Cocaine, Phencyclidine, and Procaine Inhibition of the Acetylcholine Receptor: Characterization of the Binding Site by Stopped-Flow Measurements of Receptor-Controlled Ion Flux in Membrane Vesicles†

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ABSTRACT: Noncompetitive inhibition of acetylcholine receptor-controlled ion translocation was studied in membrane vesicles prepared from both *Torpedo californica* and *Electrophorus electricus* electroplax. Ion flux was measured in the millisecond time region by using a spectrophotometric stopped-flow method, based on fluorescence quenching of entrapped anthracene-1,5-disulfonic acid by Cs^+, and a quench-flow technique using $^{86}Rb^+$. The rate coefficient of ion flux prior to receptor inactivation (desensitization), J_A, was measured at different acetylcholine and inhibitor concentrations, in order to assess which active (nondesensitized) receptor forms bind noncompetitive inhibitors. The degree of inhibition of J_A by the inhibitors studied (cocaine, procaine, and phencyclidine) was found to be independent of acetylcholine concentration. The results are consistent with a mechanism in which each compound inhibits by binding to a single site that exists with equal affinity on *all* active receptor forms. Mechanisms in which the inhibitors bind exclusively to the open-channel form of the receptor are excluded by the data. The same conclusions were reached in cocaine experiments at 0-mV and procaine experiments at −25-mV transmembrane voltage in *T. californica* vesicles. It had been previously shown that phencyclidine, in addition to decreasing J_A (by binding to active receptors), also increases the rate of rapid receptor inactivation (desensitization) and changes the equilibrium between active and inactive receptors (by binding better to inactivated receptor than to active receptor in the closed or open conformations). These effects were not observed with cocaine or procaine. Here it is shown that despite these differential effects on inactivation, cocaine and phencyclidine bind to the same inhibitory site on active receptors (in *E. electricus* vesicles). It is also shown that phencyclidine, at concentrations that significantly change the equilibrium distribution of active and inactive receptors in the presence of acetylcholine, does not appreciably change the equilibrium in *E. electricus* vesicles in the absence of acetylcholine. The implications of a preexisting binding site for these inhibitors on the active receptor, which does not require the channel to be open, are discussed.

The noncompetitive inhibition of acetylcholine receptor function by local anesthetics and other aromatic amines has been studied extensively (Popot & Changeux, 1984). Inhibition of receptor-controlled ion translocation has been accounted for in numerous electrophysiological studies by a mechanism of open-channel blockade, in which ionic channels can be blocked only after they have opened and channels can close only after unblocking (Adams, 1975, 1976, 1981; Ruff, 1977, 1982; Neher & Steinbach, 1978; Koblin & Lester, 1979; Adler et al., 1978; Gage & Hamill, 1981). The inhibition by cationic anesthetics is voltage dependent, whereas that by neutral anesthetics is not (Adams, 1981; Neher & Steinbach, 1978). From the voltage dependence of the inhibition of cationic anesthetics, it has been estimated that the drugs bind at a site near the middle of the membrane. Because a hydrophilic binding site presumably halfway across the membrane would most likely be a part of the channel, the simple, strict, sequential model (closed → open → blocked) has had intuitive appeal and does account for a number of observations in electrophysiological experiments.

Some deviations from the simple sequential model have been observed, however, in electrophysiological measurements with frog muscle cells (Adams, 1977; Tiedt et al., 1979; Albuquerque et al., 1980a,b) and in single-channel current measurements with rat cultured muscle cells (Neher, 1983). In all these measurements, the concentration of channel-activating ligand at the receptor sites was either unknown or could only be varied over a small range, or the state of receptor desensitization was not known. Therefore, the consequences of two different models, inhibitors binding only to the open-channel form or to the open- and closed-channel forms of the receptor, could not be tested.

In addition to interacting with the active (nondesensitized) forms of the receptor, inhibitors have been shown to interact with desensitized forms of the receptor. Acetylcholine receptors have been shown to inactivate (desensitize) in a variety of cells in the millisecond time region (Hess et al., 1979; Sakmann et al., 1980; Aoshima et al., 1981; Walker et al., 1981; Hess et al., 1982), but slower desensitization processes that occur in the second (Sakmann et al., 1980; Walker et al., 1981) or even the hour time region [in *E. electricus* electroplax (Aoshima, 1984)] also occur in these cells. By use of a quench-flow technique to measure ion flux in the millisecond-to-second time region in membrane vesicles prepared from the *Electrophorus electricus* electroplax, it has been shown (Karpen et al., 1982) that several inhibitors (cocaine, procaine, and phencyclidine) decrease the initial rate of ion flux mediated by the active form of the receptor before it is

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inactivated (desensitized). Phencyclidine, however, also increases the rate of rapid inactivation, an effect that was not observed with cocaine or procaine. By comparison of the effects of phencyclidine on the initial flux and the equilibrium flux (after inactivation is complete), it was concluded that phencyclidine also changes the equilibrium between active and inactive receptor forms, binding *better* to rapidly inactivated receptor than to receptor in the active closed *or* open conformations. Phencyclidine also appears to accelerate the transition to the intermediate (rapid) desensitized state in *Torpedo* (Oswald et al., 1983). Many studies in the past have shown that certain inhibitors bind better in the presence of activating ligands than in their absence [(e.g., see Krodel et al. (1979)], indicating that they bind better to fully desensitized receptor than to receptor in the resting state. Recently, several inhibitors (including phencyclidine) have been shown to shift the equilibrium to the fully desensitized state in the absence of activating ligands, presumably by binding preferentially to the fraction of preexisting desensitized receptors at rest in *Torpedo californica* preparations (Heidmann et al., 1983; Boyd & Cohen, 1984).

The measurement of ion flux and inactivation rates in membrane vesicles with different activating ligands, over a wide range of concentrations, has led to the proposal of a minimum mechanism to account for receptor-controlled ion translocation [reviewed in Hess et al. (1983)]. This mechanism has recently been extended to include a regulatory (inhibitory) binding site for suberyldicholine (Pasquale et al., 1983) and acetylcholine (Takeyasu et al., 1983) dependent on voltage. This site has been shown to be distinct from the site for procaine inhibition (Shiono et al., 1984). In this paper, initial ion flux rates prior to inactivation in *T. californica* and *E. electricus* vesicles have been measured over a wide range of ligand and inhibitor (cocaine, procaine, and phencyclidine) concentrations by using both a stopped-flow method (Karpen et al., 1983), based on the quenching of entrapped anthracene-1,5-disulfonic acid fluorescence by Cs^+, and a quench-flow technique using $^{86}Rb^+$ (Cash & Hess, 1981). The following two questions about noncompetitive inhibitor mechanisms are addressed in this paper: (i) Do the noncompetitive inhibitors studied bind selectively to the open-channel form of the receptor, or do they bind to all active (nondesensitized) forms of the receptor? (ii) Do compounds that accelerate rapid inactivation (desensitization) bind to the same site on active receptors as compounds that do not?

Experimental Procedures

The development and 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^+, have been described previously in detail (Karpen et al., 1983). The advantages of the technique over a similar stopped-flow method based on Tl^+ (Moore & Raftery, 1980), and a quench-flow technique (Hess et al., 1979; Cash & Hess, 1981) using $^{86}Rb^+$, have also been described (Karpen et al., 1983). The method has been used in the present study with the following changes and improvements.

(*i*) *Anthracene-1,5-disulfonic Acid (ADS) Synthesis.* The dye was synthesized from anthraquinone-1,5-disulfonic acid by the procedure of Lampe (1909), which resulted in higher yields than the conditions previously used (Karpen et al., 1983; Rohatgi & Singh, 1971). To 600 mL of 5% NH_4OH were added 60 g of Zn dust and 60 g of anthraquinone-1,5-disulfonic acid (Pfaltz & Bauer, Inc., Stamford, CT). The suspension was heated at 70 °C with stirring for 4 h. The suspension was filtered to remove Zn, treated with charcoal for 1 h, filtered, and rotary-evaporated. The residue was redissolved in approximately 300 mL of H_2O at 100 °C, and the solution was filtered to remove undissolved material. The dye was recrystallized twice from H_2O. The pure anthracene-1,5-disulfonic acid has an extinction coefficient of 8200 M^{-1} cm^{-1} at 365 nm (Rohatgi & Singh, 1971).

(*ii*) *Vesicle Purification To Improve Quenching Signal Fourfold.* In *E. electricus* membrane preparatons, functional receptors have been shown to exist primarily in vesicles that are well sealed and exchange internal ions slowly ($t_{1/2}$ = 6 h) with ions in the external solution (Hess & Andrews, 1977). A large-scale, CsCl–Percoll density centrifugation method has been reported (Sachs et al., 1982), in which vesicles were separated on the basis of their density, after they had exchanged internal Na^+ for external Cs^+ to varying degrees. The procedure has been adapted here to isolate well-sealed, dye-loaded vesicles from both *E. electricus* and *T. californica* preparations, a higher percentage of which respond to acetylcholine than vesicles in the native preparations. *E. electricus* and *T. californica* vesicles were loaded and frozen in 5 mM ADS (with 176 mM NaCl, 5 mM KCl, and 1.5 mM sodium phosphate buffer, pH 7, for *E. electricus* or 250 mM NaCl and 5 mM sodium phosphate buffer, pH 7, for *T. californica*) as described previously (Karpen et al., 1983). Vesicles to be used in an experiment were thawed at 4 °C. From 20 to 70 mg of vesicle protein has been successfully separated on one Percoll gradient. Vesicles were pelleted for 30 min at 40000 rpm in a 50 Ti rotor and resuspended in 20 mL of 190 mM CsCl, 1 mM sodium phosphate buffer, and 5 mM ADS, pH 7 (*E. electricus*), or 250 mM CsCl, 5 mM sodium phosphate buffer, and 5 mM ADS, pH 7 (*T. californica*). After a 90-min incubation at 4 °C, vesicles were mixed with CsCl, ADS, sodium phosphate buffer, and Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) to give a solution (38 mL) that contained 15% Percoll, 190 mM CsCl, 1 mM sodium phosphate buffer, and 5 mM ADS, pH 7 (*E. electricus*) or 15% Percoll, 250 mM CsCl, 5 mM sodium phosphate buffer, and 5 mM ADS, pH 7 (*T. californica*). The vesicle suspension was centrifuged for 30 min at 15000 rpm in a Sorvall SS-34 rotor on an RC-2B centrifuge.

After gradient fractionation (see Results), to remove Cs^+ and Percoll, in order to be able to use the Cs^+ stopped-flow method, vesicles were pelleted at 40000 rpm in a 50 Ti rotor for 45 min. Vesicles pelleted on top of the Percoll pellet. The Cs^+ buffer was removed, and the vesicles were resuspended in approximately 5 mL of 176 mM NaCl, 5 mM KCl, 5 mM ADS, and 1.5 mM sodium phosphate buffer, pH 7 (*E. electricus*), or 250 mM NaCl, 5 mM ADS, and 5 mM sodium phosphate buffer, pH 7 (*T. californica*). The vesicles were pelleted again under the same conditions to remove residual Percoll and Cs^+ and resuspended in the same buffer. The vesicles were allowed to sit for 1 day at 4 °C to allow Cs^+, which had accumulated inside the vesicles during the procedure, to leak out. Just prior to an experiment, external ADS and residual Cs^+ were removed as described previously (Karpen et al., 1983).

The purified vesicles typically contain more than twice the internal dye concentration on the average than dye-loaded vesicles in an unpurified preparation. This makes the method convenient in terms of vesicle recovery. Although only approximately one-third of the vesicle protein is pooled in the purification (Figure 1a), less than one-half of the vesicle concentration is used in the stopped-flow technique, to achieve the same degree of fluorescence. For *E. electricus* vesicles,

150 μg of protein/mL was mixed with an equal volume of Cs^+–eel Ringer's solution–6 mM $CaCl_2$ (176 mM CsCl, 6 mM $CaCl_2$, 1.5 mM sodium phosphate buffer, pH 7) as described previously (Karpen et al., 1983; see also Figure 1b). For *T. californica* vesicles, 200 μg of protein/mL was mixed with an equal volume of Cs^+ buffer (250 mM CsCl, 6 mM $CaCl_2$, 5 mM sodium phosphate buffer, pH 7). Vesicle protein was routinely determined by light scattering, which had been previously correlated to a Folin–Lowry protein determination (Cash & Hess, 1981).

(*iii*) *Instrumentation.* The stopped-flow apparatus and mixing designs for single mixing and sequential mixings have been described previously (Karpen et al., 1983). The electronics of detection were improved to result in reduced noise. The output of the photomultiplier tube was amplified 50–100-fold and filtered with a Krohn–Hite Model 3321 variable filter (Krohn-Hite Corp., Avon, MA), to reduce high frequency noise. A cutoff frequency of 50 Hz was typically used in the ion flux experiments, with no distortion of the signal for ion flux rates below $\sim 30\ s^{-1}$. Higher cutoff frequencies were used when faster rates were being measured. The data were digitized, stored, and analyzed with a PDP 11/23 computer and a DT1761-DMA-SE-C interface subsystem (Digital). A quench-flow technique was used for some of the ion flux experiments which were performed before the stopped-flow method was developed for use with *E. electricus* vesicles, and for experiments in which a transmembrane voltage was established in *T. californica* vesicles. The quench-flow technique has been described in detail (Cash & Hess, 1981). The establishment of a transmembrane voltage in *E. electricus* and *T. californica* vesicles for quench-flow experiments has also been described (Takeyasu et al., 1983; Shiono et al., 1984).

All experiments were performed at pH 7 and 1 °C, and vesicles were treated with 50 μM Tetram to inhibit acetylcholinesterase. Tetram at 50 μM has been shown to have no effect on carbamoylcholine–dependent ion flux (Cash et al., 1981).

Procaine was purchased from Schwarz/Mann. Cocaine and phencyclidine were kindly provided by Professor Leo Abood, University of Rochester Medical School.

Results

Purification of Vesicles Using CsCl–Percoll Density Centrifugation. The CsCl–Percoll gradient described under Expermental Procedures was fractionated into 43 0.9-mL fractions. The vesicle protein profile of the top of a typical gradient is shown in Figure 1a. The top of the gradient represents well-sealed vesicles (which are less dense because they have accumulated very little Cs^+), and the fractions just past the protein peak are most active in terms of specific flux response to acetylcholine, where the internal volume that responds to acetylcholine approaches the total internal volume of the vesicle fraction (Sachs et al., 1982). An average of 9–10 fractions were typically pooled for an ion flux experiment (shown in Figure 1a).

The vesicle purification results in a fourfold increase in the quenching signal, as a percentage of total fluorescence. This increase is observed with both *E. electricus* and *T. californica* membrane vesicles. Some of the enhancement (2–3-fold) results from the fact that a higher percentage of well-sealed vesicles responds to acetylcholine (Hess & Andrews, 1977; Sachs et al., 1982). The selection of well-sealed vesicles that do not lose their dye on the Sephadex G-25 column (which is used to separate dye-loaded vesicles from external dye) or leak dye after the column accounts for the remainder of the enhancement.

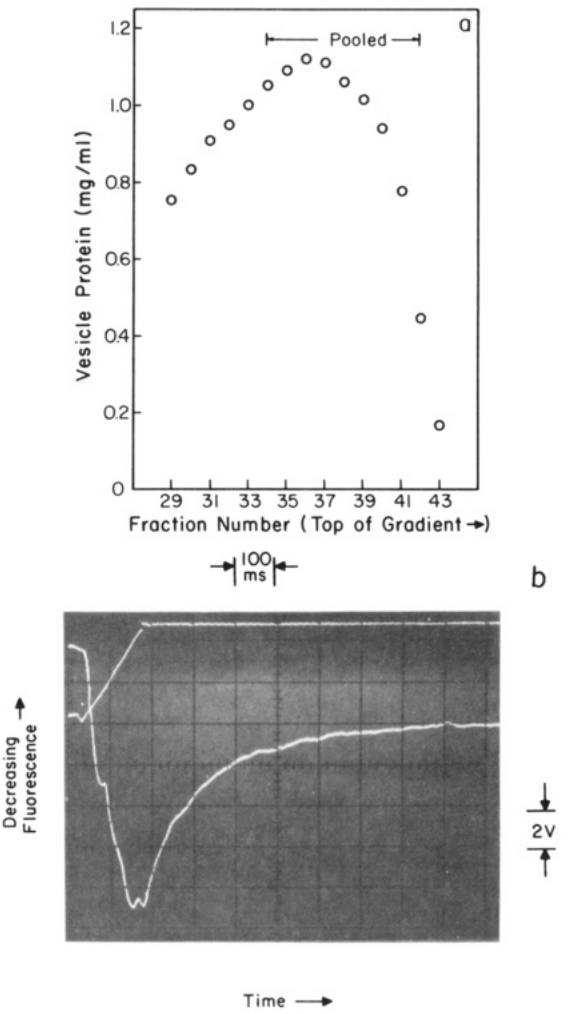


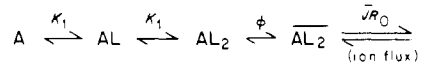
FIGURE 1: (a) Purification of well-sealed, functional vesicles from *E. electricus* electroplax. ADS-loaded membrane vesicles, which had been allowed to exchange internal Na^+ for external Cs^+ for 90 min, were separated on a CsCl–Percoll density gradient as described under Experimental Procedures. Well-sealed vesicles, which have been shown to contain functional acetylcholine receptors (Hess & Andrews, 1977; Sachs et al., 1982), do not exchange much internal Na^+ for Cs^+ in 90 min and run near the top (less dense) portion of the gradient. Vesicle protein was determined as described previously (Cash & Hess, 1981). The fractions pooled for an ion flux experiment are shown in the figure. A similar method was used for *T. californica* electroplax vesicles (Experimental Procedures). (b) Oscilloscope trace from a stopped-flow ion flux experiment with purified vesicles. Dye-loaded *E. electricus* vesicles were purified as shown in (a) and described under Experimental Procedures. In the experiment (pH 7, 1 °C), vesicles (150 μg of protein/mL) were mixed with an equal volume of Cs^+–eel Ringer's solution with 6 mM $CaCl_2$ (Experimental Procedures) containing 10 mM acetylcholine (final acetylcholine concentration is 5 mM). The upper trace monitors the progress of the piston that drives the syringes. The ion flux profile (lower trace) begins where the piston stroke ends. Increases in the ordinate (2 V/division) represent a decrease in fluorescence, as Cs^+ quenches internal dye fluorescence. An active low pass filter (cutoff frequency 50 Hz) was used to reduce high-frequency noise. The base line (–acetylcholine) was horizontal on this sensitivity scale. The signal-to-noise ratio has been improved over traces obtained previously (Karpen et al., 1983) as a result of vesicle purification and improved noise filtering.

Stopped-Flow Experiments. With vesicle purification and improved noise filtering, signal-to-noise ratios of 30:1 or greater are typically obtained in the stopped-flow experiments. An oscilloscope trace of an ion flux profile obtained with *E. electricus* vesicles at 5 mM acetylcholine is shown in Figure 1b. The base line (–acetylcholine) was horizontal on the sensitivity scale shown.

The conversion of the degree of fluorescence quenching at

Table I: Inhibitor Mechanisms[a]

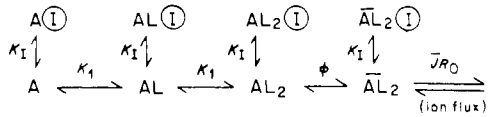
Mechanism I: No Inhibition



$$J_A = \bar{J}R_0[\bar{AL}_2]_0 \quad (2)$$

$$[\bar{AL}_2]_0 = \frac{1}{1 + \phi + 2K_1\phi/L + K_1^2\phi/L^2} = \frac{1}{D}$$

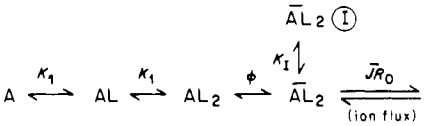
Mechanism II: Inhibitor Binds All Active (Nondesensitized) Receptor Forms with Equal Affinity



$$J_{A(I)} = \bar{J}R_0/[D(1 + I/K_I)] \quad (3)$$

$$J_A/J_{A(I)} = 1 + I/K_I \quad (4)$$

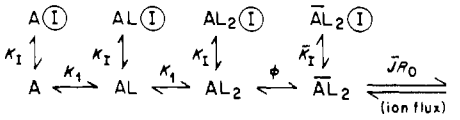
Mechanism III: Inhibitor Binds Exclusively to the Open-Channel Form of the Receptor



$$J_{A(I)} = \bar{J}R_0/(D + I/K_I) \quad (5)$$

$$J_A/J_{A(I)} = 1 + I/(K_I D) \quad (6)$$

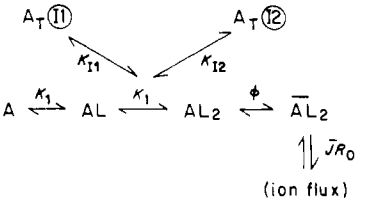
Mechanism IV: Inhibitor Binds Better to the Open-Channel Form of the Receptor Than to the Closed-Channel Form



$$J_{A(I)} = \bar{J}R_0/[D(1 + I/K_I \frac{L^2(K_I/\bar{K}_I + \phi) + 2K_1L\phi + K_1^2\phi}{L^2(1+\phi) + 2K_1L\phi + K_1^2\phi})] \quad (7)$$

$$J_A/J_{A(I)} = 1 + I/K_I \frac{L^2(K_I/\bar{K}_I + \phi) + 2K_1L\phi + K_1^2\phi}{L^2(1+\phi) + 2K_1L\phi + K_1^2\phi} \quad (8)$$

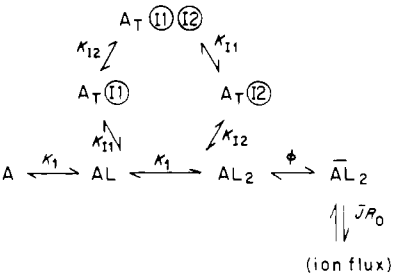
Mechanism V: Two Inhibitors, Same Binding Site



$$J_{A(I1 + I2)} = \bar{J}R_0/[D(1 + I1/K_{I1} + I2/K_{I2})] \quad (9)$$

$$J_{A(-I1)}/J_{A(+I1)} = 1 + I1/[K_{I1}(1 + I2/K_{I2})] \quad (10)$$

Mechanism VI: Two Inhibitors, Different Binding Sites



$$J_{A(I1 + I2)} = \bar{J}R_0/[D(1 + I1/K_{I1})(1 + I2/K_{I2})] \quad (11)$$

$$J_{A(-I1)}/J_{A(+I1)} = 1 + I1/K_{I1} \quad (12)$$

[a] Mechanism I, proposed previously (Cash & Hess, 1980), accounts for receptor-controlled ion translocation induced by three activating ligands (acetylcholine, carbamoylcholine, and suberyldicholine) over a 2000-fold range of ligand concentrations [reviewed in Hess et al. (1983)]. In the minimum mechanism, the receptor can exist in three states, active closed (A), active open ($\bar{A}$), and inactive (desensitized). L represents the activating ligand. The inactive forms have been left out of the mechanisms in the table, in order to focus on the effects of inhibitors on the active forms. The rate coefficient for influx prior to inactivation, J_A, can be measured independently of the rate coefficient for the inactivation process (α) (Aoshima et al., 1981). $J_A = \bar{J}R_0[\bar{AL}_2]_0$, where $\bar{J}$ is the specific reaction rate for receptor-controlled ion translocation (characteristic of the receptor and independent of the type of activating ligand), R_0 represents the moles of receptor sites per liter of internal vesicle volume, and $[\bar{AL}_2]_0$ is the fraction of active receptors in the open-channel form (Hess et al., 1981). L and I (the concentration of inhibitor) are much larger than the receptor concentration and are assumed not to change. All binding steps and isomerizations are assumed to be rapid compared to the ion translocation process and are treated as rapid equilibria. $\phi = AL_2/\bar{AL}_2$, K_1 is the intrinsic ligand dissociation constant, and K_I is the inhibitor dissociation constant of the active forms of the receptor. Bound inhibitor is represented by a circled I. Only the open form without inhibitor bound is assumed to allow ion flux. In mechanisms V and VI, I1 and I2 (different inhibitors) are assumed to bind to all active forms (A, AL, AL_2, and $\bar{AL}_2$), represented as A_T, with dissociation constants K_{I1} and K_{I2}, respectively. In mechanism I, the denominator of the expression for J_A has been called D, to simplify the expressions for J_A in mechanisms II–VI. A plot of the ratio of J_A in the absence of inhibitor to J_A in the presence of inhibitor ($J_A/J_{A(I)}$) vs. inhibitor concentration at two ligand concentrations allows one to distinguish mechanisms II and III (compare eq 4 and 6). For mechanism II (all active forms), the slope ($1/K_I$) will be independent of the ligand concentration. For mechanism III (open-channel form only), the slope is a function of D, which is dependent on ligand concentration (eq 2). To distinguish mechanisms V and VI (two inhibitors binding to the same site vs. different sites), a plot of $J_{A(-I1)}/J_{A(+I1)}$ vs. I1 at two different concentrations of I2 will give the same slope for mechanism VI and different slopes for mechanism V (compare eq 10 and 12). It should be noted that the model and the equation pertaining to it is only valid at low concentration of acetylcholine where the occupancy of the acetylcholine-specific inhibitory site is negligible.

various times (Figure 1b) to the fractional equilibration of the vesicle internal volume with metal ions (M_t/M_∞) has been described in detail previously (Karpen et al., 1983). The rate coefficients of ion flux prior to inactivation, J_A, were obtained by fitting the M_t/M_∞ profiles to eq 1 (Cash & Hess, 1980)

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

by using a nonlinear least-squares computer program. The constants are defined in the legend to Table I.

Noncompetitive Inhibitor Binding: Open Channel vs. All Active Forms. Two different mechanisms involving noncompetitive inhibition are shown in Table I. In one mechanism the inhibitor binds with equal affinity to all active (nondesensitized) receptor forms (mechanism II), and in the other inhibitor binds exclusively to the open-channel form of the receptor (mechanism III). The expressions for J_A, the rate

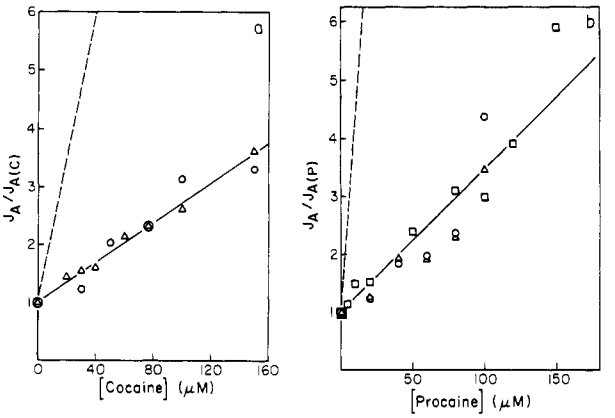


FIGURE 2: Effect of cocaine and procaine on the initial rate of ion flux in *T. californica* membrane vesicles, pH 7, 1 °C. (a) The ratios of the initial rates of ion flux in the absence and presence of cocaine ($J_A/J_{A(C)}$) are plotted vs. cocaine concentration. The Cs^+ stopped-flow method (Figure 1b) was used at zero transmembrane voltage. The two sets of points represent $J_A/J_{A(C)}$ values measured at two ligand concentrations, 7 μM acetylcholine (Δ) and 22.5 μM acetylcholine (O). The solid line represents the best fit to eq 4 for both sets of points (K_I = 58 ± 5 μM). The dashed line was generated by using eq 6 and represents the line on which the open circles (22.5 μM acetylcholine) would fall if mechanism III (Table I) were correct (inhibitor binding exclusively the open-channel form of the receptor). $K_I D$ (eq 6) for the dashed line was calculated to be 8.6 μM, assuming $K_I D$ for the 7.5 μM acetylcholine points (Δ) is the measured 58 μM. The ratio of D values (slopes) is given by the ratio of J_A values for 7.5 μM acetylcholine and 22.5 μM acetylcholine in the absence of inhibitor (eq 2), which was experimentally determined to be a factor of 6.7 [J_A(7.5 μM acetylcholine) = 3.5 s^{-1}; J_A(22.5 μM acetylcholine) = 23.5 s^{-1}]. (b) The ratios of the initial rates of ion flux in the absence and presence of procaine ($J_A/J_{A(P)}$) are plotted vs. procaine concentration. J_A's were determined by measuring $^{86}Rb^+$ influx at a transmembrane voltage of −25 mV using a quench-flow technique. The establishment of a transmembrane voltage in quench-flow measurements (Takeyasu et al., 1983; Shiono et al., 1984) and the evaluation of J_A from measurements of the $^{86}Rb^+$ content of the vesicles after a constant period of influx, M_t, in *T. californica* vesicles (Hess et al., 1982; Shiono et al., 1984) have been described. The three sets of points represent 10 μM acetylcholine (M_t at 100 ms) (Δ), 25 μM acetylcholine (M_t at 47 ms) (□), and 60 μM acetylcholine (M_t at 22 ms) (O). The solid line represents the best fit of all of the measurements to eq 4 (K_I = 40 ± 3 μM). The dashed line was generated by using eq 6 and represents the line on which the open circles (60 μM acetylcholine) would fall on the basis of the data at 10 μM acetylcholine if mechanism III were correct [according to the same analysis presented in more detail in (a)].

coefficient for ion flux prior to inactivation, are given in Table I for both mechanisms, as well as definitions for the constants in the mechanisms (in the footnotes to Table I). Mechanism I (activation without inhibitors) has been shown to account for the ligand concentration dependence of J_A over a wide range of concentrations of acetylcholine and other ligands (Hess et al., 1983). In mechanism II, J_A is decreased by the same factor ($1 + I/K_I$) at every ligand concentration. For mechanism III, the decrease in J_A is dependent on the ligand concentration, with a much smaller decrease in J_A expected at low ligand concentrations than at high concentrations.

The mechanisms are distinguished by the data in Figure 2 for cocaine and procaine binding to *T. californica* receptors. In Figure 2a the Cs^+ stopped-flow method was used to measure J_A in the presence and absence of cocaine at two different acetylcholine concentrations. The ratio of J_A in the absence and presence of cocaine ($J_A/J_{A(C)}$) is plotted vs. cocaine concentration for both 7 μM acetylcholine and 22.5 μM acetylcholine. Equation 4 (mechanism II) predicts that the slope of this plot ($1/K_I$) should be independent of ligand concentration. This is what is observed. The best fit for both sets of data points indicates a K_I of 58 μM for cocaine. If mechanism III (exclusive open-channel binding) were correct, the data at the higher acetylcholine concentration (22.5 μM) would fall on the dashed line, according to eq 6. The slope (apparent $1/K_I$) in eq 6 is a function of D, the denominator of the expression for J_A in the absence of inhibition (eq 2). Therefore, in comparing inhibition for mechanism III at two acetylcholine concentrations, the apparent K_I should decrease by the same factor as J_A is increased (or as the fraction of receptor in the open-channel form is increased) at the higher acetylcholine concentration (a factor of 6.7). In Figure 2b, similar experiments were performed with procaine by using a quench-flow technique (Cash & Hess, 1981), in which a transmembrane voltage of −25 mV was established in *T. californica* vesicles (Takeyasu et al., 1983; Shiono et al., 1984). The effect of procaine concentration on J_A was measured at three acetylcholine concentrations (10, 25, and 60 μM). The analysis and conclusions are the same as those of Figure 2a for cocaine. The inhibition (measured K_I) for procaine is independent of acetylcholine concentration. The dashed line in Figure 2b indicates where the data at 60 μM acetylcholine should fall if procaine binds exclusively to the open-channel form (the slope would differ by a factor of 13 from the slope of the solid line, which reflects the difference in J_A values at 10 and 60 μM acetylcholine). The data also show that, in the presence of a negative transmembrane voltage, which is known to increase the affinity of charged inhibitor for the receptor (Adams, 1981), procaine still does not preferentially bind to the open-channel form.

The data in Figure 2 are also consistent with a *single* inhibitory site for cocaine and procaine on the *T. californica* receptor, over the range of inhibitor concentration studied. Although multiple binding sites might exist [e.g., see Heidmann et al. (1983)], the present study is concerned with those sites that inhibit the ion flux response of the receptor. If multiple binding sites of equal or similar affinity were causing inhibition, J_A would be decreased by $(1 + I/K_I)^n$, where n is the number of sites; see mechanism VI and eq 11. Under these conditions, the plots in Figure 2 would not be linear. The linear inhibitor concentration-dependent decrease in J_A is, therefore, consistent with a single inhibitory site. Additional low-affinity inhibitory sites might be revealed at very high inhibitor concentrations.

In Figure 3, the all active forms vs. open channel binding question is addressed with cocaine and the *E. electricus* receptor. In Figure 2, the inhibitor concentration dependence of J_A was measured at two or three low ligand concentrations, because of the difficulties in measuring ion flux rates at high ligand concentrations in *T. californica* vesicles (Hess et al., 1982). In Figure 3, inhibition by a single concentration of cocaine was measured over a wide range of acetylcholine concentrations in *E. electricus* vesicles. The degree of inhibition was again observed to be independent of ligand concentration. The lower solid line (in the presence of 20 μM cocaine) differs from the upper solid line (the best fit of eq 2 to J_A measured at different acetylcholine concentrations in the absence of cocaine) by a constant factor at every ligand concentration. This is the prediction from eq 3 if mechanism II is correct. The factor ($1 + I/K_I$) was calculated from the data at 1 mM acetylcholine. The dashed line represents the J_A values expected at low acetylcholine concentrations if mechanism III were correct, given the observed inhibition at 1 mM acetylcholine. The dashed line was generated by using eq 5, with K_I being calculated from the data at 1 mM acetylcholine. It can be seen from eq 5 that, for mechanism III, much less inhibition is expected at low ligand concentrations than at high

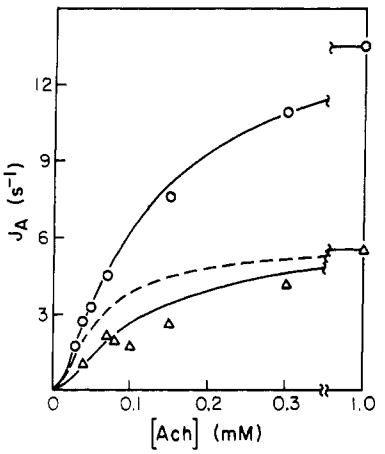


FIGURE 3: Acetylcholine concentration dependence of cocaine inhibition (20 μM) in *E. electricus* membrane vesicles, pH 7, 1 °C. Ion flux was measured by using the Cs^+ stopped-flow method, and J_A values at various ligand concentrations, in the presence (△) and absence (○) of 20 μM cocaine, were evaluated as described under Experimental Procedures. The upper solid line is the best fit to eq 2, as described previously (Cash et al., 1981) where K_I = 80 μM, Φ = 1.5, and JR_0 = 37 s^{-1}. The lower solid line was calculated by using eq 3 and differs from the upper solid line by a constant factor, $1 + I/K_I$ (=2.4, implying K_I = 14 μM), which was calculated from the data at 1 mM acetylcholine. The dashed line was generated from eq 5 and represents the J_A values that should be obtained at low ligand concentrations if mechanism III (inhibitor binding exclusively to the open-channel form of the receptor) were correct. The K_I value in eq 5 was calculated from the data at 1 mM acetylcholine (K_I = 5.2 μM) and used to generate the dashed line at lower ligand concentrations.

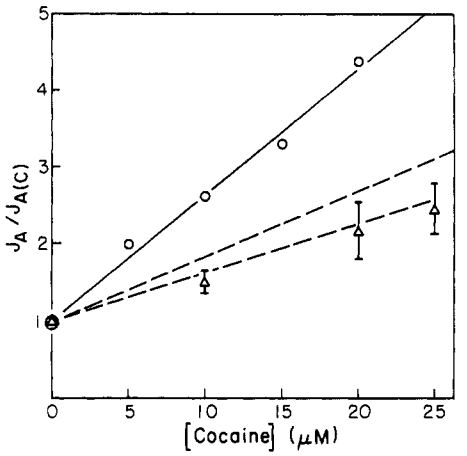


FIGURE 4: Determination of whether PCP and cocaine bind to a common inhibitory site on the *E. electricus* receptor. The ratios of J_A values in the absence and presence of cocaine ($J_A/J_{A(C)}$) were measured at 5 mM acetylcholine by using the Cs^+ stopped-flow method, without PCP (○) or in the presence of 5 μM PCP (△). The solid line is the best fit of the data without PCP (○) to eq 4, indicating a K_I of 6 μM for cocaine. (The values of K_I for the inhibitors studied have been found to be preparation dependent. The experiments in this figure were done with a different preparation than the experiments in Figure 3, in which K_I for cocaine was found to be 14 μM. There is no variation in K_I between experiments using the same preparation.) If PCP and cocaine bind to different sites, the triangles should fall on the solid line, according to eq 12. For different sites, the measured K_I for cocaine inhibition is predicted to be unaffected by the presence of PCP. PCP has previously been shown to increase α, the rate coefficient for inactivation (Karpen et al., 1982). If cocaine (which does not affect α for acetylcholine) binds to the same site, it would be expected to partially alleviate the increase in α caused by PCP. Because α is changing, there is some uncertainty in knowing α precisely and therefore a small uncertainty in determining J_A by fitting the data to eq 1. The error bars indicate the uncertainty in the J_A values obtained in the presence of both PCP and cocaine. The dashed lines indicate the range in which the data should fall, according to eq 10, if PCP and cocaine bind to the same inhibitory site. The range is presented due to the uncertainty in determining the J_A (and therefore K_I from eq 4) for PCP in the absence of cocaine, for the same reasons described above (K_I for PCP = 4 ± 1 μM, determined from the data at the intercept in the absence of cocaine).

concentrations. The conclusions regarding the mechanism of cocaine inhibition are therefore the same for two different acetylcholine receptors.

The data in Figures 2 and 3 indicate that inhibitor binding to all active forms with *equal* affinity successfully accounts for the data. Even if the inhibitor binds the open channel preferentially, but binding to closed forms is not negligible, the data at the higher concentration would fall between the solid and dashed lines in the figures, which is not the case. The experimental error in the determination of the apparent dissociation constants of the inhibitors is less than a factor of 2. Inspection of eq 8 indicates that a 3-fold difference in the dissociation constants of the inhibitor and the active forms of the receptor in the closed (K_I) and open-channel forms ($\bar{K}_I$), respectively, will change the observed dissociation constant by a factor of 2 as the acetylcholine concentration is increased in the experiments from 20 μM to 1 mM. In this range of acetylcholine concentration, the observed values of K_I for cocaine or procaine appear to be the same, indicating that the ratio of $K_I/\bar{K}_I$ has a maximum value of 3.

The main difference between the two tissues is that cocaine is a better inhibitor of the *E. electricus* receptor (K_I = 14 μM, Figure 3) than of the *T. californica* receptor (K_I = 58 μM, Figure 2a). The significance of this difference is unclear, although functional differences between the two receptors have been noted previously, particularly with regard to desensitization (Hess et al., 1982) and the values of K_R, the dissociation constant of the voltage-dependent acetylcholine-specific inhibitory site (Takeyasu et al., 1983, 1986; Shiono et al., 1984). In addition to species differences, inhibition by all the compounds studied is preparation dependent. The measured K_I for cocaine inhibition in the *E. electricus* experiments of Figure 4 is 6 μM, which differs from the K_I measured with the preparation in Figure 3 (14 μM). Experiments performed on different days with the same preparation gave identical K_I values. Consequently, the experiments within a figure were always performed on the same membrane preparation.

Determination of Whether PCP and Cocaine Bind to the Same or Different Inhibitory Sites (Active Receptor). Mechanisms V and VI (Table I) and the corresponding equations for J_A allow the two possibilities to be distinguished. In Figure 4, the ratios of J_A's in the absence and presence of cocaine ($J_A/J_{A(C)}$) are plotted vs. cocaine concentration (I1) in the presence of 0 and 5 μM PCP (I2) (acetylcholine = 5 mM). If mechanism V (same binding site) is correct, eq 10 predicts that the slope of the plot should be dependent on PCP (I2) concentration. This is what is observed in Figure 4. The apparent K_I for cocaine inhibition changes in the presence of PCP. The data fall within the expected range predicted by eq 10. The uncertainty in the range as well as the data points is due to the small uncertainty in determining J_A independently of α, the rate coefficient for inactivation, due to the effects of PCP on α (see Figure 4 legend). If mechanism VI (different binding sites) were correct, the measured K_I for cocaine inhibition would be independent of PCP concentration, and the data in the presence of PCP would fall on the same line as the data in the absence of PCP. The data are, therefore, consistent with PCP and cocaine binding to the same site on active receptors. The data literally indicate that the binding of one of the compounds prevents inhibition by the other compound. The possibility exists that this could occur through an allosteric interaction, in which a conformational change completely

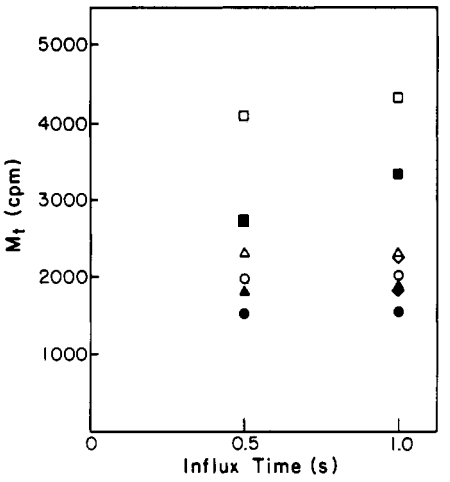


FIGURE 5: Effect of preincubation of the *E. electricus* receptor with PCP alone on ion flux. A quench-flow technique (Cash & Hess, 1981) was used to measure $^{86}Rb^+$ influx, pH 7, 1 °C. M_t represents cpm after subtracting background cpm (−acetylcholine). Preincubation with PCP was performed out of the machine. Each point is the mean of three determinations: 1 mM acetylcholine (Ach) (□); 1 mM Ach + 5 μM PCP (△); 1 mM Ach + 5 μM PCP, after a 60-s preincubation with 5 μM PCP (◇); 1 mM Ach + 5 μM PCP, after a 1.5-h preincubation with 5 μM PCP (○); 80 μM Ach (■); 80 μM Ach + 5 μM PCP (▲); 80 μM Ach + 5 μM PCP, after a 60-s preincubation with 5 μM PCP (◆); 80 μM Ach + 5 μM PCP, after a 1.5-h preincubation with 5 μM PCP (●).

prevents the binding or inhibition by the second compound. Since the displacement must be *complete* for the data to agree with eq 10 and the two dissociation constants measured independently, such an allosteric mechanism is unlikely.

Because cocaine has been shown to inhibit the receptor by binding to a single site with apparently equal affinity on all active receptor forms, the data in Figure 4 imply that PCP, in binding to the same site as cocaine, also binds all active forms. This conclusion has been corroborated by measuring the inhibition by 5 μM PCP at two acetylcholine concentrations (80 μM and 1 mM) in *E. electricus* vesicles. The same degree of inhibition of J_A was observed at both ligand concentrations (data not shown). The same observations were made for cocaine in Figure 3.

Effect of Preincubation of the E. electricus Receptor with PCP Alone. We have previously shown that PCP, in addition to binding to active receptor, increases α, the rate of rapid receptor inactivation (desensitization), and changes the equilibrium between active and inactive (desensitized) receptors by binding better to the desensitized receptor than to active receptor forms (Karpen et al., 1982). Here we show that PCP does not appreciably desensitize the *E. electricus* receptor in the absence of acetylcholine, i.e., PCP does not appear to significantly change the equilibrium between active and inactive receptors in the absence of acetylcholine. This is demonstrated in Figure 5. Preincubation of the receptor for 60 s with 5 μM PCP did not alter the rate of ion flux in a 1-s quench-flow measurement in the presence of 80 μM or 1 mM acetylcholine. Preincubation for 1.5 h resulted in a slight decrease. This may be an unspecific effect, because a similar result was obtained with cocaine (data not shown), which does not change the equilibrium between active and inactive forms in the presence of acetylcholine (Karpen et al., 1982). The result indicates that there is probably a negligible amount of preexisting desensitized receptor in *E. electricus* preparations, in contrast to recent studies with *T. californica* preparations in which certain inhibitors (including phencyclidine) were found to stabilize desensitized receptor conformations in the absence of agonist (Heidmann et al., 1983; Boyd & Cohen, 1984). The difference may be a reflection of the properties of desensitization in the two tissues (Hess et al., 1982; Changeux et al., 1984; Aoshima, 1984).

Discussion

Chemical kinetic measurements of acetylcholine receptor-mediated ion flux in membrane vesicles using rapid mixing techniques have been useful in obtaining information about those steps in the ion translocation process that are dependent on ligand concentration [reviewed in Hess et al. (1983)]. This approach has been used in the present study to determine which active (nondesensitized) receptor forms bind noncompetitive inhibitors. The ability to study inhibition by measuring effects on the initial rate of ion flux associated with active receptors (prior to inactivation) at different known ligand concentrations, where the distribution of receptor forms (between resting, singly liganded, doubly liganded closed, and open forms) is known, makes the method well suited to determining which active forms bind an inhibitor.

The results indicate that the degree of inhibition of J_A is independent of ligand concentration for cocaine, procaine, and phencyclidine. The results are consistent with mechanism II, Table I, in which the compounds inhibit receptor-controlled flux by binding to a single site that exists with equal affinity on all active receptor forms. As described earlier, multiple inhibitory binding sites would not be consistent with the linear concentration dependence of inhibition observed in Figure 2. The data are consistent with the inhibitors studied binding equally well to the closed and open-channel forms of the receptor. The results were the same in cocaine experiments at 0-mV and procaine experiments at −25-mV transmembrane voltage in *T. californica* vesicles. For the inhibitors studied, the conclusion that the receptor contains a preexisting inhibitory site that is not formed during channel opening is independent of the tissues used (*E. electricus* or *T. californica* electroplax) or the techniques used to measure ion flux (Cs^+ stopped-flow method or quench-flow technique using $^{86}Rb^+$).

Previous studies that utilized single-channel recording techniques (Neher & Steinbach, 1978; Neher, 1983) or end-plate current analysis [e.g., see Adams (1976), Ruff (1977) and Albuquerque et al. (1980b)] to study noncompetitive inhibition have focused primarily on the interactions of the inhibitors with the open-channel form of the receptor. This is partly due to the fact that these methods are best suited to studying channel-activating ligand concentration independent processes such as the lifetime of open channels. The single-channel recording method in particular has provided valuable information on the rates of blocking and unblocking the open channel by local anesthetics, the voltage and inhibitor concentration dependencies of these processes, and the conductance of blocked channels (Neher & Steinbach, 1978; Neher, 1983). In single-channel recording, the range of channel-activating ligand concentrations that can be used is limited because desensitization abolishes the signal at high ligand concentrations. In end-plate current studies the ligand concentration is unknown and changing. In one study that utilized a voltage-jump relaxation method (Adams, 1977), the ligand concentration was varied, and inhibition by procaine appeared to be independent of suberyldicholine concentration. The intent of this previous study was similar to that of the present study. The conclusions from the voltage-jump relaxation study are speculative, however, for two reasons. First, the impact of the presence of desensitized receptor was not assessed. "Equilibrium" currents such as those measured in the study would be strongly influenced by the binding of the inhibitor

to desensitized forms of the receptor. Second, the ligand dissociation constant for suberyldicholine was not determined or considered in the experiment. These experiments, therefore, do not allow one to differentiate between models in which the inhibitors bind to (i) all active forms of the receptor, (ii) only the open-channel form, or (iii) all forms but preferentially to the open-channel form. As we have just demonstrated, in order to make these distinctions, it is not only necessary to be able to make measurements of receptor function prior to the onset of desensitization but also to know the concentration of activating ligand, to be able to vary it over a large concentration range and to know its dissociation constant (see eq 6 and 8).

In attempts to locate the binding sites for noncompetitive inhibitors, several groups have photoaffinity-labeled *Torpedo* receptors efficiently with azido or mustard derivatives of noncompetitive inhibitors (Oswald & Changeux, 1981a; Kaldany & Karlin, 1983; Haring et al., 1984) or weakly by UV irradiation of the unmodified inhibitors (Oswald & Changeux, 1981b; Heidmann et al., 1983). Different inhibitors label different subunits, although the ability of chlorpromazine to label all four subunits with roughly equal efficiency and the displacement of chlorpromazine by other inhibitors suggest that the binding site for the inhibitors may exist in an area that is accessible to all receptor subunits, possibly in or near the central hydrophilic crevice, where the distances to all five receptor subunits are minimal (Changeux et al., 1984).

Several groups have recently employed the technique of rapid photoaffinity labeling as a means of identifying transient receptor forms that bind noncompetitive inhibitors (Cox et al., 1984; Heidmann & Changeux, 1984; Muhn et al., 1984). Two of the groups (Cox et al., 1984; Heidmann & Changeux, 1984) first interpreted their data in terms of a mechanism in which the inhibitors selectively label the open-channel form of the receptor. Subsequently Cox et al. (1985) suggested inhibitor binding to the desensitized state. These conclusions are based on the observations that the labeling is rapid, agonist specific, develops in the high agonist concentration range, and is transient. Here we offer two interpretations for these results: (1) The inhibitors bind to the rapidly inactivated (desensitized) receptor. The time course of photolabeling (Heidmann & Changeux, 1984; Muhn et al., 1984) follows the time course of rapid inactivation (Aoshima et al., 1981; Hess et al., 1982) more closely than it does the formation of open channels, which are believed to develop within 1 ms at high agonist concentrations. In addition, rapid inactivation rates are known to be increased by certain inhibitors (Karpen et al., 1982) and are also considerably more rapid at room temperature (unpublished observations), at which the photolabeling experiments were done, than at the temperature (1 °C) used in the previously published ion flux studies in which fast inactivation rates were measured (Aoshima et al., 1981; Hess et al., 1982; Karpen et al., 1982). Evidence has also been presented previously (Karpen et al., 1982) that phencyclidine binds to rapidly inactivated receptor with higher affinity than to receptor in the active closed *or* open conformations. In both the previous (Karpen et al., 1982) and the present studies, the effects of inhibitors on active and rapidly inactivated receptor conformations have been separated. [A detailed description of the separation of activation and inactivation parameters using rapid ion flux methods has appeared recently (Hess et al., 1983).] (2) The results from the photolabeling studies may also arise because the ratio of receptor–inhibitor dissociation constants for the active receptor in the closed- and open-channel forms may be different. Chemical kinetic measurements can differentiate between these possibilities and may be useful in the interpretation of these experiments.

The conclusion from the present study, that the channel does not have to be open to reveal the binding site for inhibitors, is also not inconsistent with conclusions from studies on the voltage dependence of inhibition, in which the binding site for inhibitors was estimated to be half to three-fourths through the membrane (Neher & Steinbach, 1978; Adams, 1981). The present study simply demonstrates that the channel (or "gate" of the channel) does not have to be open to reveal the site. In other words, there is a preexisting site for these inhibitors on a resting receptor. The small cationic aromatic amines, therefore, do not simply get stuck in the open channel and block it but bind to a site that exists prior to channel opening.

Here we have also demonstrated that an inhibitor that can increase 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 (cocaine). The simplest mechanism for phencyclidine action is that the active forms with phencyclidine bound isomerize more rapidly to inactive (desensitized) receptor, than either active receptor with no inhibitor bound or active receptor with cocaine bound to the same site. The two inhibitors, therefore, exert different effects on receptor inactivation from the same binding site. A similar phenomenon has been shown to occur with activating ligands. Different ligands are known to have different effects on the lifetime of receptor channels (Neher & Stevens, 1977) and the channel-opening equilibrium (Hess et al., 1983), even though they bind to the same ligand-binding sites. The binding of functionally diverse, noncompetitive inhibitors to the same site suggests that most known aromatic amine noncompetitive inhibitors would bind to this site and therefore interact with all active forms of the receptor. It is entirely possible, however, that some inhibitors not considered here might bind to distinct sites and even interact preferentially with the open-channel form of the receptor. It has already been shown that the activating ligands suberyldicholine (Pasquale et al., 1983) and acetylcholine (Takeyasu et al., 1983) bind to voltage-dependent regulatory sites and inhibitor receptor function at high concentrations. This regulatory site has been shown to be distinct from the binding site for procaine (Shiono et al., 1984).

Finally, it is of interest to note that the two limiting inhibition mechanisms discussed in this paper (open channel binding vs. binding to all active receptor forms) may have different physiological consequences. In the exclusive open-channel blocking mechanism, some signal might be transmitted before blocking occurs (depending on the inhibitor concentration and the rates of blocking). In the all active forms mechanism, the receptor can be blocked or inhibited before channel opening occurs. The two mechanisms are also conceptually different. One mechanism indicates that unspecific organic cations of appropriate size can block the receptor channel. A preformed binding site for cationic inhibitors suggests that this site may have a function in signal transmission, perhaps by being modulated by an as yet unidentified endogenous compound. In this regard it is important to notice that acetylcholine does not bind to this site (Shiono et al., 1984). The functional significance of these different inhibition mechanisms, and of the different inhibitory sites for ligands and aromatic amine noncompetitive inhibitors and their structural location, is the subject for further study. The experiments presented have indicated the use of chemical kinetic measurements employing fast reaction techniques in such investigations.

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Registry No. Cocaine, 50-36-2; procaine, 59-46-1; phencyclidine, 77-10-1.

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