Acetylcholine Receptor Inhibition by *d*-Tubocurarine Involves both a Competitive and a Noncompetitive Binding Site As Determined by Stopped-Flow Measurements of Receptor-Controlled Ion Flux in Membrane Vesicles†

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ABSTRACT: The issue of whether *d*-tubocurarine, the classical acetylcholine receptor inhibitor, inhibits the receptor by a competitive or noncompetitive mechanism has long been controversial. *d*-Tubocurarine, in this study, has been found to be both a competitive (K_C = 120 nM) and a noncompetitive (K_{NC} = 4 μM) inhibitor of receptor-mediated ion flux at zero transmembrane voltage in membrane vesicles prepared from *Electrophorus electricus* electroplax. A spectrophotometric stopped-flow method, based on fluorescence quenching of entrapped anthracene-1,5-disulfonic acid by Cs^+, was used to measure both the rate coefficient of ion flux prior to receptor inactivation (desensitization) and the rate coefficient of the rapid inactivation process. Inhibition by *d*-tubocurarine of the initial rate of ion flux decreased with increasing acetylcholine concentration, consistent with competitive inhibition, but the inhibition by micromolar concentrations of *d*-tubocurarine could not be overcome with saturating concentrations of acetylcholine, consistent with noncompetitive inhibition. A minimum mechanism is proposed in which *d*-tubocurarine competes for one of the two acetylcholine activating sites and also binds to a noncompetitive site. The present data do not distinguish between one or two competitive sites, although one successfully accounts for all of the data. By variation of the acetylcholine concentration, the two types of sites could be studied in isolation. Binding of *d*-tubocurarine to the noncompetitive site does not change the rate of rapid receptor inactivation, whereas binding of *d*-tubocurarine to the competitive site decreases the rate of rapid inactivation by displacing acetylcholine, in agreement with the observation that *d*-tubocurarine does not inactivate (desensitize) the *E. electricus* receptor by itself. In addition, evidence is presented showing that *d*-tubocurarine and cocaine bind to the same noncompetitive inhibitory site on active (nondesensitized) receptors.

For many years, *d*-tubocurarine has been regarded as a competitive inhibitor of the acetylcholine receptor-mediated permeability response (Jenkinson, 1960; Higman et al., 1963). The original conclusions of Jenkinson were based on the observation, in electrophysiological experiments with vertebrate skeletal muscle, that *d*-tubocurarine causes a parallel shift of the dose–response relationship for receptor activation toward higher concentrations of activating ligand. A number of groups have come to the same conclusions and account for inhibition exclusively by a competitive mechanism (Moreau & Changeux, 1976; Popot et al., 1976; Sheridan & Lester, 1977; Armstrong & Lester, 1979; Neubig & Cohen, 1979; Sine & Taylor, 1981).

Beginning in 1976, several groups have observed effects of *d*-tubocurarine that were not consistent with competitive inhibition. Decamethonium-induced $^{22}Na^+$ efflux from *Electrophorus electricus* vesicles was inhibited by *d*-tubocurarine in a manner consistent with noncompetitive inhibition (Hess et al., 1976). The inhibition by *d*-tubocurarine was not overcome by high concentrations of decamethonium, indicating that decamethonium could not displace *d*-tubocurarine from its binding site(s). In *Aplysia* neurons (Marty et al., 1976; Ascher et al., 1978), and at the frog neuromuscular junction (Manalis, 1977), voltage-dependent effects of *d*-tubocurarine were observed in electrophysiological experiments, which were proposed to result from a "channel-blocking" action of *d*-tubocurarine, rather than from a competitive effect at the acetylcholine binding sites. Subsequent electrophysiological measurements with cells (Katz & Miledi, 1978; Colquhoun et al., 1979; Lambert et al., 1980; Shaker et al., 1982) indicated that *d*-tubocurarine can alter the open-channel lifetime in a manner similar to that observed with local anesthetics and other aromatic amine noncompetitive inhibitors [reviewed in Adams (1981) and discussed in the preceding paper (Karpen & Hess, 1986)]. Because voltage-insensitive effects were also observed in electrophysiological measurements (Colquhoun et al., 1979), it was concluded in several studies that *d*-tubocurarine has two different actions, competition for acetylcholine binding (voltage insensitive) and a noncompetitive blockade of the open channel (voltage sensitive).

d-Tubocurarine has also been shown to be a weak receptor channel-activating ligand, but almost exclusively in embryonic preparations (Ziskind & Dennis, 1978; Trautmann, 1982; Jackson et al., 1982; Takeda & Trautmann, 1984). In single-channel recording studies on rat myotubes (Trautmann, 1982; Takeda & Trautmann, 1984), *d*-tubocurarine was able to both open channels and decrease the mean channel-open time at high *d*-tubocurarine concentrations, presumably by the blocking action proposed in the studies described above.

Despite the observations of different effects, the mechanism of *d*-tubocurarine inhibition remains controversial and poorly understood. Within the same tissue type (*Torpedo*) electro-

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plax, one group (Neubig & Cohen, 1979) observed two widely separated affinities for *d*-tubocurarine in binding studies and concluded that both sites compete with acetylcholine. Another group (Shaker et al., 1982) characterized a site in *Torpedo* that they concluded to be noncompetitive for acetylcholine on the basis of the observation that *d*-tubocurarine displaced another noncompetitive inhibitor, histrionicotoxin, under conditions in which the competitive sites for *d*-tubocurarine had presumably been blocked by α-bungarotoxin. In the clonal muscle cell line BC_3H1, one group (Sine & Taylor, 1981) has characterized two sites of widely different affinities for various receptor inhibitors, including dimethyl-*d*-tubocurarine, on the basis of binding and $^{22}Na^+$ flux studies. The conclusion was that both sites are competitive for channel-activating ligands. One contention has been that the putative noncompetitive sites observed by electrophysiologists are of significance only for inhibition at negative (inside the cell) membrane potentials.

The sites have remained poorly characterized for several reasons. Receptor inactivation (desensitization) has recently been shown to be rapid (subsecond) in all the tissues studied thus far (Hess et al., 1979, 1982; Sakmann et al., 1980; Aoshima et al., 1981; Walker et al., 1981). In almost all the previous studies on *d*-tubocurarine inhibition referenced (including all studies involving slow application of acetylcholine and electrophysiological equilibrium dose ratios, voltage-jump relaxation, ion flux, and binding), the receptor was exposed to ligand for a considerable period of time and was presumably either largely or fully desensitized. Because channel-activating ligands and many noncompetitive inhibitors bind with much higher affinity to inactive (desensitized) receptor (Krodel et al., 1979; Karpen et al., 1982; Karpen & Hess, 1986), it is necessary in characterizing inhibition to separate effects on active and inactive receptor. A second problem has been that, in those studies which *may* not have been complicated by desensitization, the activating ligand concentration was either unknown or could only be varied over an extremely low concentration range (to avoid desensitization). In order to properly separate and characterize competitive and noncompetitive binding sites, the ligand concentration must be varied over a wide range. For example, at very low ligand concentrations, competitive and noncompetitive inhibition of steady-state responses are indistinguishable. At a given concentration of ligand, and in the absence of desensitization, the open-channel form of the receptor, the concentration of which determines the rate of the receptor-controlled ion flux, can be assumed to be in a steady state.

The independent measurement, in membrane vesicles, of ion flux rates prior to receptor inactivation and inactivation rates, over a wide range of ligand concentrations, has led to the proposal of a minimum mechanism to account for receptor-controlled ion translocation [reviewed in Hess et al. (1983)]. In the preceding paper (Karpen & Hess, 1986), rates of ion flux prior to inactivation were measured over a wide range of ligand and noncompetitive inhibitor (cocaine, procaine, and phencyclidine) concentrations, in order to determine which active (nondesensitized) receptor forms are involved in inhibition by noncompetitive inhibitors. The results were consistent with the inhibitors binding to all the active receptor forms with equal affinity, and not preferentially to the open-channel form of the receptor. It was also determined that an inhibitor that enhances the rate of rapid receptor inactivation, phencyclidine (Karpen et al., 1982), binds to the same site on active receptors as an inhibitor that does not influence rapid inactivation, cocaine.

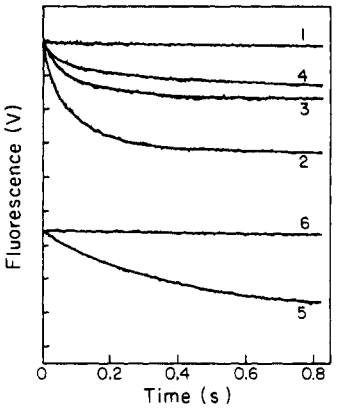


FIGURE 1: Effect of *d*-tubocurarine on receptor-controlled ion translocation in *E. electricus* vesicles, pH 7, 1 °C. Traces were obtained by using a Cs^+ stopped-flow method (Karpen et al., 1983) with improvements in vesicle purification and noise filtering described in the preceding paper (Karpen & Hess, 1986). Purified, ADS-loaded membrane vesicles (150 μg of protein/mL) were mixed with an equal volume of Cs^+ eel Ringer's solution with 6 mM $CaCl_2$ (Karpen & Hess, 1986) containing acetylcholine and *d*-tubocurarine at various concentrations. Final concentrations were as follows: curve 1, base line (no additions); curve 2, 1 mM acetylcholine; curve 3, 1 mM acetylcholine + 5 μM *d*-tubocurarine (5 mM acetylcholine + 5 μM *d*-tubocurarine, virtually superimposable); curve 4, 1 mM acetylcholine + 10 μM *d*-tubocurarine (5 mM acetylcholine + 10 μM *d*-tubocurarine and 10 mM acetylcholine + 10 μM *d*-tubocurarine, virtually superimposable); curve 5, 50 μM acetylcholine; curve 6, 50 μM acetylcholine + 5 μM *d*-tubocurarine and 50 μM acetylcholine + 10 μM *d*-tubocurarine (superimposable with base line).

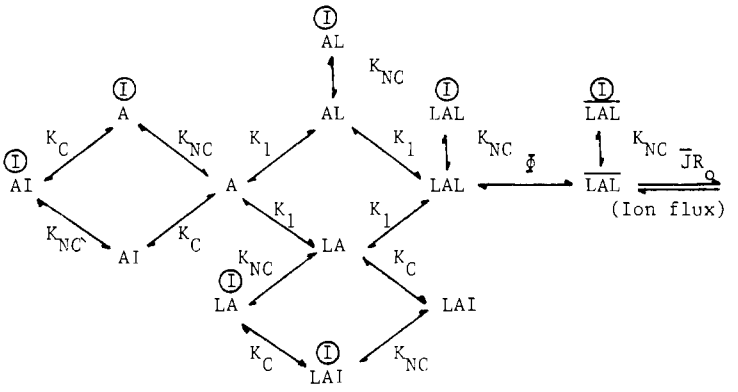
In this paper, ion flux rates associated with active (nondesensitized) receptor and inactivation rates over a wide range of acetylcholine and *d*-tubocurarine concentrations have been measured in membrane vesicles prepared from the *E. electricus* electroplax and are described. A stopped-flow method, based on the quenching of entrapped anthracene-1,5-disulfonic acid by Cs^+ (Karpen et al., 1983), was used to measure ion flux. The existence of both competitive and noncompetitive inhibition of ion flux by *d*-tubocurarine at zero transmembrane voltage was demonstrated. The sites were characterized in terms of the affinities for *d*-tubocurarine, the effects of *d*-tubocurarine binding at each site on rapid inactivation, and the competition of *d*-tubocurarine at its noncompetitive site with other known noncompetitive inhibitors.

Experimental Procedures

The use of a stopped-flow spectrophotometric method to measure ion flux and inactivation rates in membrane vesicles, based on fluorescence quenching of an entrapped dye (anthracene-1,5-disulfonic acid, ADS) by Cs^+, has been described in detail (Karpen et al., 1983; Karpen & Hess, 1986). Cocaine was kindly supplied by Professor Leo Abood, University of Rochester.

Results

Competitive and Noncompetitive Inhibition of Ion Flux by d-Tubocurarine prior to Inactivation. Figure 1 illustrates the effects of *d*-tubocurarine on receptor-controlled ion flux over a wide range of acetylcholine concentrations. There are two important observations about the data in Figure 1. The first is that the degree of inhibition of ion flux by *d*-tubocurarine is highly dependent on the acetylcholine concentration used. Concentrations of *d*-tubocurarine (5 μM) that reduce ion flux amplitudes in 1 s by approximately 50% at high acetylcholine concentrations (1 mM) completely abolish the flux induced by 50 μM acetylcholine (compare curves 3 and 6 in Figure



$$J_{A(+dTc)} = \frac{\bar{J}R_o}{(1 + I/K_{NC})\,[1 + \Phi + 2K_1\Phi/L + K_1^2\Phi/L^2 + (I/K_C)(K_1\Phi/L + K_1^2\Phi/L^2)]} \quad (1)$$

$$J_{A(-dTc)} = \frac{\bar{J}R_o}{1 + \Phi + 2K_1\Phi/L + K_1^2\Phi/L^2} \quad (2)$$

$$J_A/J_{A(+dTc)} = (1 + I/K_{NC})\left[1 + (I/K_C)\left(\frac{K_1\Phi L + K_1^2\Phi}{L^2(1 + \Phi) + 2K_1\Phi L + K_1^2\Phi}\right)\right] \quad (3)$$

$$J_{A\,(2\ \text{competitive sites})} = \frac{\bar{J}R_o}{(1 + I/K_{NC})[1 + \Phi + (2K_1\Phi/L)(1 + I/K_C) + (K_1^2\Phi/L^2)(1 + I/K_C)^2]} \quad (4)$$

FIGURE 2: Minimum mechanism to account for *d*-tubocurarine inhibition. The mechanism accounts for the effects on J_A, the rate coefficient for ion flux, over a wide range of ligand and *d*-tubocurarine concentrations. A description of the activation mechanism in the absence of inhibitors, the assumptions, and definitions of the constants (J_A, $\bar{J}R_0$, K_1, and Φ) is in the legend to Table I of the previous paper (Karpen & Hess, 1986). In the present mechanism, the inhibitor *d*-tubocurarine (I) is assumed to compete for one of the two acetylcholine-binding sites (designated by L in the figure) characterized by the dissociation constant K_C (competitive). The inhibitor also binds to a noncompetitive site, distinct from the two acetylcholine-binding sites, characterized by the dissociation constant K_{NC}. Binding of *d*-tubocurarine to the noncompetitive site is represented by a circled I. Equation 1 is the expression for J_A relating to the mechanism, eq 2 is the expression for J_A in the absence of inhibitor (Hess et al., 1983; Karpen & Hess, 1986), and eq 3 is the ratio of eq 2 to eq 1. Equation 4 is the expression for J_A for a mechanism in which there are *two* competitive sites of equal affinity (characterized by K_C) and a single noncompetitive site (characterized by K_{NC}).

1; note that curve 6 has the same slope as curve 1 which represents an experiment in which acetylcholine was absent). Ligand concentration dependent inhibition of this type (decreasing inhibition with increasing ligand concentration) indicates a competitive inhibition mechanism. The second observation is that although the inhibition is less at high acetylcholine concentrations, inhibition by concentrations of *d*-tubocurarine greater than about 1 μM could not be *completely* overcome by saturating concentrations of acetylcholine. [Acetylcholine at 1 mM saturates the activating ligand sites, i.e., results in maximum ion flux rates in the absence of *d*-tubocurarine (Cash et al., 1981)]. This is demonstrated by curves 3 and 4 in Figure 1. In the presence of 5 or 10 μM *d*-tubocurarine, raising the concentration from 1 to 10 mM acetylcholine only slightly increased the rate of ion flux. If the inhibition by *d*-tubocurarine at 1 mM acetylcholine was being caused primarily by a competitive mechanism (to be described), raising the acetylcholine concentration 10-fold would almost completely alleviate the inhibition (making the curves in the presence of *d*-tubocurarine and 10 mM acetylcholine virtually superimposable with curve 2). The inability to overcome inhibition with saturating concentrations of acetylcholine indicates noncompetitive inhibition by *d*-tubocurarine.

Recently, a voltage-dependent regulatory site for suberyldicholine and acetylcholine has been characterized (Pasquale et al., 1983; Takeyasu et al., 1983; Shiono et al., 1984). Binding to this site results in inhibition of ion flux by the ligand at high concentrations. The experiments in this paper have been performed at zero transmembrane voltage, where the affinity of acetylcholine for the regulatory site is very small. Acetylcholine at 10 mM causes only a small decrease in the ion flux rate compared to 1 mM acetylcholine [<20%, see accompanying paper by Takeyasu et al. (1986)]. This effect does not begin to account for the inability of 5 or 10 mM acetylcholine to overcome inhibition by 5–10 μM *d*-tubocurarine.

A minimum mechanism that accounts for all of the above effects of *d*-tubocurarine on initial ion flux rates is presented in Figure 2. The constants are defined in the figure legend and the footnotes to Table I of the preceding paper (Karpen & Hess, 1986). The mechanism in the absence of inhibition was proposed previously (Cash & Hess, 1980) and accounts for the ligand concentration dependence of the rate coefficient for ion flux prior to inactivation, J_A (eq 2; Figure 2), for different activating ligands over a wide concentration range [reviewed in Hess et al. (1983)]. The essential features of the mechanism in Figure 2 are a single competitive site for *d*-

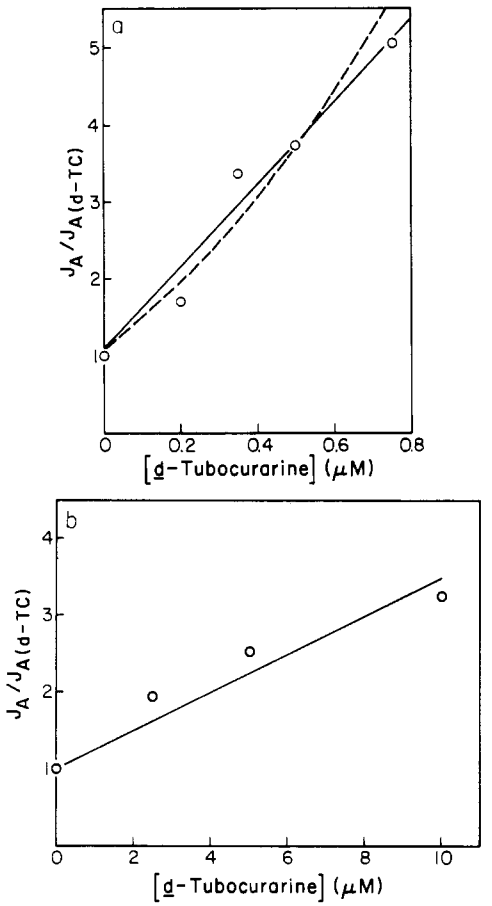


FIGURE 3: Effects of *d*-tubocurarine on the rate coefficient for ion flux at low (50 μM) and high (5 mM) concentrations of acetylcholine (*E. electricus* vesicles, pH 7, 1 °C). (a) The ratios of J_A in the absence and presence of *d*-tubocurarine ($J_A/J_{A(d\text{-TC})}$) at 50 μM acetylcholine are plotted vs. *d*-tubocurarine concentration. J_A was evaluated from stopped-flow ion flux traces as described previously (Karpen et al., 1983). The solid line represents a fit of the data to eq 3 in Figure 2 in which the expression has been reduced to a linear equation in I, $J_A/J_{A(d\text{-TC})} = 1.1 + 0.62I/K_C$, by assuming $1 + I/K_{NC} = 1.1$ and does not vary appreciably over the concentration range in this figure (<10%; see part b, $K_{NC} = 4$ μM) and substituting known values of K_1 (80 μM), Φ (1.5) [see Hess et al. (1983) and Table I of the preceding paper (Karpen & Hess, 1986)], and *L* (50 μM). The best fit of the expression to the data indicates $K_C = 120 \pm 10$ nM. The dashed line represents the best fit of the data to equation 4 of Figure 2 by assuming two competitive sites of equal affinity for *d*-tubocurarine, where K_C for each site is 340 nM. (b) The same ratio as in (a) is plotted vs *d*-tubocurarine concentration, where the acetylcholine concentration is 5 mM. The solid line represents the best fit to eq 3, Figure 2, in which the expression was reduced to the equation $(J_A/J_{A(d\text{-TC})})/(1 + I/12.8) = 1 + I/K_{NC}$ by substituting known values for K_1 (80 μM), Φ(1.5) (Hess et al., 1983), *L* (5 mM), and K_C (120 nM, part a). The measured values of $J_A/J_{A(d\text{-TC})}$ were adjusted for competitive site inhibition (part a) by dividing them by the factor (1 + $I/12.8$), shown in the left side of the equation, and the resulting values were plotted vs. the concentration of *d*-tubocurarine (I). The best fit indicates that $K_{NC} = 4 \pm 0.4$ μM. The equation reflects the fact that, at 5 mM acetylcholine, approximately 20% of the observed inhibition is due to the competitive site (see text).

tubocurarine (competition with one of two activating ligand sites; the possibility of two competitive sites will be considered) and a noncompetitive site which exists on all active receptor forms (this feature is also addressed below). The sites are characterized respectively by the dissociation constants K_C and K_{NC}. Equation 1 represents the expression for J_A for the mechanism. Equation 3 represents the ratio of the J_A expressions in the absence and presence of *d*-tubocurarine, and is used as will be discussed to determine the values of the two dissociation constants.

In Figure 3a, the *d*-tubocurarine concentration dependence of inhibition was determined at 50 μM acetylcholine in order to evaluate K_C on the basis of the mechanism in Figure 2. In the figure, the ratio of J_A in the absence and presence of *d*-tubocurarine is plotted vs. *d*-tubocurarine concentration. Equation 3 can be reduced to a simple linear equation at 50 μM acetylcholine by substituting known values for constants and the ligand concentration, as described in the legend to Figure 3a. The solid line in Figure 3a represents the best fit of the reduced form of eq 3 to the data, where $K_C = 120$ nM. The dashed line represents the best fit of the data to a mechanism in which it is assumed that there are two competitive binding sites of equal affinity where $K_C = 340$ nM (eq 4; Figure 2). The present data, therefore, cannot be used to distinguish whether there are one or two competitive binding sites for *d*-tubocurarine. The mechanisms might be distinguished at much lower ligand concentrations, where the curvature of the dashed line would be greater. However, since the ion flux amplitude is small at very low ligand concentrations, the mechanisms are not easily distinguished.

In Figure 3b, the *d*-tubocurarine concentration dependence of inhibition was determined at 5 mM acetylcholine in order to evaluate K_{NC} on the basis of the mechanism in Figure 2. The plot is the same as Figure 3a. Equation 3 is again reduced to a linear plot by substituting known values for constants and the ligand concentration, as described in the Figure 3b legend. The plotted J_A ratios have been adjusted for the inhibition by the competitive site (20%) that still occurs at 5 mM acetylcholine (see Figure 3b legend). The contribution of competitive inhibition could be eliminated by using higher concentrations of acetylcholine, but under these conditions, the inhibition caused by acetylcholine binding to its regulatory site in the absence of a transmembrane voltage would become significant [Takeyasu et al., 1983; see accompanying paper by Takeyasu et al. (1986)]. Acetylcholine at 5 mM was used to study the noncompetitive *d*-tubocurarine binding site throughout this paper, because the contributions of both *d*-tubocurarine competitive site inhibition and acetylcholine regulatory site inhibition are small at this concentration. The solid line in Figure 3b represents the best fit of the reduced form of eq 3, Figure 2, to the data, where $K_{NC} = 4$ μM.

d-Tubocurarine was found to have no detectable "agonist" properties (activation or inactivation) in the range 2–10 μM. *d*-Tubocurarine alone did not induce measurable ion flux in *E. electricus* vesicles. In addition, preincubating *E. electricus* vesicles with *d*-tubocurarine alone for 20 min did not result in a lower ion flux rate after mixing the vesicles with Cs^+ buffer, acetylcholine, and the same concentration of *d*-tubocurarine than when ion flux was measured in the presence of acetylcholine and *d*-tubocurarine at the same concentration without preincubation. This indicates that *d*-tubocurarine does not inactivate (desensitize) the receptor by itself. The concentration range (2–10 μM) was such that the competitive site(s) would be saturated to result in the maximum possible activating ligand effects, and the noncompetitive site would be occupied to various extents (33–71%).

Effects of d-Tubocurarine on the Rate of Rapid Receptor Inactivation. In Figure 4, the rate coefficient of rapid receptor inactivation, α, was measured at 5 mM acetylcholine with and without 5 μM *d*-tubocurarine (Figure 4a) and at 50 μM acetylcholine with and without 0.35 μM *d*-tubocurarine (Figure 4b). In the experiments, the rate coefficient of ion flux, J_A, was measured after different preincubation times as described in detail previously (Karpen et al., 1983). The first-order decay of the flux rate coefficient associated with

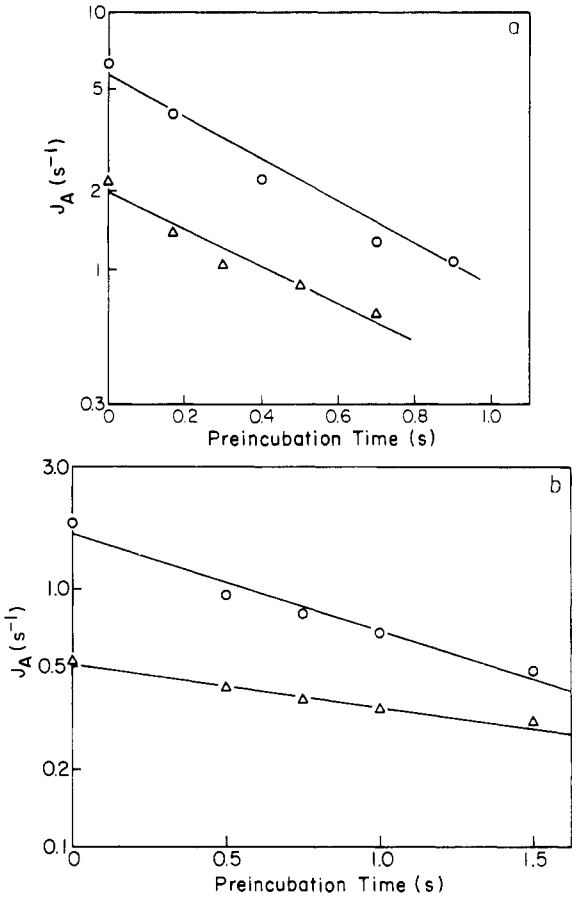


FIGURE 4: Effect of *d*-tubocurarine at its noncompetitive and competitive binding sites on the rate of rapid receptor inactivation (desensitization), pH 7, 1 °C, *E. electricus* vesicles. The measurements of α, the rate coefficient for rapid receptor inactivation, using the Cs^+ stopped-flow method with two sequential mixings has been described in detail (Karpen et al., 1983). (a) Purified ADS-loaded *E. electricus* vesicles (Karpen & Hess, 1986) were mixed with an equal volume of eel Ringer's solution, pH 7, 1 °C, such that the mixtures contained, as final concentrations, 5 mM acetylcholine (○) or 5 mM acetylcholine + 5 μM *d*-tubocurarine (△). After the times shown on the abscissa, the first mixtures were mixed with an equal volume of Cs^+ eel Ringer's solution (Karpen et al., 1983) containing the same concentrations of acetylcholine and *d*-tubocurarine. Fluorescence (ion flux) was monitored after the second mixing event. J_A values after different preincubation times were evaluated as described (Karpen et al., 1983). α is given by the slope of the semilogarithmic plot $[J_{A(T)} = J_{A(0)} \exp(-\alpha T)$ where T is preincubation time (Aoshima et al., 1981)]: 5 mM acetylcholine (○), α = 2.0 s^{-1}; 5 mM acetylcholine + 5 μM *d*-tubocurarine (△), α = 1.7 s^{-1}. (b) Procedure indentical with (a) with the following preincubation and incubation conditions: 50 μM acetylcholine (○), α = 0.9 s^{-1}; 50 μM acetylcholine + 0.35 μM *d*-tubocurarine (△), α = 0.4 s^{-1}.

active receptors represents the inactivation process (Aoshima et al., 1981; Karpen et al., 1983). α is given by the slopes of the semilogarithmic plots in Figure 4. The results indicate that the binding of *d*-tubocurarine to its noncompetitive site (Figure 4a) has no effect on α within experimental error. The binding of *d*-tubocurarine (0.35 μM) to its competitive site (Figure 4b) decreases α by a factor of 2.3. Since *d*-tubocurarine does not desensitize the *E. electricus* receptor by itself, the simplest way of accounting for the result is that *d*-tubocurarine decreases α by displacing acetylcholine. The value of α has been shown to be dependent on acetylcholine concentration (Cash et al., 1981; Hess et al., 1983), and therefore on the degree of receptor occupancy by acetylcholine. The effect of *d*-tubocurarine at its competitive site on α is probably simply due to a decrease in receptor occupancy by acetylcholine.

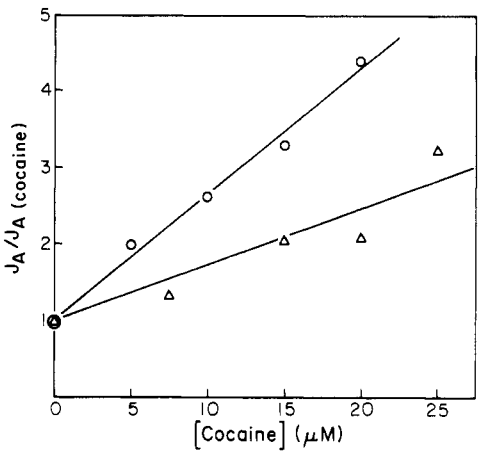


FIGURE 5: Determination of whether the noncompetitive inhibitory site for *d*-tubocurarine is the same as the site for cocaine inhibition (*E. electricus* receptor). The ratios of J_A values in the absence and presence of cocaine ($J_A/J_{A(cocaine)}$) were measured at 5 mM acetylcholine minus *d*-tubocurarine (○) and in the presence of 5 μM *d*-tubocurarine (△). The upper solid line is the best fit of the data in the absence of *d*-tubocurarine to eq 4 in Table I of the preceding paper (Karpen & Hess, 1986), indicating a K_I of 6 μM for cocaine. The lower solid line is the expected curve for cocaine inhibition in the presence of 5 μM *d*-tubocurarine, if the two inhibitors bind to the same site, according to eq 10 in Table I of the preceding paper (Karpen & Hess, 1986), assuming K_{NC} for *d*-tubocurarine (corresponding to K_{I2} in the equation) = 4 μM (Figure 3b). The line also represents the best fit to the data in the presence of *d*-tubocurarine (△): $K_{Iapp} = K_{I1}(1 + I2/K_{I2})$ = 13.8 μM [see eq 10 of the preceding paper (Karpen & Hess, 1986); I1 and I2 represent cocaine and *d*-tubocurarine, respectively].

Determination of Whether d-Tubocurarine and Cocaine Bind to the Same Noncompetitive Inhibitory Site (Active Receptor). The two alternative mechanisms, same or different binding sites, are shown as mechanisms V and VI, respectively, in Table I of the preceding paper (Karpen & Hess, 1986). In Figure 5, the ratio of J_A in the absence and presence of cocaine is plotted vs. the cocaine concentration (I1) in the presence of 0 and 5 μM *d*-tubocurarine (I2) (acetylcholine = 5 mM). If mechanism V is correct, eq 10 of the preceding paper predicts that the slope of the plot should be dependent on the *d*-tubocurarine (I2) concentration. This is what is observed in Figure 5. The lower solid line is the calculated line [using eq 10 of Karpen & Hess (1986)] for cocaine inhibition in the presence of 5 μM *d*-tubocurarine, assuming $K_{I2} = K_{NC} = 4$ μM (Figure 3b). The lower solid line is also the line that best fits the lower data set. If mechanism VI (different binding sites) were correct, the measured K_I for cocaine inhibition would be independent of the *d*-tubocurarine concentration [eq 12 of Karpen & Hess (1986)], and the data in the presence of *d*-tubocurarine would also fall on the upper solid line. The data are consistent with cocaine and *d*-tubocurarine binding to the same noncompetitive inhibitory site on active receptors.

Discussion

Having the time resolution to measure the rate of ion flux prior to inactivation and the rate of the inactivation process, over a wide range of ligand concentrations, has allowed the investigation of the mechanism of *d*-tubocurarine action in detail. As described in the introduction, previous studies either have not allowed one to separate effects on active and inactive receptors or have not varied the ligand concentration over a sufficient range to distinguish competitive and noncompetitive inhibitory effects and thus to study them in isolation. It is shown here that the effects of *d*-tubocurarine on the rapid ion flux associated with active receptors are acetylcholine concentration dependent (less inhibition with increasing acetyl-

choline concentration), but that they are not overcome by saturating acetylcholine concentrations. These results indicate both competitive and noncompetitive inhibition of receptor-controlled ion flux.

By measurement of the effects of *d*-tubocurarine at two widely separated ligand concentrations (50 μM and 5 mM acetylcholine), the competitive and noncompetitive effects could be studied largely in isolation (at 50 μM acetylcholine the effects at the competitive site dominate, with the converse being true at 5 mM acetylcholine). The data are accounted for by a single competitive site (K_C = 120 nM) and a single noncompetitive site (K_{NC} = 4 μM). Several previous studies have also reported two widely separated affinities for *d*-tubocurarine (Eldefrawi et al., 1971; Neubig & Cohen, 1979; Sine & Taylor, 1981), although it was believed that both sites were competitive with acetylcholine. The present study indicates that, at a minimum, there are two sites with widely separate affinities, one (the higher affinity site) which is competitive with acetylcholine and the other which is not. As described under Results, the present data do not distinguish whether there are one or two competitive sites. It seems likely that *d*-tubocurarine would compete for both ligand binding sites, although if the affinities for the competitive sites were widely separated, the lower affinity competitive site would be very difficult to detect in an inhibition study. The mechanism in Figure 2 represents a minimum mechanism that accounts for all the present data.

It has been shown that the binding of *d*-tubocurarine to the competitive site(s) in the absence of acetylcholine does not result in detectable activation or inactivation of the receptor. Weak agonist effects of *d*-tubocurarine have been detected, but primarily in embryonic systems, as described earlier. The single-channel recording technique, which was used in some of the studies (Trautmann, 1982; Takeda & Trautmann, 1984), may also be better suited to detecting very weak agonist effects, if the frequency and lifetime of open channels is small but the conductance of an open channel is similar to that induced by other ligands, as appears to be the case with *d*-tubocurarine in rat myotubes. An effect of *d*-tubocurarine at its competitive site was observed on the rate of rapid receptor inactivation induced by acetylcholine. The rate of inactivation observed at 50 μM acetylcholine was reduced by a factor of 2.3 in the presence of 0.35 μM *d*-tubocurarine. Since *d*-tubocurarine does not inactivate the receptor by itself, and the rate of inactivation is acetylcholine concentration dependent (Aoshima et al., 1981; Hess et al., 1983), the simplest explanation for the result is that *d*-tubocurarine at its competitive site decreases the rate of inactivation by reducing the degree of receptor occupancy by acetylcholine.

d-Tubocurarine binds to a noncompetitive inhibitory site on active *E. electricus* receptor at zero transmembrane voltage with an affinity (K_{NC} = 4 μM) similar to that observed with other high-affinity noncompetitive inhibitors (Karpen & Hess, 1986). Evidence is presented in this study showing that *d*-tubocurarine binds to the same noncompetitive inhibitory site on active receptors as cocaine. The possibility exists that the binding of one compound prevents inhibition by the other compound through an allosteric mechanism. Since the displacement would have to be *complete* for the data to agree with eq 10 of the preceding paper (Karpen & Hess, 1986), by use of the two dissociation constants measured independently (Figure 3b, *d*-tubocurarine; Figure 5, cocaine), such an allosteric mechanism is unlikely. In addition to binding to the same site, the noncompetitive inhibitory actions of *d*-tubocurarine and cocaine are similar in two other respects. The compounds inhibit with a very similar dissociation constant (on the same membrane preparation, for cocaine, 6 μM, and for *d*-tubocurarine, 4 μM). Second, *d*-tubocurarine at its noncompetitive site was shown not to change α, the rate coefficient of rapid receptor inactivation. This was previously shown to be true for cocaine (Karpen et al., 1982) and procaine (Shiono et al., 1984).

In the preceding paper (Karpen & Hess, 1986), it was demonstrated that inhibition of the initial rate of ion flux by cocaine was independent of the acetylcholine concentration. The data showed that cocaine binds to all active receptor forms (at a single site) with equal affinity, and not only to the open-channel form of the receptor. It is not possible to do the same experiments to study the noncompetitive *d*-tubocurarine site, because the competitive site dominates inhibition at low ligand concentrations. However, because cocaine and *d*-tubocurarine bind to the same site in the presence of high acetylcholine concentrations, this strongly suggests that *d*-tubocurarine at its noncompetitive site also interacts with all active receptor forms, and not exclusively with the open-channel form as previously postulated (Marty et al., 1976; Manalis, 1977). The fact that phencyclidine also competes with cocaine and binds to all active receptor forms (Karpen & Hess, 1986) provides further support for the same conclusion about *d*-tubocurarine. Phencyclidine has been shown to increase the rate of receptor inactivation at the same site, however, and to change the equilibrium between active and inactive receptor forms (Karpen et al., 1982), in contrast to both cocaine and *d*-tubocurarine.

Several aromatic amine noncompetitive inhibitors have been shown to interact weakly with activating ligand binding sites (Heidmann et al., 1983). The present study shows that *d*-tubocurarine has the structural features necessary to interact strongly with *both* acetylcholine and known noncompetitive inhibitor sites.

The application of fast reaction techniques to the investigation of transmembrane processes permits one to investigate the effects of inhibitors on acetylcholine receptor function at known concentrations of reactants (ligand and acetylcholine receptor) over a wide range of ligand concentrations. Furthermore, measurements can be made before and after receptor inactivation (desensitization), and the rate coefficients for the inactivation process can be obtained in separate experiments. We can now explain a multitude of effects of inhibitors by a simple mechanism: a number of diverse compounds (cocaine, procaine, phencyclidine, and *d*-tubocurarine) bind to a common inhibitory site that preexists on the acetylcholine receptor (channel opening is not required to reveal the site). The inhibitory site is different from the regulatory site for the activating ligands suberyldicholine and acetylcholine. Inhibitors binding to the inhibitory site may affect the rate of receptor inactivation (desensitization) (e.g., phencyclidine) or have no effect on the inactivation rate (cocaine, procaine, and *d*-tubocurarine). In addition, an inhibitor like *d*-tubocurarine can both bind to the inhibitory site and compete with the channel-activating ligand-binding site. If such an inhibitor does not inactivate (desensitize) the receptor by itself, it can decrease the rate of receptor inactivation in the presence of activating ligand by decreasing the binding of the activating ligand to its site. The simple mechanisms presented here accommodate many seemingly contradictory results obtained in electrophysiological and biochemical studies in the past.

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