anesthetic procaine (Shiono et al., 1984; Figure 6). The receptors in *T. californica* membranes have a site density of $\sim 2 \times 10^4$ receptor molecules/μm^2, characteristic of the receptor density at the junctions (synapses) between nerve terminals and vertebrate skeletal muscles (Fambrough, 1979). With the assumption that the properties of receptors at the neuromuscular junctions and in *T. californica* membranes are similar, we have enough information (Table I) to calculate the change in the number of receptor molecules that can form transmembrane channels as the transmembrane voltage changes at a given concentration of acetylcholine. The acetylcholine concentration rises to about 300 μM during the transmission of a signal between cells (Kuffler & Yoshikamo, 1975). We calculate that at −70 mV, the average V_m value for muscle cells, all but 1% of the receptors present are inhibited, leaving only ~ 200 receptor sites/μm^2 that can form transmembrane channels. At the acetylcholine concentration that gives rise to signal transmission, a change in the V_m value of 5 mV changes the number of receptor molecules by a factor of 2. This factor of 2 may be critical for the transmission process because it has been estimated that between 50 and 400 receptor channels/μm^2 are required for signal transmission (Junge, 1981). In this regard it is of interest to note that the release of small amounts of acetylcholine from nerve terminals, independent of signal transmission, has recently been discovered (Thesleff & Molgo,

1984). The release changes the transmembrane potential of the adjacent receptor-containing cells by 0.5–10 mV, a V_m change that is insufficient to cause propagation of the signal. Interestingly enough, the density of receptor sites in the *E. electricus* membranes is only about 1% of that of the *T. californica* membranes, while the K_R value is 100-fold larger.

In summary, it has been shown that the number of receptor molecules that can form transmembrane channels depends critically not only on the concentration of acetylcholine during the signal transmission but also on the transmembrane voltage of the cell membrane containing the receptors. It has been suggested that a change in the amount of acetylcholine released from the nerve terminal is an important factor in controlling signal transmission between cells (Kandel & Schwarz, 1982). The studies presented here and previously (Takeyasu et al., 1983; Shiono et al., 1984) suggest that even when the amount of acetylcholine released remains constant, small changes in the transmembrane voltage of the cell can critically affect the number of receptors capable of forming transmembrane channels, and thereby regulate signal transmission between cells. Chemical kinetic investigations, which allow one to measure the effect of wide concentration ranges of acetylcholine on J_A and α separately and prior to receptor desensitization, in combination with fast reaction techniques have been useful in discovering and characterizing the effect of the voltage-dependent, acetylcholine-specific regulatory site of the receptor in the receptor-controlled translocation of ions across the membrane and may be equally successful in investigations of other transmembrane processes.

APPENDIX

Equations. Equations 2 and 3 are used to determine $(J_A)_{T=0}$ and α from the inactivation experiments (Aoshima et al., 1981; Hess et al., 1982). T represents the time for which the vesicles

$$(J_A)_T = (J_A)_{T=0}e^{-\alpha T} \tag{2}$$

$$\frac{\ln(1 - (M_t)_T/M_\infty)}{\ln(1 - (M_t)_{T=0}/M_\infty)} = e^{-\alpha T} \tag{3}$$

are preincubated with an agonist before the influx measurement is made, and $(M_t)_T$ is the radioactivity inside the vesicles when flux is measured after time t. J_A is given by eq 4 for

$$J_A = JR_0 \frac{L_2}{L^2(1 + \Phi) + 2K_1\Phi L + K_1\Phi^2}\frac{K_R}{K_R + L} \tag{4}$$

the general model (Pasquale et al., 1983). K_1 represents the intrinsic dissociation constant for the activating ligand before inactivation, K_R is the dissociation constant for the binding of ligand to the regulatory site, Φ^{-1} is the channel-opening equilibrium constant, L and R_0 are the concentrations of the activating ligand and the receptor, respectively, and J is the specific reaction rate for ion translocation. JR_0, K_1, and Φ can be obtained from eq 5 (Hess et al., 1981).

$$(JR_0/J_A - 1)^{1/2} = \Phi^{1/2} + \frac{\Phi^{1/2}K_1}{L} \tag{5}$$

K_R values can be calculated by using eq 6

$$\bar{J}_A/J_A = 1 + L/K_R \tag{6}$$

which is derived from eq 4. $\bar{J}_A$ is the calculated J_A value which would be obtained in the absence of a regulatory site.

J_A values can be obtained from influx experiments by using eq 7 (Hess et al., 1983). M_t and M_∞ are the concentrations

$$\frac{M_t}{M_\infty} = 1 - \exp\left[-\left(J_A\frac{1 - e^{-\alpha t}}{\alpha} + J_I t\right)\right] \tag{7}$$

of a particular inorganic ion at time t and at equilibrium inside the membrane vesicles, and J_I is the rate constant for ion flux after inactivation of the receptor is complete.

The equations that can be used to distinguish, on the basis of kinetic measurements, between identical or separate inhibitory sites for acetylcholine and a local anesthetic have been derived (Shiono et al., 1984):

$$J_A/J_{A(P)} = 1 + P_0/K_P \tag{8}$$

$$J_A/J_{A(P)} = 1 + (P_0/K_P)[K_R/(K_R + L_0)] \tag{9}$$

$J_{A(P)}$ is the rate coefficient for ion translocation in the presence of procaine, the local anesthetic used, at a given concentration of acetylcholine, and J_A is the rate coefficient obtained in the absence of procaine. P_0 and L_0 represent the initial concentrations of procaine and acetylcholine, respectively. P_0 and L_0 are much greater than R_0 so that the initial concentration of these two ligands can be considered to be constant.

$$\alpha \approx k_{34}\Phi\,\frac{L^2}{L^2(1 + \Phi) + 2K_1\Phi L + K_1\Phi^2} \tag{10}$$

when $L_0 > K_2, K_1$ (Hess et al., 1983).

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