Procaine Rapidly Inactivates Acetylcholine Receptors from *Torpedo* and Competes with Agonist for Inhibition Sites†

Stuart A. Forman

Committee on Higher Degrees in Biophysics, Harvard University, Cambridge, Massachusetts 02138, and Program in Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Keith W. Miller*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and Department of Anaesthesia, Massachusetts General Hospital, Boston, Massachusetts 02114

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ABSTRACT: The relationship between the high-affinity procaine channel inhibition site (apparent dissociation constant $K_P \simeq 200$ μM) and the agonist self-inhibition site on acetylcholine receptors (AChRs) from *Torpedo* electroplaque was investigated by using rapid $^{86}Rb^+$ quenched-flux assays at 4 °C in native AChR-rich vesicles on which 50–60% of ACh activation sites were blocked with α-bungarotoxin (α-BTX). In the presence of channel-activating acetylcholine (ACh) concentrations (10 μM–10 mM) alone, AChR undergoes one phase of inactivation (fast desensitization, rate = k_d) in under a second. Addition of procaine produces two-phase inactivation similar to that seen with self-inhibiting (>10 mM) ACh concentrations [Forman & Miller (1988) *Biophys. J. 54*, 149–158]—rapid inactivation (rate = k_r) complete in 30–75 ms is followed by fast desensitization at the same k_d observed without procaine. The dependence of k_r on [procaine] is consistent with a bimolecular association between procaine and its AChR site with $k_{on} = 2.5 \times 10^5$ M^{-1} s^{-1}, $k_{off} = 36$ s^{-1}, and $K_P = 145 \pm 36$ μM. Inhibition of AChR function by mixtures of procaine (up to $12K_P$) plus self-inhibiting concentrations of ACh or suberyldicholine ([SubCh] up to 13 × the 50% self-inhibiting agonist concentration, K_B) was studied by reducing the level of α-BTX block in vesicles. The apparent K_B increased in the presence of procaine, and the apparent K_P increased linearly with [SubCh], indicating mutually exclusive actions at a common AChR site. Our data support a mechanism where procaine binds preferentially to the open-channel AChR state, since no procaine-induced inactivation is observed without agonist and k_r's dependence on [ACh] in the channel-activating range closely parallels that of $^{86}Rb^+$ flux response to ACh.

Both local anesthetics (LA's)[1] and cholinergic agonists inhibit nicotinic acetylcholine receptor (AChR) function at a site or sites distinct from the receptor protein structures that bind ACh and trigger the transmembrane cation flux response [for reviews, see Adams (1981) and Udgaonkar and Hess (1986)]. Indeed, a number of features of "self-inhibition" by cholinergic agonists are similar to LA actions at the AChR cation channel. Electrophysiological studies reveal rapid, brief closures of open channels in the presence of either LA's (Neher & Steinbach, 1978) or high concentrations of acetylcholine (ACh), carbamylcholine (CCh), and suberyldicholine (SubCh) (Sine & Steinbach, 1984; Ogden & Colquhoun, 1985). The inhibitory potencies of LA's (Kordas, 1970; Adams, 1977) and agonists (Takeyasu et al., 1983, 1986; Sine & Steinbach, 1984; Ogden & Colquhoun, 1985) at the AChR are both enhanced by increased negative membrane potential (hyperpolarization). Furthermore, inhibitory concentration–response curves for agonists and LA's are characterized by Hill coefficients near 1.0 (Ogden & Colquhoun, 1985), and cation flux inhibition by both families of drugs is unassociated with spectroscopically detectable membrane perturbations (Forman et al., 1987).

The similarity of LA's and agonists' inhibitory actions suggests that these agents may inhibit cation flux via similar mechanisms or at the same site on the AChR. Indeed, one study has shown that [^{3}H]histrionicotoxin binding to *Torpedo* AChR is enhanced at low (channel-activating) concentrations of (−)-nicotine and decamethonium but reduced by high concentrations of these partial agonists (Eldefrawi et al., 1982) that inhibit channel function. On the other hand, two studies (Shiono et al., 1984; Takeyasu et al., 1986) concluded that both in the presence and in the absence of a membrane voltage, procaine did not compete with ACh for inhibitory sites.

This paper reports studies on two aspects of procaine's channel-inhibiting action at AChR from *Torpedo*. We initially measured the time course of this action in *Torpedo* vesicles using quenched-flux assays with better (3 ms) time resolution than that used in previous studies. These experiments reveal that procaine, like self-inhibitory concentrations of ACh, causes rapid inactivation of AChR within milliseconds of agonist exposure (Forman & Miller, 1988). Details of procaine's inhibition mechanism are derived from analysis of inactivation kinetics at various procaine and agonist concentrations. Second, we took advantage of the high density of AChR on native *Torpedo* vesicles to measure additive inhibitory effects of procaine plus self-inhibitory concentrations of either ACh or suberyldicholine using much higher inhibitor concentrations than those used by Shiono et al. (1984) or Takeyasu et al. (1986). Our results indicate that procaine and agonists interact competitively at a common inhibitory site.

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* Address correspondence to this author at the Department of Anaesthesia, Massachusetts General Hospital, Boston, MA 02114.

[1] Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; SubCh, suberyldicholine; α-BTX, α-bungarotoxin; LA, local anesthetic.

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Table I: Definitions of Variables

variable	definition
F_A	integrated flux response in presence of ACh concn A
F_{eq}	flux response corresponding to full equilibration of $^{86}Rb^+$ across AChR-containing vesicle membranes
$F^{0,0,0}_{A,P,T}$	integrated flux response ([ACh] = A, time = T) in presence of procaine concn P, without preincubation
$F^{a,p,t}_{A,P,T}$	integrated flux response after preincubation period t in presence of ACh concn a and procaine concn p
k_f	$^{86}Rb^+$ flux rate constant
k_d	fast desensitization rate constant
k_r	rapid inactivation rate constant
K_A	ACh concn eliciting F_A(max)/2 response
K_B	ACh concn causing 50% self-inhibition
K_r	ACh concn causing rapid inactivation rate k_r(max)/2
V_m	transmembrane voltage

Materials and Methods

Torpedo Vesicle Preparations. Purification of AChR from freshly killed *Torpedo Nobiliana* (Biofish Associates, Georgetown, MA) and determinations of receptor concentration (6–11 μM in [^{3}H]ACh sites) and purity were performed as described previously (Forman et al., 1987). Aliquots of receptor-rich membrane were suspended in *Torpedo* physiological saline (TPS: 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 5 mM PO_4^{2-}, and 0.02% NaN_3, pH 7.0), frozen in liquid nitrogen, and thawed up to 48 h before use. Vesicles were incubated for 20 min with 0.1 mM diisopropyl fluorophosphate (DFP) at 4 °C to inhibit acetylcholinesterase activity prior to flux assays. Control experiments using carbamylcholine, which is not hydrolyzed by acetylcholinesterase, showed that flux responses were not altered by DFP treatment.

α-Bungarotoxin Blockade. α-Bungarotoxin (α-BTX) was repurified as described in Forman et al. (1987). Partial (50–60% for quenched-flux) blockade of [^{3}H]ACh sites on AChR vesicles, in order to prevent complete equilibration of $^{86}Rb^+$ during flux assays, was achieved by overnight incubation with appropriate stoichiometric amounts of α-BTX at 4 °C.

$^{86}Rb^+$ Flux Studies. All $^{86}Rb^+$ flux assays were done at 3–5 °C in a cold room. Ten-second and 20-ms integrated $^{86}Rb^+$ efflux assays were performed as described in Forman et al. (1987). Inactivation kinetic experiments using double-mix and pulsed quenched-flow $^{86}Rb^+$ influx assays were performed as described (Forman & Miller, 1988). AChR ion channel activity for these experiments is reported as $F^{a,p,t}_{A,P,T}$ (see Table I for definitions of variables). Superscript parameters in this variable denote the ACh (a) and procaine (p) concentrations and time (t) of preincubation, while subscript parameters represent equivalent conditions during $^{86}Rb^+$ influx. The ACh concentration during influx (A) was generally 1 mM, except when preincubation was with ACh concentrations (a) above 1 mM. In the latter case, flux conditions were established where $A = a$. Procaine concentrations during $^{86}Rb^+$ influx (P) were two-thirds times the preincubating concentration (p) because of dilution with flux medium. Flux times (T) estimated from tracings of pneumatic ram movements were generally 3–4 ms. Longer flux periods (up to 15 ms) were sometimes used, and data were normalized to control flux responses ($F_{control}$, measured in the presence of 1 mM ACh without preincubation) at both 3–4 ms and the longer time. Procedures for separation of $^{86}Rb^+$ entrapped in vesicles from that in external medium and analysis of raw data (scintillation counts per minute) have also been previously described (Forman et al., 1987; Forman & Miller, 1988).

Chemicals. Diisopropyl fluorophosphate (DFP) was from Aldrich Chemical Co. (Milwaukee, WI). α-Bungarotoxin was from Miami Serpentarium (Miami, FL). Acetylcholine chloride, procaine hydrochloride, and buffer reagents were from Sigma Chemical Co. (St. Louis, MO).

Radiochemicals. [^{3}H]ACh was from New England Nuclear (Boston, MA) or Amersham (Arlington Heights, IL). Specific activities were determined by isotope dilution assays (Neubig & Cohen, 1979). ^{86}RbCl was from New England Nuclear.

Results

Concentration–response curves for ACh and a number of other agonists are well described by a biphasic logistic equation:

$$F_A = F_A(\max)\frac{[A]^{N_1}}{[A]^{N_1} + K_A{}^{N_1}}\left(1 - \frac{[A]^{N_2}}{[A]^{N_2} + K_B{}^{N_2}}\right) \quad (1)$$

where F_A is the $^{86}Rb^+$ efflux response, K_A is the 50% activating agonist concentration, N_1 is the Hill coefficient for activation, K_B is the concentration causing 50% self-inhibition, and N_2 is the Hill coefficient for self-inhibition. Our earlier studies (Forman et al., 1987), using 15-ms $^{86}Rb^+$ efflux assays in α-BTX-treated *Torpedo* vesicles, established that for ACh, $K_B = 110 \pm 12$ mM and $N_2 = 0.9 \pm 0.1$. In order to study procaine's actions in the absence of agonist self-inhibition, we initially restricted ACh concentrations to below 1 mM. Procaine's inhibitory concentration–response curve for agonist-mediated efflux can also be described by a simple logistic function:

$$F_A = F_A(\max)\left(1 - \frac{[P]^{N_P}}{[P]^{N_P} + K_P{}^{N_P}}\right) \quad (2)$$

where K_P, the apparent dissociation constant for procaine, was found to be 220 ± 50 μM and $N_P = 1.2 \pm 0.2$ (Forman et al., 1987).

Procaine Effects on Inactivation Kinetics. Figure 1 shows results of a series of rapid quenched-flux experiments in which *Torpedo* vesicles were preincubated with 1 mM ACh and/or 200 μM procaine for various times prior to measuring flux responses. Pulsed quenched-flux data demonstrate that preincubation with buffer (TPS) alone (●) caused no time-dependent inactivation, while preincubation with 1 mM ACh (■) caused fast desensitization at a rate (k_d) of 6.5 ± 0.3 s^{-1}. Preincubation with 200 μM procaine alone (○) also caused no time-dependent inactivation, although the presence of 133 μM procaine during the 3-ms $^{86}Rb^+$ flux assay produced a 27% drop from $F_{control}$. Similar experiments with up to 2.5 mM procaine for periods up to 15 min of incubation (not shown) also showed that procaine alone does *not* cause a time-dependent loss of flux response. The presence of 133 μM procaine during $^{86}Rb^+$ flux did not change the apparent desensitization rate when vesicles were preincubated in the presence of 1 mM ACh alone (□). Nor did preincubation with 1 mM ACh plus 200 μM procaine for periods over 75 ms (▲) significantly alter the apparent rate of ACh-induced fast desensitization—the fitted straight line (–·–) indicates $k_d = 5.2 \pm 0.5$ s^{-1}. In other experiments (not shown), apparent k_d's at 10, 32, and 100 μM ACh were similar in the presence and absence of 200 μM procaine. Without procaine, k_d's were 0.3 $\pm$ 0.1, 1.2 $\pm$ 0.2, and 2.8 $\pm$ 0.2 s^{-1}, respectively, and with procaine, they were 0.4 $\pm$ 0.1, 0.8 $\pm$ 0.3, and 3.0 $\pm$ 0.5 s^{-1} (see Table II).

In contrast to the monophasic desensitization observed with ACh alone, preincubation with ACh plus procaine produced biphasic inactivation kinetics. One feature of the pulsed quenched-flow data in Figure 1 with 200 μM procaine plus 1 mM ACh was that the line fitted to data at $t > 75$ ms (▲; –·–) projects a $0.27F_{control}$ response at $t = 0$, while the mea-

Table II: Fitted Parameters from Biphasic Inactivation Experiments[a]

preincubation conditions		fitted parameters				
[ACh]	[procaine] (μM)	$F^{0,0,0}_{A,P,T}/F_{control}$ (%)	I_r	k_r (s^{-1})	I_d	k_d (s^{-1})
32 μM	200	74 ± 3	0.23 ± 0.02 (0.31)	17 ± 4	0.51 ± 0.02 (0.69)	0.8 ± 0.3
50 μM	200	73 ± 8	0.35 ± 0.06 (0.48)	60 ± 15	0.38 ± 0.05 (0.52)	1.5*
100 μM	100	90 ± 4	0.34 ± 0.03 (0.38)	59 ± 5	0.56 ± 0.03 (0.62)	3.0*
100 μM	200	74 ± 6	0.39 ± 0.04 (0.52)	90 ± 17	0.35 ± 0.04 (0.48)	3.0 ± 0.5
100 μM	400	52 ± 3	0.41 ± 0.02 (0.79)	135 ± 15	0.11 ± 0.02 (0.21)	3.0*
320 μM	200	73 ± 3	0.47 ± 0.02 (0.64)	137 ± 14	0.26 ± 0.02 (0.36)	4.5*
1 mM	200	73 ± 3	0.48 ± 0.02 (0.65)	144 ± 12	0.25 ± 0.02 (0.35)	4.8 ± 0.5
10 mM	200	73 ± 3	0.50 ± 0.03 (0.68)	165 ± 27	0.23 ± 0.02 (0.32)	5.0 ± 0.4

[a] Fitted parameters were derived from nonlinear least-squares fits to eq 3. k_d's marked with an asterisk were constrained during nonlinear least-squares fitting—values were estimated from $k_d = k_d(\max) \times [A]^{N_1}/([A]^{N_1} + K_A^{N_1})$ with K_A = 79 μM, $k_d(\max)$ = 5.0 s^{-1}, and N_1 = 1.8 (Forman & Miller, 1988). I_r and I_d values in parentheses were renormalized so that $I_r + I_d = 1.0$ (see text above eq 4a).

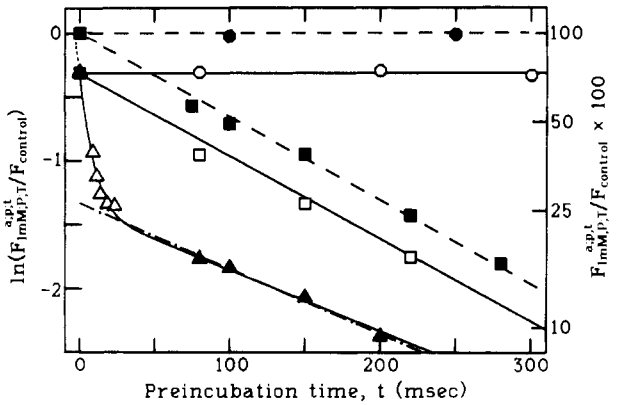


FIGURE 1: Inactivation kinetics with 1 mM ACh and procaine. Points represent averages of at least three quenched-flow $^{86}Rb^+$ influx assays in the presence of 1 mM ACh for times from 3.1 to 3.6 ms. When procaine was present in the preincubation, it was also present during $^{86}Rb^+$ influx at $^2/_3$ × the preincubating concentration. Preincubations shorter than 75 ms were achieved with the continuous double-mix technique, while longer periods were obtained with the pulsed method. Straight lines through data were calculated by linear least squares. $F_{control}$ was determined without preincubation using 1 mM ACh during $^{86}Rb^+$ influx. Preincubation conditions are given in italics along with fitted parameters: *preincubation with TPS* (●), intercept = 0.05 ± 0.06, slope = 0.01 ± 0.03 s^{-1}; *preincubation with 1 mM ACh* (■), intercept = 0.01 ± 0.06, slope = −6.5 ± 0.1 s^{-1}; *preincubation with 200 μM procaine* (○), intercept = −0.30 ± 0.02, slope = −0.05 ± 0.04 s^{-1}; *preincubation with 1 mM ACh, flux with 1 mM ACh + 133 μM procaine* (□), intercept = −0.30 ± 0.05, slope = −6.5 ± 0.4 s^{-1}. *Preincubation with 1 mM ACh + 200 μM procaine* (△, ▲); only points after preincubation times (t) greater than 75 ms (▲) were used in the linear fit (−·−), giving intercept = −1.3 ± 0.1 and slope = −5.2 ± 0.3 s^{-1}. The curve drawn through all triangles represents a nonlinear least-squares fit to eq 3 in the text with I_r = 0.48 ± 0.02, k_r = 144 ± 12 s^{-1}, I_d = 0.25 ± 0.01, and k_d = 4.8 ± 0.5 s^{-1}.

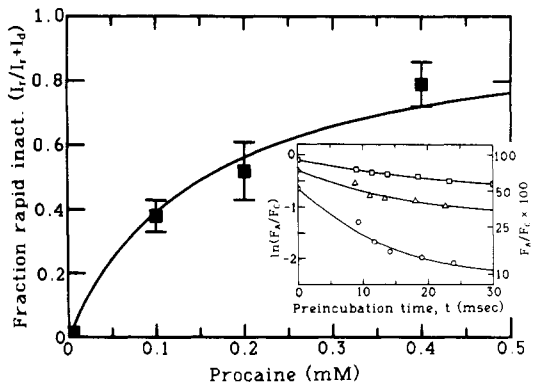


FIGURE 2: Procaine concentration effect on rapid inactivation with 100 μM ACh. The fraction of AChR function lost due to rapid inactivation $[I_r/(I_r + I_d)]$ was calculated from parameters fitted to inactivation curves (inset; Table II) and plotted against procaine concentration. The curve through data represents a nonlinear least-squares fit to eq 4c with K_P = 155 ± 20 μM. Inset: Points represent averages of at least three quenched-flow assays. ACh in the preincubations was fixed at 100 μM, and procaine concentrations were 100 μM (□), 200 μM (△), and 400 μM (○). Flux activity ($F_A/F_C = F^{a,p,t}_{1mM,P,T}/F_{control}$) was assessed with 1 mM ACh plus $^2/_3$ × the preincubating procaine concentration. Zero time points were measured by preincubating with TPS and adding procaine during the $^{86}Rb^+$ flux period. Data were normalized to control flux responses measured with 1 mM ACh alone ($F_{control}$) and fitted to eq 3 using nonlinear least squares. Fitted parameters for biphasic inactivation curves are reported in Table II.

sured response without preincubation but under the same final flux conditions ($F^{0,0,0}_{1mM,133\mu M,T}$) was $0.73F_{control}$. This discrepancy between projected and measured initial flux responses indicates the presence of a second inactivation phase occurring within 75 ms. Rapid inactivation on this time scale has been observed in experiments with self-inhibitory concentrations of ACh alone (Forman & Miller, 1988). Quantitative information on rapid inactivation in the presence of ACh plus procaine was obtained by using the double-mix quenched-flow method, which enabled preincubations as short as 8 ms (Figure 1, △). Data from both double-mix and pulsed experiments were combined after normalization and fitted to a biphasic inactivation function using nonlinear least squares:

$$F^{a,p,t}_{A,P,T}/F_{control} = I_r \exp(-k_r t) + I_d \exp(-k_d t) \quad (3)$$

where I_r and I_d are the fractions of inactivation caused by rapid inactivation and fast desensitization, respectively ($I_r + I_d = F^{0,0,0}_{A,P,T}/F_{control}$), and k_r and k_d are the rates of the two processes. This analysis indicates that, in addition to fast desensitization, a rapid inactivation at 144 s^{-1} occurs in the presence of procaine. Extrapolation of the biphasic fitted curve to negative abscissa values (dotted line) reaches $F_{control}$ at −2.7 ms, close to the time used to assay flux activity.

Procaine Dependence of Rapid Inactivation. At fixed ACh concentration (100 μM), the rate (k_r) and extent (I_r) of rapid inactivation increased with procaine concentration. The inset to Figure 2 shows that 3-ms flux responses without preincubation ($F^{0,0,0}_{A,P,T}$) are $0.90F_{control}$ at 100 μM procaine, $0.75F_{control}$ at 200 μM, and $0.52F_{control}$ at 400 μM and that the responses dropped to $0.57F_{control}$, $0.35F_{control}$, and $0.12F_{control}$, respectively, after 30-ms preincubation. Parameters from nonlinear least-squares fits to eq 3 are reported in Table II. Since $F^{0,0,0}_{A,P,T}$ is decreased in the presence of procaine, plotted I_r values in Figure 2 were renormalized to $F^{0,0,0}_{A,P,T}/F_{control} = I_r + I_d$ (Table II).

The following equations describe the expected relationships for a bimolecular association between procaine (P) and a receptor site (R):

$$P + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PR \qquad K_P = k_{off}/k_{on} \quad (4a)$$

$$k_r = Pk_{on} + k_{off} \quad (4b)$$

$$I_r = P/(P + K_P) \quad (4c)$$

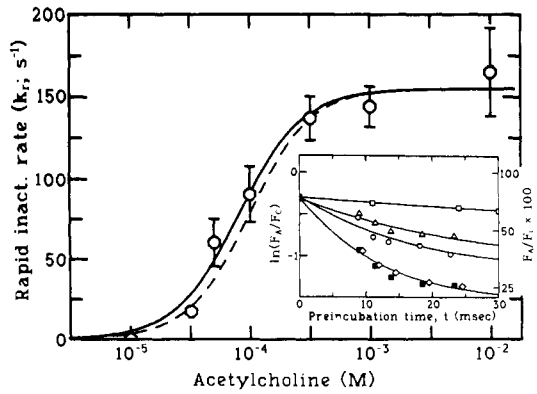


FIGURE 3: ACh concentration dependence of rapid inactivation rates with 200 μM procaine. Rapid inactivation rates (k_r's) fitted to inactivation curves (inset) at varying ACh concentrations are plotted. Vertical bars represent 95% confidence ranges for the values. The solid curve through data points is a nonlinear least-squares fit to eq 5 with $k_r(\max) = 155 \pm 8\ s^{-1}$, $N_r = 1.6 \pm 0.3$, and $K_r = 81 \pm 12\ \mu M$. The dashed line represents initial $^{86}Rb^+$ flux rate data from Forman and Miller (1988), plotted by using eq 1 with $N_1 = 1.7 \pm 0.3$, $K_A = 100 \pm 16\ \mu M$, $K_B = \infty$, and $F_A(\max)$ set so that the curves overlap at high ACh. Inset: Points represent averages of three or more double-mix quenched-flow assays ($F_A/F_C = F^{app}_{1mM,P,T}/F_{control}$) and analyzed as described in the legend to Figure 2. Preincubations were in the presence of 200 μM procaine plus ACh at the following concentrations: 32 μM (□), 50 μM (△), 100 μM (○), 320 μM (◇), and 1 mM (■). Flux activity was assessed with 3–15-ms assays in the presence of 1 mM ACh + 133 μM procaine. 10 mM ACh was also tested (not shown) and flux measured with 1 mM ACh + 133 μM procaine. Fitted parameters are reported in Table II.

Data from Figure 2 were fitted to eq 4c by nonlinear least squares, resulting in $K_P = 155 \pm 20\ \mu M$. The k_r's derived from the inset to Figure 2 (Table II) were fitted to eq 4b by linear least squares, resulting in $k_{on} = 2.5\ (\pm 0.2) \times 10^5\ M^{-1}\ s^{-1}$ and $k_{off} = 36 \pm 6\ s^{-1}$ ($K_P = 145 \pm 36\ \mu M$). These K_P values are within one standard deviation of the K_P estimated directly from procaine concentration–response curves ($220 \pm 50\ \mu M$; Forman et al., 1987).

ACh Dependence of Rapid Inactivation. In the presence of 200 μM procaine, the observed rate of rapid inactivation (k_r) varied with the ACh concentration as shown in Figure 3. Preincubation with 10 μM ACh plus procaine caused monophasic inactivation at a rate of 0.4 s^{-1}. At 32 μM and higher ACh concentrations, two inactivation processes were observed when procaine was present (Figure 3, inset shows rapid phases), whereas only one was observed with ACh alone (Forman & Miller, 1988). Results of nonlinear least-squares fits of data to eq 3 are listed in Table II. The heavy solid line drawn through data in Figure 3 represents a nonlinear least-squares fit to a logistic function:

$$k_r = k_r(\max)\frac{[A]^{N_r}}{[A]^{N_r} + K_r^{N_r}} \quad (5)$$

where K_r is the ACh concentration at which $k_r = k_r(\max)/2$ and N_r is the Hill coefficient for the relationship. The solid line was drawn with the following fitted parameters: $k_r(\max) = 155\ s^{-1}$, $K_r = 81\ \mu M$, and $N_r = 1.6$. For comparison, the dashed line represents initial $^{86}Rb^+$ flux rate data from a previous study (Forman & Miller, 1988), plotted by using eq 1 with $N_1 = 1.7$ and $K_A = 100\ \mu M$ so that the maximum overlaps that of the k_r curve.

Interactions between Agonists and Procaine at Inhibitory Sites. If procaine and agonists inhibit AChR at the same receptor site, then the presence of inhibiting concentrations of one drug should reduce the apparent inhibitory potency of the other. For example, the apparent dissociation constant for ACh self-inhibition (K_B in eq 1) should depend on procaine according to

$$K_B^{app} = K_B(1 + P/K_P) \quad (6a)$$

where K_B is the dissociation constant for agonist at the self-inhibition site and K_P is the dissociation constant for procaine. Conversely, the apparent inhibition constant for procaine should vary with agonist at self-inhibiting concentrations according to

$$K_P^{app} = K_P(1 + A/K_B) \quad (6b)$$

To test this hypothesis, efflux of $^{86}Rb^+$ from AChR-rich vesicles was used to establish concentration–response curves for both agonists and procaine in the presence of inhibitory concentrations of the presumed competitors.

Several experimental limitations made a systematic study with ACh and procaine difficult. First, the K_B for ACh is near 100 mM—using concentrations of ACh above the range used in previous studies (up to 320 mM) would introduce factors such as ionic strength, osmolarity, and viscosity as important experimental variables. In order to avoid this complexity, we used suberyldicholine (SubCh) as the agonist for most of these studies. SubCh elicits a maximum flux response close to that of ACh in 10-s integrated flux experiments but self-inhibits in the low millimolar range ($K_B \simeq 2.5$ mM; Forman et al., 1987).

Second, eq 6a and 6b predict that large shifts in K_B^{app} or K_P^{app} can be observed only if the competing inhibitor is present at many times its dissociation constant for the inhibitory site. By reducing the amount of α-BTX blockade in AChR-rich vesicles, we were able to extend the inhibitor concentration range for additivity studies without changing the maximum $^{86}Rb^+$ flux response. Usually, we block a fraction of [^{3}H]ACh sites on vesicles with α-BTX in order to reduce the number of active channels and limit the transmembrane $^{86}Rb^+$ flux rate at maximum agonist stimulation. This technique prevents full equilibration of $^{86}Rb^+$ during the flux assays and provides integrated flux response data that are directly proportional to flux rates (Forman et al., 1987). In some of the following additivity experiments no α-BTX blockade was used, but *maximum $^{86}Rb^+$ flux rates were limited by high inhibitor (i.e., procaine or SubCh) concentrations*, which reduced the amount of flux per channel noncompetitively to the same rate observed in the α-BTX-treated vesicles.

Figure 4A both demonstrates this technique and shows that procaine causes an increase in K_B^{app} for SubCh. The control experiment (○, dashed line) was a concentration–response curve for SubCh measured with a 10-s efflux assay in vesicles that had 82% of their [^{3}H]ACh sites blocked with α-BTX. Responses were fitted by nonlinear least squares to eq 1. With the addition of 200 μM procaine (△), another concentration–response curve measured by using the same α-BTX-treated vesicles showed a 50% decrease in $F_A(\max)$, no significant change in K_A, N_1, or N_2, but a 1.7-fold increase in K_B^{app}. A much larger change in K_B^{app} was seen in a SubCh concentration–response curve measured in the presence of 2.5 mM procaine (■) using vesicles that were not treated with α-BTX. $F_A(\max)$ under these conditions was close to that in the control experiment, K_A was lower than the control value, N_1 was the same, and K_B^{app} *was 13-fold higher than the control value.* This increase in K_B^{app} is predicted by eq 6a, because at 2.5 mM procaine with $K_p \simeq 200\ \mu M$, $P/K_P \simeq 12$.

Figure 4B shows similar effects when ACh was used in 10-s integrated flux assays. The dashed line and (○) symbols represent the biphasic ACh concentration responses measured in α-BTX-treated vesicles. Measurements were repeated in

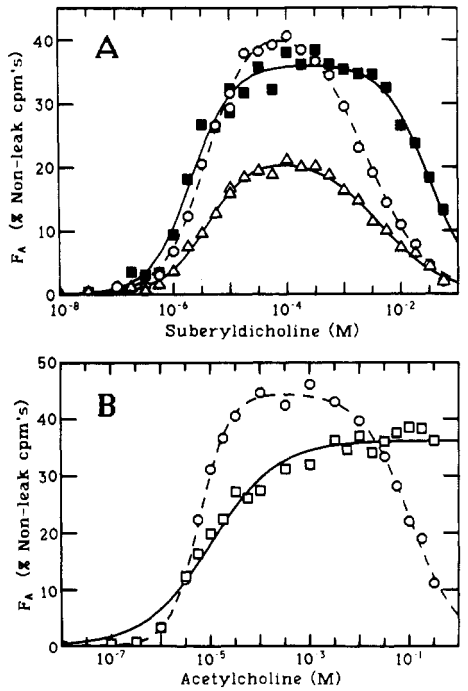


FIGURE 4: Effect of procaine on ACh and SubCh concentration–response curves. (A) Ten-second integrated $^{86}Rb^+$ efflux responses with SubCh over a 10^7-fold range encompassing both activating and self-inhibiting concentrations were measured under three different conditions of procaine and α-BTX blockade. Results were fitted to a biphasic logistic function (eq 1 in text) using nonlinear least squares. *82% α-BTX, no procaine* (○): $F_A(\max) = 43.6 \pm 1.3\%$, $K_A = 4.0 \pm 0.3$ μM, $N_1 = 1.2 \pm 0.1$, $K_B = 2.5 \pm 0.3$ mM, $N_2 = 1.1 \pm 0.1$. *82% α-BTX + 200 μM procaine* (△): $F_A(\max) = 21.9 \pm 0.7\%$, $K_A = 3.9 \pm 0.3$ μM, $N_1 = 1.1 \pm 0.1$, $K_B = 4.4 \pm 0.5$ mM, $N_2 = 0.8 \pm 0.1$. *No α-BTX + 2.5 mM procaine* (■): $F_A(\max) = 36.2 \pm 0.8\%$, $K_A = 2.1 \pm 0.2$ μM, $N_1 = 1.2 \pm 0.1$, $K_B = 32 \pm 4$ mM, $N_2 = 1.1 \pm 0.2$. (B) Ten-second integrated efflux responses with ACh. *85% α-BTX, no procaine* (○): $F_A(\max) = 44.7 \pm 0.6\%$, $K_A = 6.0 \pm 0.5$ μM, $N_1 = 1.4 \pm 0.1$, $K_B = 100 \pm 10$ mM, $N_2 = 0.9 \pm 0.1$. *No α-BTX + 2.7 mM procaine* (□): $F_A(\max) = 38.8 \pm 0.9\%$, $K_A = 10 \pm 1$ μM, $N_1 = 0.6 \pm 0.1$, $K_B > 1000$ mM (value did not converge).

untreated vesicles in the presence of sufficient procaine (2.7 mM) to bring $F_A(\max)$ near the control level. In the presence of 2.7 mM procaine (□), no self-inhibition was observed at up to 320 mM ACh. The effect of high procaine concentrations on ACh self-inhibition was confirmed by using 20-ms $^{86}Rb^+$ efflux assays (not shown). In the control (α-BTX treated) vesicles, F_{100mM}/F_{1mM} was 0.52 ± 0.06, consistent with $K_B \simeq 100$ mM ACh. In untreated vesicles with 1 mM procaine ($5K_P$) present, F_{100mM}/F_{1mM} was 0.9 ± 0.1, indicating only a small amount of self-inhibition at 100 mM ACh. Results of both 10-s and 20-ms experiments are consistent with the interpretation that K_B^{app} for ACh increases in the presence of high procaine concentration.

Having established that procaine causes an increase in the K_B^{app} for agonists, we investigated the converse interaction—the effect of agonists on apparent procaine potency. Procaine concentration–response curves were established with both 10-s and 20-ms integrated $^{86}Rb^+$ efflux measurements using SubCh as agonist. With up to 5 mM SubCh, α-BTX-treated vesicles were used, and at higher [SubCh], unblocked vesicles were used. K_P^{app} values were determined from nonlinear least-squares fits to eq 2 and are plotted in Figure 5 against SubCh concentration. The linear least-squares fitted line through the points ($r = 0.99$) has a slope of 0.057 and a y intercept (K_P) of 220 ± 20 μM. The K_B for SubCh calculated from this line (eq 6b) is 3.9 ± 0.5 mM, in reasonable agreement with the value derived from control SubCh concentration–response curves (2.5 ± 0.3 mM; Figure 4A).

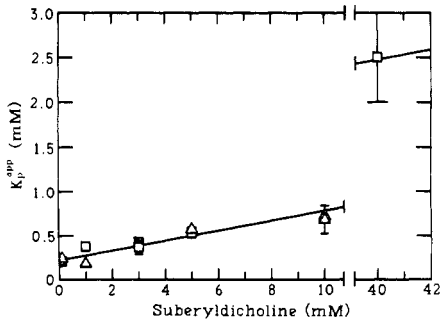
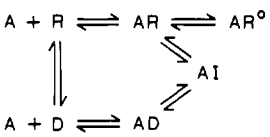


FIGURE 5: Procaine's potency varies with SubCh concentration. Procaine concentration–response curves in the presence of fixed SubCh concentrations (abscissa) were measured by using either 10-s integrated $^{86}Rb^+$ efflux (□) or 20-ms quenched-flow efflux (△) assays. For [SubCh] below 5 mM, α-BTX-treated vesicles were used (80% block at 10 s, 50% block at 20 ms). Experiments at 10 mM SubCh (20 ms) and at 40 mM SubCh (10 s) were performed with untreated vesicles. F_A vs [procaine] data (eight or more points per curve, not shown) were analyzed by fitting to eq 2 using nonlinear least squares. Fitted K_P^{app} vaues are plotted against SubCh concentration (vertical bars represent 95% confidence limits when larger than plotted symbols). The linear least-squares line (eq 6b) through data points gives a y intercept (K_P) = 0.22 ± 0.02 mM and slope = 0.057 ± 0.002 (r = 0.99). K_B for SubCh (negative x intercept) is 3.9 ± 0.5 mM.

Discussion

A modified cyclic scheme (Katz & Thesleff, 1957), incorporating the transient inactive state (AI) of Heidmann and Changeux (1984) and Neubig and Cohen (1980) illustrates the known state transitions of the AChR:



(7)

Briefly, resting state receptors (R) are in equilibrium with inactive (desensitized; D) receptors in the absence of cholinergic ligands, and the rate of transitions between R and D states is slow (minutes to hours; Cohen & Boyd, 1979). When cholinergic ligands (A) rapidly bind receptor (AR), the open-channel state (AR°) is formed, resulting in translocation of cations and depolarization of postsynaptic membranes. Under conditions of prolonged agonist exposure, a fast desensitization (milliseconds) leading to a transient inactive state (AI) occurs, followed by slow desensitization (seconds to minutes) to the highly stable agonist-bound desensitized state (AD).

A number of noncompetitive inhibitors, including LA's, bind more tightly to D than to R in eq 7, shifting the equilibrium in the absence of agonists further toward the inactive D state (Blanchard et al., 1979; Sine & Taylor, 1982; Heidmann et al., 1983; Boyd & Cohen, 1984). Local anesthetics also directly modulate the conductivity of the AChR cation channel, an action which occurs faster than the R → D transition and in most cases at lower concentrations than those which bind to the allosteric desensitizing site (Changeux & Revah, 1987). Indeed, procaine competes with other ligands at a "low affinity" allosteric site with an apparent dissociation constant of 2–3 mM, but unlike many other LA's does not itself alter the R ⇌ D equilibrium (Blanchard et al., 1979; Medynski, 1983; Cohen et al., 1985). Thus, the interaction of procaine with its high-affinity channel inhibition site could be studied in the absence of slow allosterically induced transitions. In addition, our kinetic experiments were performed on a time scale that eliminated slow desensitization effects. Our analysis

therefore deals mostly with procaine's effects on channel activation ($R \rightarrow AR^o$), rapid inactivation, and fast desensitization ($R \rightarrow AI$).

Procaine-Induced Inactivation at the AChR. Our kinetic experiments show that procaine, like ACh at self-inhibiting concentrations, causes a rapid inactivation phase *before* agonist-induced fast desensitization (Figure 1). Both the rate (k_r) and extent (I_r) of rapid inactivation increase with procaine concentration in a manner fully consistent with a bimolecular interaction at a single procaine binding site (or multiple equivalent sites) characterized by $K_P = 150 \pm 40$ μM (data in Figure 2). Rates of rapid inactivation increase linearly with procaine concentration over the experimental range (100–400 μM), and eq 4b predicts that k_r will increase without limit as procaine concentration is increased. This prediction is supported by our observation that 50 mM procaine effectively "quenches" AChR-mediated $^{86}Rb^+$ flux in native *Torpedo* vesicles, which are characterized by maximal $^{86}Rb^+$ flux rates of 300–600 s^{-1} (Neubig & Cohen, 1980; Takeyasu et al., 1986). On the basis of the k_{on} and k_{off} values derived from Figure 2, k_r at 50 mM procaine should be over 1.3×10^4 s^{-1}, sufficiently fast to account for effective quenching.

Procaine Inhibits after ACh Binds. In our study on ACh-induced inactivation (Forman & Miller, 1988), we suggested that self-inhibition might occur only *after* ACh binds to receptor activation sites. However, because ACh both activates and inhibits flux at high concentrations, we were unable to test whether ACh inhibits resting-state AChR. With procaine, which has no agonist activity, we could test directly whether inactivation occurs *before* agonist binding. Preincubation with either 200 μM (Figure 1) or 2.5 mM procaine caused no time-dependent fast or slow inactivation, confirming the conclusion of Cohen et al. (1985) that procaine does not induce desensitization ($R \rightarrow D$ transition) in the absence of agonists, and also proving that procaine-induced rapid inactivation requires prior ACh binding.

When we used our shortest flux period (3.5 ms), 200 μM procaine reduces the integrated flux response to 1 mM ACh by about 27% and subsequently causes rapid inactivation at an apparent rate (k_r) of 144 s^{-1}. Extrapolation of the biphasic curve in Figure 1 to negative abscissa values (dotted line) intersects $F_{control}$ at −2.7 ms, suggesting that the initial 27% drop in flux may be attributed to rapid inactivation during the flux assay. To test this possibility, we calculated the effects of biphasic inactivation on integrated flux using the following equation (Forman & Miller, 1988):

$$F_{A,T} = F_{eq}\left(1 - \exp\left\{-k_f\left[\frac{I_r}{k_r}[1 - \exp(-k_r T)] + \frac{I_d}{k_d}[1 - \exp(-k_d T)]\right]\right\}\right) \quad (8)$$

where I_r, I_d, k_r, and k_d are defined as in eq 3, F_{eq} is the maximum possible flux signal in the absence of desensitization, and k_f is the $^{86}Rb^+$ influx rate constant. Equation 8 predicts only a 14% drop in $F^{0,0,0}_{A,P,3.5ms}$ using a k_f of 80 s^{-1} [estimated by using methods described in Forman and Miller (1988)] and other parameters from the legend to Figure 1 (setting $I_r = 1 - I_d$). The additional inhibition may represent an inactivation process faster than quenched-flow methods can detect (at least 300 s^{-1}). However, our results (Figure 2, inset) indicate that the amount of "instantaneous block" increases in parallel with the extent of rapid inactivation (I_r) as [ACh] varies. Thus, this instantaneous component of procaine's action is almost certainly triggered by the same site which causes rapid inactivation. Underestimation of the actual flux period (T) or overestimation of k_f may explain why eq 8 fails to predict the correct amount of instantaneous block.

In contrast to our conclusions, previous studies of procaine's inhibition at AChR (Karpen et al., 1982; Karpen & Hess, 1986), using methods similar to ours, found that apparent initial $^{86}Rb^+$ flux rates were reduced in the presence of procaine and no rapid inactivation phase was reported. That such similar experiments should lead to opposite conclusions is at first surprising, but consideration of experimental techniques shows that previous investigations of both agonist self-inhibition (Takeyasu et al., 1986) and LA actions (Karpen et al., 1982) failed to identify the rapid inactivation process, because they used longer preincubations (t) and longer integrated flux periods (T) than we did. Karpen et al. (1982) used flux periods from 20 ms to 1 s, while Karpen and Hess (1986) used periods from 27 to 100 ms. Rapid inactivation is essentially complete within these flux periods, resulting in the apparent drop in initial flux rates.

Finally, our pulsed quenched-flow experiments showed that at four different ACh concentrations (10 μM–1 mM), procaine did not significantly alter the rate of ACh-induced fast desensitization. These results extend those from previous studies (Karpen et al., 1982; Shiono et al., 1984) that had examined procaine's effect at only one ACh concentration.

Procaine and the Agonist Self-Inhibition Site. The similarities between AChR inhibition by LA's and self-inhibition by cholinergic agonists are enumerated in the introduction to this paper. Our data add two more items to this list: (1) neither procaine nor self-inhibiting ACh concentrations alter the apparent rate of fast desensitization, and (2) both drugs cause rapid inactivation within milliseconds of agonist binding to AChR. In the second part of this study, we investigated the interaction between procaine and the self-inhibited AChR state by measuring the additive inhibitory effects of procaine and agonists on $^{86}Rb^+$ efflux from receptor-rich vesicles.

Analysis of concentration–response relationships for both SubCh and ACh in the presence of high concentrations of procaine reveals that this local anesthetic does not affect the ability of agonists to activate AChR (K_A and N_1 are essentially unchanged in Figure 4) but does reduce their apparent affinity for the self-inhibitory site. Complementary experiments showed that procaine's apparent affinity for its inhibitory site was reduced in the presence of millimolar SubCh concentrations (Figure 5), confirming that agonist self-inhibition and inhibition by procaine are mutually exclusive events. In both types of experiments, the changes in inhibitory potency were closely predicted by eq 6a and 6b using the K_B and K_P values measured in the absence of competing inhibitors. The simplest explanation for these results is that procaine and agonists compete for a single inhibitory site on the AChR protein.

Two previous studies, one under transmembrane voltage (V_m) clamp conditions (Shiono et al., 1984) and another in the absence of a transmembrane voltage (Takeyasu et al., 1986), reported no effect of self-inhibitory ACh concentrations on procaine's potency and concluded that the self-inhibition site was distinct from the procaine site of *Torpedo* AChR, apparently in contradiction to our results. However, Takeyasu et al. (1986) used only 5 mM ACh as their self-inhibiting concentration, because they estimated K_B for ACh to be 1 mM at $V_m = 0$. In the same paper, a K_B of 50 mM was reported for *Electrophorus* vesicles, which agrees more closely with our K_B measured in α-BTX-treated *Torpedo* vesicles (100 mM; Forman et al., 1987) and another estimate from voltage dependence studies of self-inhibition in patch-clamped BC3H1

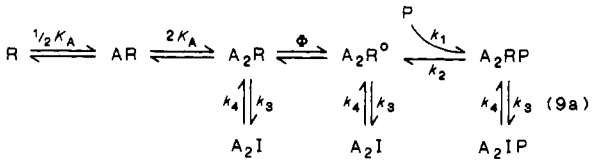
cells (50 mM; Sine & Steinbach, 1984). A possible source of Takeyasu's underestimated K_B has been discussed in a previous report (Forman et al., 1987). On the basis of higher K_B estimates (50–100 mM), 5 mM ACh should only cause a 5–10% increase in the apparent K_P for procaine. This change would not be reliably detected using quenched-flow methods.

An important advantage of our additivity studies was the wide range of inhibitor concentrations we were able to use by exploiting the high receptor density in AChR-rich vesicles from *Torpedo*. Because of this high level of "spare" receptors (Furchgott & Burystyn, 1967; Neubig & Cohen, 1980; Forman et al., 1987), we could use up to 12 times the 50% blocking concentrations of procaine or SubCh (Figures 4 and 5), resulting in greater than 12-fold increases in apparent inhibition constants for the competing inhibitor. Our conclusion that procaine and agonists are mutually exclusive inhibitors over a wide range of concentrations makes an allosteric interaction between separate inhibitory sites for agonists and procaine unlikely.

Mechanism of Procaine Inhibition. Several aspects of our results indicate that procaine binds preferentially to the open-channel AChR state (AR^o in eq 7). As already discussed, our results show directly that procaine-induced channel inactivation occurs only after ACh binding and, presumably, channel opening. Analogous results have been provided by AChR photolabeling experiments using radiolabeled LA's (Oswald et al., 1983; Cox et al., 1985; Heidmann & Changeux, 1984, 1986). These agents bind slowly to *Torpedo* AChR protein in the absence of agonists, but simultaneous addition of labeling agents and agonists greatly enhances labeling rates.

More evidence for the open-channel block model is found in our observation that the dependence of k_r on ACh concentration (Figure 3) is similar to that of cation channel activation by ACh (Forman & Miller, 1988). Both Hill coefficients ($N_r = 1.6 \pm 0.3$, $N_1 = 1.7 \pm 0.3$) and ACh concentrations at half-maximal effect (K_r in eq 4 = 81 ± 12 μM, K_A in eq 1 = 100 ± 16 μM) for the relationships are close. Thus, as ACh concentration increases, the availability of procaine sites increases in parallel with the number of open channels, suggesting that rapid procaine-induced inactivation occurs from the open-channel state and/or another doubly liganded AChR state in rapid equilibrium with the open-channel state (our data do not exclude the possibility of procaine binding to a preopened ACh-bound state). Karpen and Hess (1986) point out that if procaine binds exclusively to open channels, then procaine's apparent blocking potency should increase with ACh concentration over the activating range. Indeed, relative I_r increases with ACh (Table II), consistent with this prediction.

Our data are most compatible with a modified open-channel block mechanism in which fast desensitization proceeds from all doubly liganded states (A_2R, A_2R^o, and A_2RP) at equal rates. Similar models have been suggested by Pasquale et al. (1983) for self-inhibition by SubCh, by Clapham and Neher for substance P (1984a), and by us for ACh-induced self-inhibition at the AChR (Forman & Miller, 1988). The scheme in eq 9a illustrates the model:



We have explicitly included two agonist binding steps leading to channel activation. In addition, we define $K_P = k_2/k_1$ and the parameter $\Phi = A_2R/A_2R^o$ as used by Hess et al. (1983). For ACh, we assume $\Phi < 1$. Slow desensitization steps have been omitted. The following relationships for cation flux rate (k_f), rapid inactivation rate due to procaine block (k_r), and fast desensitization (k_d) are derived:

$$k_f = k_f(\max)\frac{A^2}{A^2(1+\Phi) + 2AK_A\Phi + K_A^2\Phi} \quad (9b)$$

$$k_r = \frac{k_1PA^2}{A^2(1+\Phi) + 2AK_A\Phi + K_A^2\Phi} + k_2 \quad (9c)$$

$$k_d = \frac{k_3A^2(1+\Phi+P/K_P)}{A^2(1+\Phi+P/K_P) + 2AK_A\Phi + K_A^2\Phi} + k_4 \quad (9d)$$

Equations 9b and 9c correctly predict that k_f and k_r will have parallel sigmoidal dependences on ACh throughout the activating concentration range. Under the experimental conditions used for k_d measurements in this study ($P/K_P \simeq 1$), eq 9d also predicts that procaine will not produce significant changes in k_d, as observed in this and other studies (Karpen et al., 1982; Shiono et al., 1984). Furthermore, since the blocked state can desensitize directly, this mechanism explains why flux integrated for long periods is reduced by LA's, as seen in both electrophysiological (Neher, 1983) and vesicle flux studies (Forman et al., 1987).

The k_{on} rate for procaine (2.5×10^5 M^{-1} s^{-1}) derived from our studies is 40–100-fold lower than other estimated bimolecular on rates for LA's from both electrophysiological experiments (Adams, 1977; Neher & Steinbach, 1978; Ruff, 1982) and photolabeling studies (Oswald et al., 1983; Heidmann & Changeux, 1984). The higher reported rates ($\simeq 10^7$ M^{-1} s^{-1}) are thought to represent diffusion-limited binding in a somewhat sterically hindered aqueous environment. Part of this discrepancy may be because these experiments were performed at higher temperatures and, in the case of electrophysiological studies, in the presence of a membrane voltage, which increases the association rate for charged inhibitors (Woodhull, 1973). Hindered diffusion in the membrane lipid phase (Gage et al., 1983; Clapham & Neher, 1984b; Blanton et al., 1988) may also explain procaine's inhibition kinetics.

Another "slow blocking" reaction at nicotinic AChRs by low concentrations of trifluoperazine has been reported (Clapham & Neher, 1984b), while concentrations of chlorpromazine that apparently label the AChR channel efficiently (Heidmann & Changeux, 1984) do not produce alterations in single-channel traces that are associated with channel "plugging" and "unplugging" events (Changeux et al., 1986). These results and our low k_{on} suggest another possible mechanism where procaine binds to an open-channel site at the diffusion limit, thereby triggering a rate-limiting unimolecular transition to an inactive state (Neher & Steinbach, 1978). In addition to explaining the binding rates for radiolabeled LA's, this model explains why ACh at $1K_B$ produces the same k_r (150 s^{-1}, Forman & Miller, 1988) as procaine at $1K_P$, even though the apparent k_{on} rates for the two inhibitors differ by a factor of 100. This model predicts a hyperbolic relationship between k_r and [procaine] with a projected $k_r(\max)$ of 400–600 s^{-1}, as opposed to the linear relationship used to analyzed data from Figure 2 (see text under Results). Our ability to measure k_r's at higher procaine concentrations (and thereby check for a hyperbolic maximum rate) is limited by our quenched-flow equipment's time resolution (shortest preincubation period = 8 ms). If there is a unimolecular step limiting rapid inactivation rates, one must then postulate additional actions of procaine in the millimolar range in order to account for the

effective "quenching" of $^{86}Rb^+$ flux in *Torpedo* vesicles (see discussion of "quenching" above).

Conclusions

There is evidence that multiple noncompetitive inhibitor sites exist on the AChR from *Torpedo* (Oswald et al., 1983; Heidmann et al., 1983; Karpen & Hess, 1986). Procaine does not allosterically desensitize AChR at the low-affinity site on resting-state receptors and therefore provides a useful probe of the high-affinity channel inhibition site. Our data indicate that (1) the high-affinity procaine site is the same as the agonist self-inhibition site, (2) procaine acts at this site only *after* ACh binds to receptor, and (3) as [ACh] increases, the availability of these sites increases in parallel with the number of open channels. Further studies are required to determine whether the high- and low-affinity procaine sites are spatially separated on the AChR or are the same site displaying different characteristics in different receptor conformations.

Acknowledgments

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Registry No. Procaine, 59-46-1.

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