Acetylcholine Receptor: Evidence for a Regulatory Binding Site in Investigations of Suberyldicholine-Induced Transmembrane Ion Flux in *Electrophorus electricus* Membrane Vesicles†

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ABSTRACT: Suberyldicholine-induced ion translocation in the millisecond time region in acetylcholine receptor rich membrane vesicles prepared from the electric organ of *Electrophorus electricus* was investigated in eel Ringer's solution, pH 7.0, 1 °C. A quench-flow technique with a time resolution of 5 ms was used to measure the transmembrane flux of a radioactive tracer ion ($^{86}Rb^+$). J_A, the rate coefficient for ion flux mediated by the active form of the receptor, and α, the rate coefficient for the inactivation of the ion flux, increase with increasing suberyldicholine concentrations and reach a plateau value at about 15 μM. At higher suberyldicholine concentrations (>50 μM), a concentration-dependent decrease in the ion flux rate was observed without a corresponding decrease in the rate of receptor inactivation. This regulatory effect was not observed with acetylcholine or carbamoylcholine. The minimal kinetic scheme previously presented for acetylcholine and carbamoylcholine, modified by the inclusion of an additional regulatory ligand-binding site for suberyldicholine and characterized by a single dissociation constant, K_R, is consistent with the results obtained over a 10000-fold concentration range of this ligand. Rate and equilibrium constants pertaining to this scheme were elucidated. Suberyldicholine binds to the regulatory site (K_R = 500 μM) approximately 100-fold less well than to its activating sites, and the binding to the regulatory site has no effect on the inactivation (desensitization) rate coefficient α [α(max) = 5.7 s^{-1}], which is comparable to that observed with acetylcholine. The maximum influx rate coefficient [J_A(max) = 18.5 s^{-1}] is approximately twice that obtained when carbamoylcholine is the activating ligand and somewhat higher than when acetylcholine is used. Previous interpretations of the specific reaction rate, $\bar{J}$, for the receptor-controlled ion translocation process required that while the maximum observed flux rates can depend on the activating ligand used, $\bar{J}$ is the same for all activating ligands. Here we find that $\bar{J}$ for suberyldicholine ($\bar{J} = 3 \times 10^7$ M^{-1} s^{-1}) has the same value as was found earlier for carbamoylcholine and acetylcholine. This is the first report of chemical kinetic investigations of the inhibitory effect exerted by suberyldicholine at high concentrations.

Changes in the permeability of the membranes of nerve and muscle cells are induced upon binding of specific ligands to receptor proteins that are inserted in the membrane and can form transmembrane ion-conducting channels. The consequent modification of the transmembrane potential in a cell constitutes information that is eventually communicated to other cells (Hodgkin & Huxley, 1952; Schoffeniels & Nachmansohn, 1957; Katz, 1966). Comparative studies of the action of various activating ligands of the acetylcholine receptor showed that some features of the receptor-controlled ion translocation process depend on the activating ligand used [for instance, Katz & Miledi (1973), Colquhoun (1975), and Cash et al. (1981)]. For example, in muscle cells the lifetime of an open receptor channel is longer in the presence of suberyldicholine than in the presence of acetylcholine or carbamoylcholine (Dunin-Barkovskii et al., 1969; Katz & Miledi, 1973; Colquhoun, 1975; Neher & Sakmann, 1976a; Neher & Stevens, 1977). The detection of electrical signals due to channel opening induced by suberyldicholine is thus relatively easy (Katz & Miledi, 1973), and this ligand has been used in a number of measurements of receptor-mediated voltage or current fluctuations in cells (Katz & Miledi, 1973; Colquhoun, 1975; Neher & Sakmann, 1976a,b; Neher & Stevens, 1977) and in other electrophysiological studies (Adams, 1977; Dionne et al., 1978; Koblin & Lester, 1978; Neher & Steinbach, 1978).

Chemical kinetic investigations of acetylcholine receptor controlled transmembrane ion fluxes in *Electrophorus electricus* membrane vesicles, using either carbamoylcholine or acetylcholine as the activating ligand (Hess et al., 1979; Cash & Hess, 1981), led to the proposal of a minimum mechanism relating ligand binding and ion fluxes (Aoshima et al., 1980; Cash & Hess, 1980; Cash et al., 1980, 1981). The effects of acetylcholine and carbamoylcholine were studied over a wide concentration range, 2000-fold for carbamoylcholine (Aoshima et al., 1980, 1981) and 5000-fold for acetylcholine (Cash et al., 1980, 1981), and the functional differences between the two ligands were elucidated (Hess et al., 1981). No evidence for a regulatory site was obtained in these investigations. The minimum scheme (Scheme I) based on these data is shown in Figure 1. The pertinent equations have been derived previously (Cash & Hess, 1980; Aoshima et al., 1981) and are given in Table I. The symbols used are defined in the legend to Figure 1 and the constants in Table I.

Here we report that the mechanism of interaction of suberyldicholine with the acetylcholine receptor is more complex than that of carbamoylcholine and acetylcholine. At high concentrations, suberyldicholine inhibits the receptor-controlled ion translocation process, presumably by binding to a regulatory site of the receptor.

The existence of regulatory binding sites has been proposed previously in order to explain the effect of local anesthetics (Adams, 1977; Heidmann & Changeux, 1978; Koblin & Lester, 1978; Neher & Steinbach, 1978), the inhibition caused

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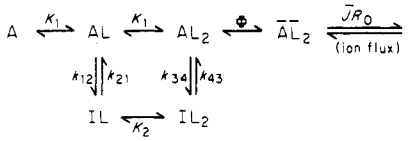
Table I: Minimum Mechanisms and Pertinent Equations[a]

Scheme I: No Inhibition (See Figure 1)

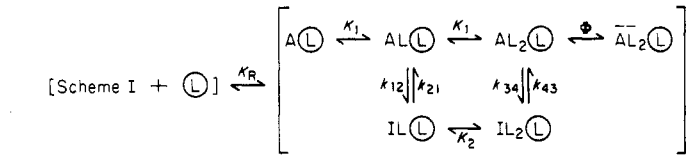


$$J_A = \bar{J}R_0L^2/[L^2(1+\Phi) + 2LK_1\Phi + K_1^2\Phi] \quad (1a\text{-I})$$

$$(\bar{J}R_0/J_A - 1)^{1/2} = \Phi^{1/2} + K_1\Phi^{1/2}/L \quad (1b\text{-I})$$

$$\alpha = \Phi(k_{12}2K_1L + k_{34}L^2)/[L^2(1+\Phi) + 2K_1\Phi L + K_1^2\Phi] + (k_{21}2K_2 + k_{43}L)/(2K_2 + L) \quad (2\text{-I})$$

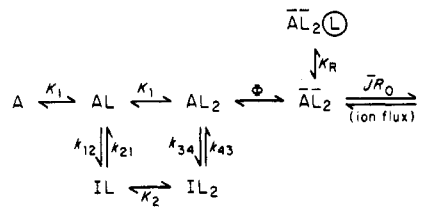
Scheme II: Suberyldicholine Inhibits by Binding to All Forms of Receptor (See Figure 1)



$$J_A = \bar{J}R_0(L^2/[L^2(1+\Phi) + 2K_1\Phi L + K_1^2\Phi])[K_R/(K_R + L)] \quad (1\text{-II})$$

α is given by eq 2-I (2-II)

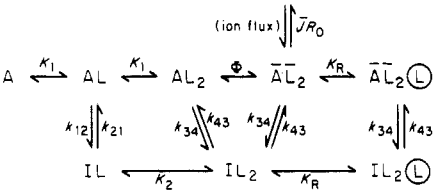
Scheme III: Suberyldicholine Inhibits by Binding to $\bar{A}\bar{L}_2$, the Open Form



$$J_A = \bar{J}R_0L^2/[L^2(1+\Phi) + 2K_1\Phi L + K_1^2\Phi + L^3/K_R] \quad (1\text{-III})$$

$$\alpha = \Phi(k_{12}2K_1L + k_{34}L^2)/[L^2(1+\Phi) + 2K_1\Phi L + K_1^2\Phi + L^3/K_R] + (k_{21}2K_2 + k_{43}L)/(2K_2 + L) \quad (2\text{-III})$$

Scheme IV: Suberyldicholine Inhibits by Binding to $\bar{A}\bar{L}_2$ and IL_2



J_A is given by eq 1-III (1-IV)

$$\alpha = [k_{12}2K_1L\Phi + k_{34}L^2(\Phi + 1 + L/K_R)]/[L^2(1+\Phi) + 2K_1\Phi L + K_1^2\Phi + L^3/K_R] + [k_{21}2K_2 + k_{43}L(2 + L/K_R)]/(2K_2 + L + L^2/K_R) \quad (2\text{-IV})$$

$$M_t/M_\infty = 1 - \exp(-[J_A[(1 - e^{-\alpha t})/\alpha] + J_I t]) \quad (3)$$

[a] J_A, the rate constant for influx before inactivation occurs, is defined by eq 1-I when acetylcholine or carbamoylcholine is the activating ligand and eq 1-II when suberyldicholine is used. At suberyldicholine concentrations lower than 15 μM, when $K_R \gg L$, eq 1-II reduces to eq 1-I. $\bar{J}$ is the specific reaction rate for the ion translocation process, reflects the properties of the receptor, and is independent of the activating ligand used or the receptor concentration (Hess et al., 1981). R_0 indicates the moles of receptor per liter of solution inside the vesicles, $\phi = AL_2/\bar{A}\bar{L}_2$, and K_1 and K_2 are the intrinsic dissociation constants of the complexes involving the active (A) or inactive (I) forms of the receptor, respectively. K_R is the dissociation constant of the complexes involving the regulatory site of the receptor. $K_R = [R][L]/[RL]$; k_{12} and k_{21} are the rate constants for interconversion between active and inactive forms of the receptor with one molecule of suberyldicholine bound. k_{34} and k_{43} are the rate constants with two suberyldicholine molecules bound. These rate constants have been assumed, for simplicity, to be the same for the isomerization processes between active and inactive forms with two molecules of suberyldicholine bound. In the expression for α given by eq 2-IV, occupancy of the regulatory site by suberyldicholine has only a small effect on the first term of the equation. The second term of the equations describing α is usually much smaller than the first since $k_{12} \gg k_{21}$ and $k_{34} \gg k_{43}$ (Aoshima et al., 1981); therefore, Scheme IV also predicts that α is not affected by occupancy of the regulatory site by suberyldicholine but the scheme is more complex than Scheme II. Equation 3 is the integrated rate equation derived from the minimum scheme (Cash & Hess, 1980) (Schemes I and II) and is used to calculate the rate constant for influx before inactivation (J_A) and the inactivation rate constant (α), from influx of radioactive tracer ions. J_I is much smaller than J_A (Hess et al., 1983a) and is obtained in separate measurements (Aoshima et al., 1981). All the constants not defined here are defined in previous publications that have been summarized recently (Hess et al., 1983a).

by high concentrations of decamethonium (Adams & Sakmann, 1978) and of dansylcholine (Cohen & Changeux, 1973), and the inactivation (desensitization) of the receptor (Dunn & Raftery, 1982; Walker et al., 1982). Here we characterize the effect of suberyldicholine concentration on the receptor-controlled transmembrane ion flux. This is the first report of chemical kinetic investigations of the inhibitory effect exerted by this activating ligand at high concentrations.

Experimental Procedures

Suberyldicholine diiodide was a gift from Dr. Ungar, University of Edinburgh. The other chemicals used and the preparation of receptor-rich vesicles from the electroplax of *E. electricus* have been described previously (Kasai &

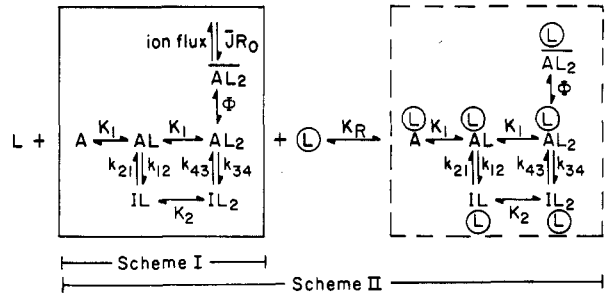


FIGURE 1: Minimum mechanism relating ligand binding to acetylcholine receptor and receptor-controlled ion flux. Scheme I was previously proposed on the basis of measurements obtained with carbamoylcholine (Cash & Hess, 1980; Aoshima et al., 1981) and acetylcholine (Cash et al., 1981). Three states of the receptor are considered in Scheme I: an active closed (A), an active open ($\bar{A}$), and an inactive (I) form. L indicates ligand bound to the receptor. Two ligand dissociation constants are involved: K_1 pertaining to the A form and K_2 pertaining to the I form. The first-order rate constants, k, are pertinent to the interconversion between the A and I states. The channel-closing equilibrium constant (Φ) and the rate constant for flux ($\bar{J}R_0$) through the open channel are also included. K_R is the dissociation constant of the regulatory site of the receptor. The dissociation constants (K_1 and K_2) and the rates of interconversion between active and inactive forms (k_{12}, k_{21}, k_{34}, k_{43}) have been assumed, for simplicity, to be the same in the inhibited and noninhibited forms of the receptor. An additional ligand-binding site is included in Scheme II to account for the measurements with suberyldicholine. For clarity, ligand molecules binding to this site are represented by Ⓛ. All the constants have been defined in Table I or in previous publications that have been reviewed recently (Hess et al., 1983a).

Changeux, 1971; Fu et al., 1977). All the measurements were carried out by using vesicles equilibrated with eel Ringer's solution (169 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM MgCl, 1.5 mM sodium phosphate, pH 7.0), at 1 °C.

The fast influx of radioactive tracer metal ions, measured before and during inactivation, was followed in the millisecond to second time region by using quench-flow techniques (Fersht & Jakes, 1975) modified for use with membrane vesicles (Hess et al., 1979; Cash & Hess, 1981).

The rate constant for receptor inactivation (desensitization), α, was determined either by fitting points obtained in influx experiments to eq 3 in Table I or by exposing the receptor to suberyldicholine before the ion flux measurements were made. This was accomplished by rapidly mixing equal volumes of the vesicle suspension (0.8 mg of protein/mL) and of a solution containing suberyldicholine. After different incubation times (160 ms to 5 s), the vesicles were mixed with an equal volume of solution containing $^{86}Rb^+$ (to give about 33 μCi/mL after mixing), and influx was allowed to proceed for a constant time (1 s). The suberyldicholine concentration was kept constant during incubation and influx. The evaluation of α from such measurements has been described (Aoshima et al., 1981).

In each experiment, measurements with a saturating (1 mM) concentration of acetylcholine were performed as a control. The values of the fast influx rate constant, J_A, were normalized to each other by using the acetylcholine control in order to correct for occasional variability in the membrane preparation. The value of J_A, in fact, depends not only on the ligand concentration but also on the number of receptors per internal volume of the vesicles, which varies somewhat for different membrane preparations (Hess et al., 1981).

Results

Figure 2 shows the concentration of $^{86}Rb^+$ in the vesicles after influx has proceeded for 1 s ($M_{t=1s}$) over a 10 000-fold range of suberyldicholine concentration. The $M_{t=1s}$ values in Figure 2 were normalized to the concentration of $^{86}Rb^+$ in the

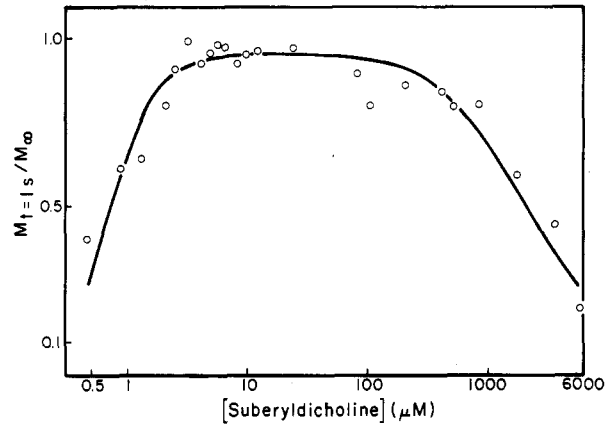


FIGURE 2: Plot of fraction of complete influx, $M_{t=1s}/M_\infty$, vs. suberyldicholine concentration. The $^{86}Rb^+$ content of the vesicles obtained after influx for 1 s, $M_{t=1s}$, in the presence of various concentrations of suberyldicholine has been normalized by the equilibrium concentration of $^{86}Rb^+$ in the vesicles, M_∞. M_∞ is the final radioactive content of the vesicles, determined in each experiment with a saturating acetylcholine concentration (1 mM), when influx is allowed to proceed for at least 1 s. The line was calculated from eq 3 (Table I), and the values of J_A and α were calculated from eq 1-II and 2-I (Table I) with the data shown in Figures 4 and 5, respectively.

vesicles at equilibrium, M_∞. It can be seen that the $^{86}Rb^+$ concentration in the vesicles first increases with increasing suberyldicholine concentration, reaches a plateau value at 10 μM ligand, and then decreases progressively as the suberyldicholine concentration is increased above 100 μM. The amplitude of the influx, $M_{t=1s}/M_\infty$, is determined by the ratio J_A/α; in fact, when $J_A \gg J_I$ and $e^{-\alpha t} \rightarrow 0$, eq 3 (Table I) reduces to

$$M_t/M_\infty = 1 - \exp(J_A/\alpha) \quad (4)$$

We have developed techniques to measure J_A, the influx rate coefficient before inactivation, and α, the inactivation rate coefficient, independently (Hess et al., 1979; Cash & Hess, 1980; Aoshima et al., 1981). We can, therefore, determine whether the observations shown in Figure 2 are due to the effect of suberyldicholine on J_A, α, or both.

The radioactive ionic content of the vesicles at different times after the addition of suberyldicholine is shown in Figure 3 and analyzed according to eq 3 in Table I. Figure 3a shows that micromolar concentrations of suberyldicholine are sufficient to induce measurable fluxes of ions into the vesicles. Both the rate constant for ion influx before inactivation (J_A) and the final amplitude of the curves after influx has occurred for 1 s ($M_{t=1s}/M_\infty$) (Figure 2) increase with increasing suberyldicholine concentration. In contrast, in the presence of more than 50 μM suberyldicholine (Figure 3b), the ion flux rate decreases with increasing ligand concentration and becomes almost undetectable at 6 mM. Also in contrast, the inactivation rate constant, α, remains constant in the millimolar range of suberyldicholine concentration, after having increased to its maximum value of 5.7 s^{-1} at lower concentrations (Figure 5).

The minimum mechanism, previously presented in order to describe the carbamoylcholine- (Cash & Hess, 1980; Aoshima et al., 1981) and acetylcholine-dependent fluxes (Cash et al., 1981; Scheme I in Figure 1), does not account for the inhibition of ion translocation that occurs at high concentrations of suberyldicholine. The additional binding of a third molecule of suberyldicholine to a regulatory site is proposed here (Scheme II in Figure 1) to explain the decrease in concentration of the open-channel form ($\bar{A}\bar{L}_2$) at concentrations of suberyldicholine higher than 50 μM. The expansion of the

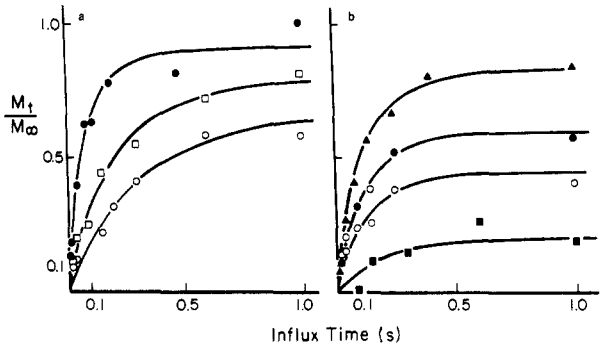


FIGURE 3: Examples of suberyldicholine-mediated flux of $^{86}Rb^+$ into membrane vesicles. Different curves represent different concentrations of suberyldicholine. M_t/M_∞ indicates the fraction of complete influx observed at different times. Each point is the average of three experimental determinations. The lines were computed by using eq 3 (Table I). (a) Low concentrations of suberyldicholine: 1.5 (○), 2.5 (□), and 10 μM (●). (b) High concentrations of suberyldicholine: 0.5 (▲), 2 (●), 4 (○), and 6 mM (□). 1.5 μM (○), J_A = 2.3 ± 0.1 s^{-1}, α = 1.9 s^{-1}, χ^2 = 0.64; 2.5 μM (□), J_A = 4.2 ± 0.2 s^{-1}, α = 2.5 s^{-1}, χ^2 = 0.79; 10.0 μM (●), J_A = 12.9 ± 0.9 s^{-1}, α = 5.0 s^{-1}, χ^2 = 0.92; 0.5 mM (▲), J_A = 8.1 ± 0.3 s^{-1}, α = 4.5 s^{-1}, χ^2 = 0.8; 2.0 μM (●), J_A = 5.5 ± 0.2 s^{-1}, α = 6.0 s^{-1}, χ^2 = 0.6; 4.0 mM (○), J_A = 3.6 ± 0.2 s^{-1}, α = 6.0 s^{-1}, χ^2 = 0.5; 6.0 mM (□), J_A = 1.1 ± 0.2 s^{-1}, α = 5.0 s^{-1}, χ^2 = 0.20.

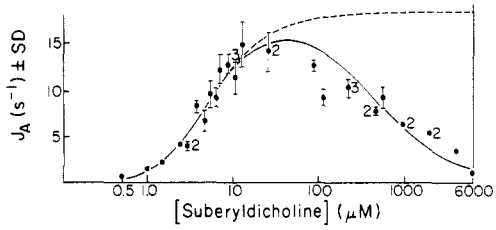


FIGURE 4: Effect of suberyldicholine concentration on rate of influx mediated by the active form of the receptor (J_A). The vertical bars show the standard deviation of the J_A values (obtained by evaluating the influx curves with a least-squares computer program), and the numbers near the points indicate how many influx curves (other than 1) were used to determine the J_A values reported. The theoretical (solid) line was calculated according to eq 1-II and Scheme II in Figure 1 by using K_1 = 4.5 μM, Φ = 1.0, and K_R = 500 μM. The dotted line indicates how J_A would change with suberyldicholine concentration in the absence of inhibition and has been included to allow a comparison with the experimentally determined line.

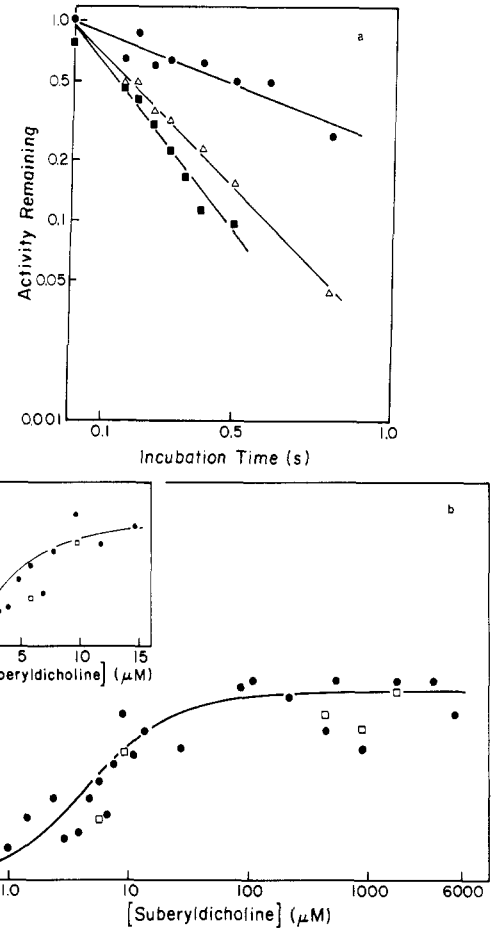


FIGURE 5: Dependence of inactivation rate constant, α, on suberyldicholine concentration. (a) Some of the incubation measurements were carried out as described under Experimental Procedures in order to determine α. "Activity remaining" corresponds to ln $[(M_\infty - M_{ls})/M_\infty]_T$/ln $[(M_\infty - M_{ls})/M_\infty]_{T=0}$ (Aoshima et al., 1981), where M_∞ is the final $^{86}Rb^+$ content of the vesicles in cpm and M_{ls} the radioactivity inside the vesicles when influx is allowed to proceed for 1 s after different incubation times (subscript T). The plot of ln (activity remaining) vs. incubation time gives a straight line according to ln (activity remaining) = αt (Aoshima et al., 1981). The slope of the lines obtained represents α [see Aoshima et al. (1981)]. The lines were fitted to the measurements by a least-squares computer program, and the following values were calculated for α, at different suberyldicholine concentrations: 6 μM (●), α = 1.5 ± 0.21 s^{-1} and χ^2 = 0.47; 10 μM (△), α = 3.8 ± 0.14 s^{-1} and χ^2 = 0.04; 1 mM (■), α = 4.6 ± 0.30 s^{-1} and χ^2 = 0.12. (b) The values of α determined from influx measurements (●) such as those illustrated in Figure 4 or from incubation measurements (□) such as those shown in (a) are plotted vs. suberyldicholine concentration. The line through the points was evaluated by using eq 2-I (Table I). The α values obtained at low suberyldicholine concentrations are shown in the inset to (b). The abscissa is a linear scale in the inset and a logarithmic scale in the main graph in (b).

minimum mechanism by including a low-affinity binding site for suberyldicholine and the corresponding definition of J_A (eq 1-II, Table I) accommodate the results obtained in the measurements of suberyldicholine-dependent influx over a 10000-fold concentration range from 0.5 μM to 6 mM. At low (<15 μM) concentrations of suberyldicholine, when $K_R \gg L$ and inhibition is not observed, eq 1-II (Table I) reduces to eq 1a-I, which can be rearranged to give eq 1b-I (Hess et al., 1981).

The dissociation constant of the regulatory sites, K_R, was evaluated by fitting the J_A values shown in Figure 4 to eq 1-II of Table I. A value of 500 μM for K_R accounted for the regulatory effect of suberyldicholine in the high concentration range (>50 μM). The solid line in Figure 4 was computed according to eq 1-II in Table I.

It should be noted that J_A reaches only about 80% of its maximum calculated value (indicated by the dashed line in Figure 4), because suberyldicholine becomes effective as an inhibitor (decreasing J_A) at concentrations at which J_A is still lower than J_A(max). The dashed line is the theoretical curve of J_A plotted vs. suberyldicholine concentrations that would be obtained if the regulatory effect due to binding of ligand to the regulatory site did not occur.

The expression for the rate constant for receptor inactivation, α, is identical in Schemes I and II and is given by eq 2-I. The values of α were fitted to eq 2-I in Figure 5b. As illustrated in Figure 5b, the value of α does not appear to be affected by the interaction of suberyldicholine with the regulatory site. The values of k_{12} (1.0 s^{-1}) and k_{34} (11.4 s^{-1}) were determined on the basis of the values of α obtained with 0.5–15 μM suberyldicholine (inset of Figure 5b).

Having determined J_A and α independently, we can now fit the line in Figure 2. The solid line in Figure 2 was computed by using eq 3, the data in Figure 4a (which give the relationship between suberyldicholine concentration and J_A), and the data in Figure 5b (which give the relationship between suberyldicholine concentration and α). A good fit between

the calculated line and the experimental measurements is obtained (see Figure 2). The inhibitory effect of suberyldicholine on the amplitude of the influx is, therefore, accounted for by the effect of the ligand on only the influx rate coefficient, J_A, according to the minimum mechanism shown in Figure 1 (Scheme II).

The rate constant for influx after inactivation (J_I) was measured at saturating concentrations of suberyldicholine (not shown), and the ratio J_A/J_I at saturation was found to about 4000. The value of J_A/J_I at saturation with acetylcholine was found to be ~1000 (Cash et al., 1981) and with carbamoylcholine 700 (Aoshima et al., 1981).

Discussion

Rapid-mixing techniques were applied to the study of transmembrane processes, in particular to investigate the interaction of carbamoylcholine, acetylcholine (Hess et al., 1979; Cash & Hess, 1980; Aoshima et al., 1981; Cash et al., 1981), and suberyldicholine with the nicotinic acetylcholine receptor and the consequent translocation of inorganic monovalent ions into membrane vesicles prepared from the electroplax of *E. electricus*. The results have been summarized in a recent review (Hess et al., 1983a). Scheme I in Figure 1 is the *simplest* mechanism that predicts the effects of carbamoylcholine and acetylcholine over the concentration range investigated (over 2000-fold). Scheme II in Figure 1 is the *simplest* mechanism that predicts the effect of suberyldicholine over the concentration range (10^4-fold) investigated. Suberyldicholine binds to a single regulatory site characterized by a dissociation constant K_R, causing a decrease in the concentration of the open-channel form of the receptor ($\bar{A}\bar{L}_2$) and, therefore, an inhibition of the ion flux. The dissociation constant for this regulatory binding to the open ($\bar{A}$) and closed (A, I) states, K_R, was assumed to be about the same for binding to all forms of the receptor and was found to be 500 μM.

With this assumption (Scheme II, Figure 1), we can account for the effect of suberyldicholine concentration on J_A (solid line, Figure 4) and α (solid line, Figure 5b), the amplitude of influx for 1 s in the presence of suberyldicholine (solid line, Figure 2). The assumptions made in arriving at the minimum model in Figure 1 also allow one to calculate the specific reaction rate, $\bar{J}$, for the receptor-controlled ion flux (Hess et al., 1981).

The high value found for K_R justifies the assumption made previously (Hess et al., 1982, 1983a) that at low concentrations of suberyldicholine (<15 μM), the regulatory effect can be neglected. It was shown that at suberyldicholine concentrations of 15 μM or less, $[(\bar{J}R_0/J_A) - 1]^{1/2}$ could be plotted vs. the reciprocal of the suberyldicholine concentration according to eq 1b-I in Table I (Hess et al., 1982). The equilibrium constant for channel opening, Φ^{-1}, evaluated from this plot had a value of 1.0, and K_I a value of 4.5 μM. The $\bar{J}$ value determined from this plot has a value of 3×10^7 M^{-1} s^{-1}, at 1 °C, pH 7.0 (Hess et al., 1982), and is the same as that found previously when acetylcholine or carbamoylcholine was used as the ligand (Hess et al., 1981). These results are consistent with the mechanism (Scheme I) proposed on the basis of results obtained with carbamoylcholine and acetylcholine and indicate (Hess et al., 1981) that in this case also, two suberyldicholine molecules bind to the active conformation of the receptor before the channel opens (Cash & Hess, 1980; Aoshima et al., 1981; Cash et al., 1981).

The specific reaction rate, $\bar{J}$, allows one to compare chemical kinetic measurements made with vesicles with electrophysiological measurements made with cells, the single-channel conductance, γ, determined in measurements with cells, is directly related to $\bar{J}$, and the values of both constants can be calculated from either chemical kinetic measurements or electrophysiological measurements with cells (Hess et al., 1983b). In experiments with muscle cells, the value of γ has been found to be independent of the activating ligand used (Neher & Stevens, 1977; Suarez-Isla et al., 1983). Furthermore, the value of γ determined in measurements with *E. electricus* electroplax cells was found to be in good agreement with the value of $\bar{J}$ determined with the vesicles that are prepared from the plasma membrane of those cells.

The interpretations of the kinetic measurements (the model in Figure 1) account for the different maximum flux rates $J_A(\max)$ that are obtained with different activating ligands (suberyldicholine, 18.5 s^{-1}; acetylcholine, 14.8 s^{-1}; carbamoylcholine, 9.7 s^{-1}). While a unique value for $\bar{J}$ is required, the different values of $J_A(\max)$ obtained with different activating ligands are accounted for by the different effects the ligands have on the channel-opening equilibrium constant, Φ^{-1}. The larger the value of Φ^{-1} the larger is the value of $J_A(\max)$ [$J_A(\max) = \bar{J}R_0(1 + \Phi)^{-1}$, Hess et al., 1981]. We find now that the channel-opening equilibrium constant is larger for suberyldicholine ($\Phi^{-1} = 1$) than for acetylcholine ($\Phi^{-1} = 0.7$). We have previously determined (Hess et al., 1981) that the value of Φ^{-1} for acetylcholine is larger than that for carbamoylcholine ($\Phi^{-1} = 0.35$).

The mean channel-open time, or the mean lifetime of the open channel that can be calculated by statistical analysis of end-plate voltage or current fluctuations (Katz & Miledi, 1973; Neher & Skamann, 1976a; Neher & Stevens, 1977) or measured directly from single-channel current recordings in muscle fibers (Colquhoun, 1975; Neher & Sakmann, 1976b) or eel electroplax cells (Hess et al., 1983b), is also dependent on the activating ligand used. The mean lifetime of the open channel is a measure of the rate constant for channel closing, the longer the mean lifetime the smaller the channel-closing rate constant. The mean lifetime of the open channel is longest for suberyldicholine and longer for acetylcholine than for carbamoylcholine (Dunin-Barkovskii et al., 1969; Katz & Miledi, 1973; Colquhoun, 1975; Neher & Sakmann, 1976; Neher & Stevens, 1977). The effect of the three activating ligands on the relative magnitude of the mean lifetimes of the open receptor channels is similar to their effect on the relative magnitudes of the channel-opening equilibrium constant. This suggests that the activating ligands exert their main effect on the rate constant for channel closing and that the rate constant for channel opening is affected to a lesser extent, if at all.

It has been suggested that local anesthetics and other inhibitors bind to the open-channel form of the receptor and block it (Adams, 1975; Ruff, 1976; Neher & Stevens, 1977; Koblin & Lester, 1978; Neher & Steinbach, 1978). The consequences of this blockage for single-channel current measurements have been analyzed in detail (Neher & Steinbach, 1978). The minimum mechanism in Figure 1 (Scheme II) is consistent with these results providing species $\bar{A}\bar{L}_2\textcircled{I}$ is impermeable to cations. Binding of suberyldicholine to species $\bar{A}\bar{L}_2$ only, see Table I (Scheme III, eq 1-III and 2-III), predicts a decrease of α at high concentrations of suberyldicholine. This is not observed (Figure 5b). Binding of suberyldicholine only to open-channel forms of the receptor is accommodated by the data providing the open-channel form, $\bar{A}\bar{L}_2$, and the inhibited open form, $\bar{A}\bar{L}_2\textcircled{I}$, can also inactivate to IL_2 and $IL_2\textcircled{I}$, respectively (Scheme IV in Table I). This scheme is more complex than Scheme II. Even in the absence of inhibition, Scheme IV requires one more isomerization process ($\bar{A}\bar{L}_2 \rightleftharpoons IL_2$) than does Scheme I (Figure 1).

The existence of a regulatory binding site in both the closed and open channels was also suggested for procaine (Adams, 1977), piperocaine (Tiedt et al., 1979), histrionicotoxin (Masukawa & Albuquerque, 1978), and phenothiazines (Carp et al., 1983) as a result of electrophysiological studies with cells. Evidence for an anesthetic binding site that is distinct from the site for the activating ligands was also provided by spectroscopic methods with a fluorescent local anesthetic, quinacrine (Grünhagen & Changeux, 1976). It was suggested that the local anesthetic site is near the channel and is present in the open as well as in the desensitized receptor (Grünhagen & Changeux, 1976). The regulatory effect of suberyldicholine observed in our studies resembles the inhibition of ion flux produced by some local anesthetics, for instance, cocaine (Karpen et al., 1982). The concomitant activating and inhibiting actions that we observed for suberyldicholine in chemical kinetic measurements with vesicles were also found by electrophysiological methods for decamethonium (Adams & Sakmann, 1978b), dansylcholine (Cohen & Changeux, 1973), and suberyldicholine (Adams & Sakmann, 1978b; Neher & Steinbach, 1978; Nelson & Sachs, 1979). In electrophysiological studies, it has not been possible to measure channel opening and channel inactivation separately or to obtain information about the constant for binding of these ligands to a regulatory site of the receptor.

In summary, chemical kinetic experiments with membrane vesicles allow one to measure three phases of the ion-translocation process (characterized by the rate coefficients J_A, J_I, and α) separately and over a wide range (10^4-fold with suberyldicholine) of activating ligand concentration. It was, therefore, possible to propose a minimum mechanism that accounts for the effect of ligand concentration on J_A and α. A minimum extension of the previously proposed mechanism (Figure 1, Scheme I) involves the binding of suberyldicholine to a single regulatory site present on all species of the receptor, characterized by a single dissociation constant, K_R (Figure 1, Scheme II). This interpretation leads to the conclusion that ion flux in the millisecond time region requires the binding of two ligand molecules to the receptor in agreement with results previously obtained with carbamoylcholine (Cash & Hess, 1980; Aoshima et al., 1981) and acetylcholine (Cash et al., 1981). The transmembrane flux characterized by the rate coefficient, J_A, reaches a maximum value and then decreases with increasing suberyldicholine concentration. This flux undergoes inactivation in the first few hundreds of milliseconds after addition of suberyldicholine, with a rate that is dependent on the ligand concentration. The inactivation rate coefficient, α, reaches a maximum value but, in contrast to J_A, does not decrease when the suberyldicholine concentration is further increased. A slow flux in the minute time region remains after the inactivation process has gone to completion.

At present, the mechanism in Scheme II (Figure 1), which relates the binding of suberyldicholine to ion translocation over a 10000-fold concentration range, is the simplest one that is consistent with both the chemical kinetic measurements with vesicles and the statistical methods, which give information about elementary steps of the channel-opening process (Katz & Miledi, 1972; Neher & Sakmann, 1976). Future studies may require an elaboration of the simple model, for example, the introduction of an inactivation of the open-channel form and the abandonment of other simplifying assumptions.

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Registry No. Suberyldicholine, 7262-79-5; acetylcholine, 51-84-3; carbamoylcholine, 462-58-8; Rb, 7440-17-7.

References

Adams, P. R. (1975) *J. Physiol. (London) 246*, 61P–63P.
Adams, P. R. (1977) *J. Physiol. (London) 268*, 271–289, 291–318.
Adams, P. R., & Sakmann, B. (1978a) *J. Physiol. (London) 283*, 621–644.
Adams, P. R., & Sakmann, B. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2994–2998.
Aoshima, H., Cash, D. J., & Hess, G. P. (1980) *Biochem. Biophys. Res. Commun. 92*, 896–904.
Aoshima, H., Cash, D. J., & Hess, G. P. (1981) *Biochemistry 20*, 3467–3474.
Carp, J. S., Aronstam, R. S., Witkop, B., & Albuquerque, E. X. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 310–314.
Cash, D. J., & Hess, G. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 842–846.
Cash, D. J., & Hess, G. P. (1981) *Anal. Biochem. 112*, 39–51.
Cash, D. J., Aoshima, H., & Hess, G. P. (1980) *Biochem. Biophys. Res. Commun. 95*, 1010–1016.
Cash, D. J., Aoshima, H., & Hess, G. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3318–3322.
Cohen, J. B., & Changeux, J.-P. (1973) *Biochemistry 12*, 4855–4864.
Colquhoun, D. (1975) *Nature (London) 253*, 204–206.
Colquhoun, D., & Sakmann, B. (1981) *Nature (London) 294*, 464–466.
Dionne, V. E., Steinbach, J. H., & Stevens, C. F. (1978) *J. Physiol. (London) 281*, 421–444.
Dunin-Barkovskii, V. L., Kovalev, S. A., Magazanik, L. G., Potapova, T. V., & Chailakhyan, L. M. (1969) *Biofizika 14*, 485–494.
Dunn, S. M. J., & Raftery, M. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6757–6761.
Fersht, A. R., & Jakes, R. (1975) *Biochemistry 14*, 3350–3362.
Fu, J-j. L., Donner, D. B., Moore, D. E., & Hess, G. P. (1977) *Biochemistry 16*, 678–684.
Grünhagen, H-h., & Changeux, J.-P. (1976) *J. Mol. Biol. 106*, 497–516, 517–535.
Heidman, T., & Changeux, J.-P. (1978) *Annu. Rev. Biochem. 47*, 317–357.
Hess, G. P., Cash, D. J., & Aoshima, H. (1979) *Nature (London) 282*, 329–331.
Hess, G. P., Aoshima, H., Cash, D. J., & Lenchitz, B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1361–1365.
Hess, G. P., Pasquale, E. B., Karpen, J. W., Sachs, A. B., Takeyasu, K., & Cash, D. J. (1982) *Biochem. Biophys. Res. Commun. 107*, 1583–1588.
Hess, G. P., Cash, D. J., & Aoshima, H. (1983a) *Annu. Rev. Biophys. Bioeng. 12*, 443–473.
Hess, G. P., Kolb, H.-A., Läuger, P., Schoffeniels, E., & Schwarze, W. (1983b) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Higman, H. B., Podleski, T. R., & Bernal, E. (1963) *Biochim. Biophys. Acta 75*, 187–193.
Hodgkin, A. L., & Huxley, A. F. (1952) *J. Physiol. (London) 117*, 500–544.
Karpen, J. W., Aoshima, H., Abood, L. B., & Hess, G. P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2994–2998.
Kasai, M., & Changeux, J.-P. (1971) *J. Membr. Biol. 6*, 1–23.

Katz, B. (1966) *Nerve, Muscle and Synapse*, McGraw-Hill, New York.
Katz, B., & Miledi, R. (1972) *J. Physiol. (London) 224*, 665–699.
Katz, B., & Miledi, R. (1973) *J. Physiol. (London) 230*, 707–717.
Koblin, D. D., & Lester, H. A. (1978) *Mol. Pharmacol. 15*, 559–580.
Masukawa, L. M., & Albuquerque, E. X. (1978) *J. Gen. Physiol. 72*, 351–367.
Neher, E., & Sakmann, B. (1976a) *J. Physiol. (London) 258*, 705–729.
Neher, E., & Sakmann, B. (1976b) *Nature (London) 260*, 799–802.
Neher, E., & Stevens, C. F. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 345–381.
Neher, E., & Steinbach, J. H. (1978) *J. Physiol. (London) 277*, 153–176.
Nelson, D. J., & Sachs, F. (1979) *Nature (London) 282*, 861–863.
Ruff, R. L. (1976) *Biophys. J. 16*, 433–439.
Schoffeniels, E., & Nachmansohn, D. (1957) *Biochim. Biophys. Acta 26*, 1–15.
Suarez-Isla, B. A., Wan, K., Lindstrom, S., & Montal, M. (1983) *Biochemistry 22*, 2319–2323.
Tiedt, T. N., Albuquerque, E. X., Bakry, N. M., Eldefrawi, M. E., & Eldefrawi, A. T. (1979) *Mol. Pharmacol. 16*, 909–921.
Walker, J. W., Takeyasu, K., & McNamee, M. G. (1982) *Biochemistry 21*, 5384–5389.

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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