Is Agonist Self-Inhibition at the Nicotinic Acetylcholine Receptor a Nonspecific Action?†

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ABSTRACT: Agonist concentration–response relationships at nicotinic postsynaptic receptors were established by measuring $^{86}Rb^+$ efflux from acetylcholine receptor rich native *Torpedo* membrane vesicles under three different conditions: (1) integrated net ion efflux (in 10 s) from untreated vesicles, (2) integrated net efflux from vesicles in which most acetylcholine sites were irreversibly blocked with α-bungarotoxin, and (3) initial rates of efflux (5–100 ms) from vesicles that were partially blocked with α-bungarotoxin. Exposure to acetylcholine, carbamylcholine, suberyldicholine, phenyltrimethylammonium, or (−)-nicotine over 10^8-fold concentration ranges results in bell-shaped ion flux response curves due to stimulation of acetylcholine receptor channel opening at low concentrations and inhibition of channel function at 60–2000 times higher concentrations. Concentrations of agonists that inhibit their own maximum $^{86}Rb^+$ efflux by 50% (K_B values) are 110, 211, 3.0, 39, and 8.9 mM, respectively, for the agonists listed above. For acetylcholine and carbamylcholine, K_B values determined from both 10-s and 15-ms efflux measurements are the same, indicating that the rate of agonist-induced desensitization increases to maximum at concentrations lower than those causing self-inhibition. For all partial and full agonists studied, Hill coefficients for self-inhibition are close to 1.0. Concentrations of agonists up to 8 times K_B did not change the order parameter reported by a spin-labeled fatty acid incorporated in *Torpedo* membranes. We conclude that agonist self-inhibition cannot be attributed to a general nonspecific membrane perturbation. Instead, these results are consistent with a saturable site of action either at the lipid–protein interface or on the acetylcholine receptor protein itself.

Acetylcholine receptors (AChR)[1] in postsynaptic membranes of vertebrate neuromuscular junctions and the electroplaque organs of electric fish possess binding sites for cholinergic drugs and cation channels that open transiently when agonist sites are occupied [for review, see McCarthy et al. (1986)]. Very high concentrations of acetylcholine and other cholinergic agonists have also been shown to inhibit AChR-mediated cation flux (Takeyasu et al., 1983; Shiono et al., 1984). In single-channel studies, high concentrations of agonists either reduce single-channel conductance or cause frequent interruptions of open channels (Sine & Steinbach, 1984; Ogden & Colquhoun, 1985). This self-inhibiting effect of agonists is enhanced by membrane hyperpolarization (Takeyasu et al., 1983, 1986; Sine & Steinbach, 1984; Ogden & Colquhoun, 1985), indicating that the inhibitory site is within the membrane's voltage gradient.

Two contrasting models might account for self-inhibition. First, self-inhibition may be the result of nonspecific perturbations in the receptor's membrane environment. In favor of this hypothesis are the high agonist concentrations needed for inhibition and recent evidence that the correct lipid environment is essential for channel function (Criado et al., 1984; Fong & McNamee, 1986). Lipophilic drugs, such as alkanols and general anesthetics, both inhibit AChR channel function (Gage & Hamill, 1976) and increase the fluidity of AChR-rich *Torpedo* membranes (Miller et al., 1986). ACh at millimolar concentrations has also been reported to perturb lipids in axonal membranes (Simpkins et al., 1972). Since membrane partitioning of charged drugs is sensitive to transmembrane potential (Raines & Cafiso, 1984), this model can account for the observed voltage dependence of self-inhibition.

Alternatively, occupation of a specific low-affinity site on the protein, either in the channel or elsewhere, may reduce AChR-mediated ion flux. This second hypothesis is supported by the observation that noncompetitive inhibition of the AChR channel by cationic drugs, such as amine local anesthetics, is also enhanced by membrane hyperpolarization (Adams, 1977). A number of these inhibitors bind at sites within the AChR–protein complex (Krodel et al., 1979; Eldefrawi et al., 1982; Cohen et al., 1986). As with agonists, single-channel recordings in the presence of cationic noncompetitive inhibitors also display frequent brief interruptions of open channels (Neher & Steinbach, 1978). However, many cationic inhibitors act at lower concentrations than do the agonists, and it has been reported that inhibition of AChR-mediated ion flux

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[1] Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; CCh, carbamylcholine; DFP, diisopropyl fluorophosphate; PTA, phenyltrimethylammonium; SubCh, suberyldicholine; TPS, *Torpedo* physiological saline; α-BTX, α-bungarotoxin; 12-DS, 12-doxylstearate; Tris, tris(hydroxymethyl)aminomethane.

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by agonists does not occur at the same site at which one such drug (procaine) acts (Shiono et al., 1984; Takeyasu et al., 1986).

To distinguish between these opposing hypotheses, we have established conditions for accurately determining apparent dissociation constants and Hill coefficients for self-inhibition in *Torpedo* AChR-rich vesicles for five agonists of widely varying efficacy. These parameters were determined in the absence of a membrane voltage so that they could be compared to the ability of the same agents to perturb the ESR spectra of spin-labeled fatty acid probes incorporated into *Torpedo* vesicles.

Materials and Methods

Preparation of Torpedo Postsynaptic Membranes. Postsynaptic membranes suspended in *Torpedo* physiological saline (TPS; 250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM NaH_2PO_4, and 0.02% NaN_3, pH 7.0) were prepared by sucrose density gradient centrifugation from freshly dissected electroplaques of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) at 4 °C, as described by Braswell et al. (1984). ACh sites on postsynaptic membranes were measured by specific [^{3}H]ACh binding, as described (Braswell et al., 1984). Vesicles were stored in liquid nitrogen and kept at 4 °C for up to 3 days after thawing.

Ten-Second Net $^{86}Rb^+$ Efflux Assay. Net efflux of $^{86}Rb^+$ from sealed AChR vesicles at 4 °C was measured after a 10-s exposure to agonist. ^{86}RbCl was brought to pH 7.0 with 1 M Tris and diluted with TPS. AChR vesicles (3–7 μM in ACh sites) were incubated overnight at 4 °C with ^{86}RbCl (100–200 μCi/mL) and desired stoichiometric amounts of α-BTX. Extravesicular $^{86}Rb^+$ was first removed by exclusion chromatography (Sephadex G-50, Pharmacia, 0.5 cm × 20 cm) and again 20 min later by rapid passage over a cation-exchange column (Dowex 50W, 20–50 mesh). When ACh and SubCh were used, vesicles were incubated with 0.2 mM DFP during the 20 min between elutions to inhibit acetylcholinesterase activity. Efflux was initiated when an aliquot of $^{86}Rb^+$-loaded vesicles was mixed with TPS containing agonist and antagonist where appropriate (final AChR was less than 50 nM in ACh sites). After 10 s, the mixture (1 mL) was filtered (Whatman GF/F) in a vacuum manifold. Filtrate (0.5 mL) was mixed with scintillation cocktail and counted. Slow, time-dependent leakage of $^{86}Rb^+$ from vesicles was measured from filtrates of experiments without agonist at intervals after elution from the ion-exchange column (Neubig & Cohen, 1980). A linear least-squares fit of leak counts vs. time gave an estimate of leak counts, cpm(leak, t), at the time of filtration for experiments done in the presence of agonist. Total counts, cpm(total), were measured from unfiltered vesicle solutions. Time-independent efflux response F_A is reported as the percentage of nonleak $^{86}Rb^+$ counts released:

$$F_A = \frac{\text{cpm(Ag, } t) - \text{cpm(leak, } t)}{\text{cpm(total)} - \text{cpm(leak, } t)} \times 100 \qquad (1)$$

Quenched-Flow $^{86}Rb^+$ Efflux Measurements. Quenched-flow efflux measurements were performed at 4 °C essentially as described by Neubig and Cohen (1980). Efflux was initiated when equal volumes of $^{86}Rb^+$-loaded vesicles and agonist solution were pneumatically driven from reservoir syringes through a ball-type mixing chamber (Berger et al., 1968) made by Research Instruments & Manufacturing Co. (San Diego, CA). The outflow from the mixing chamber passed through a variable-length delay tube before mixing again with an equal volume of 100 mM procaine, which stopped further efflux of tracer ions. Aliquots of 1 mL of the quenched solution were filtered, time-dependent leak measurements made, and calculations of F_A performed as described for the 10-s assay. Flux time (between the two mixing events) was calculated for each experiment from the known volume of the delay tube and an oscilloscope tracing of the pneumatic ram (coupled to a sliding variable resistor) movement. Calibration of flux times was performed by measuring base hydrolysis of *o*-nitrophenyl acetate, as described by Neubig and Cohen (1980).

$^{86}Rb^+$ Influx Assays. Both 10-s manual assays and quenched-flow measurements of $^{86}Rb^+$ influx into *Torpedo* vesicles at 4 °C were performed essentially as described by Hess et al. (1979). Vesicles were mixed either manually or in the rapid-mixing apparatus with a solution containing $^{86}Rb^+$, agonists, and, where appropriate, anesthetics. Influx was quenched after the required time by mixing with an equal volume of 100 mM procaine. Quenched vesicles (0.175 mL) were loaded on small cation-exchange columns (Dowex 50W, 100–200 mesh, 1-mL bed volume) and eluted with 220 mM sucrose in order to separate extravesicular $^{86}Rb^+$. Entrapped $^{86}Rb^+$ was determined by scintillation counting as described above. F_A was calculated as described for efflux measurements (eq 1), except that passive leak measurements are independent of time for influx, and cpm(total) was measured after overnight incubation of vesicles with $^{86}Rb^+$.

Electron Spin Resonance (ESR) Spectroscopy. *Torpedo* membranes were spin-labeled for ESR spectroscopy by gentle shaking (6 h, 4 °C) with 12-doxylstearate (12-DS). Membrane suspensions were 4–5 μM in [^{3}H]ACh sites, and the final concentration of 12-DS was less than 1% (mol/mol) of membrane lipid. Spin-labeled membrane suspensions were washed repeatedly with TPS containing 0.1 mM DFP, mixed with drugs, and sealed into glass capillary tubes. ESR spectroscopy was performed on a Varian E-109 spectrometer at 4.0 ± 0.1 °C with field strength = 3250 G, modulation amplitude = 3.2 G, modulation frequency = 100 kHz, microwave frequency = 9.2 GHz, and microwave power = 10 mW. Gas chromatography was used to assess 1-butanol concentrations in samples after spectra were taken. The order parameter (S) was calculated by the method of Hubbell and McConnell (1971) from the hyperfine splittings, which were corrected for solvent polarity (Gaffney, 1976).

Chemicals. Diisopropyl fluorophosphate was from Aldrich Chemical Co. (Milwaukee, WI). α-BTX was from Miami Serpentarium (Miami, FL) and was purified by CM-cellulose chromatography (Lee et al., 1972) before use. 12-Doxylstearate was from Molecular Probes (Junction City, OR). Acetylcholine chloride, carbamylcholine chloride, suberyldicholine dichloride, decamethonium bromide, (−)-nicotine di-(+)-tartrate, phenyltrimethylammonium chloride, procaine hydrochloride, butanol, and buffer reagents were from Sigma Chemical Co. (St. Louis, MO). [^{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

Our first aim was to establish the concentrations at which agonists inhibit ion flux across *Torpedo* AChR-rich vesicle membranes. Previous studies of agonist self-inhibition at nicotinic AChRs (Takeyasu et al., 1983; Sine & Steinbach, 1984; Ogden & Colquhoun, 1985) have mostly been performed in the presence of a membrane voltage, which enhances the inhibitory potency of agonists. However, spectroscopic measurements of the membrane order parameter take several

Table I: Fitted Parameters from Agonist Concentration–Response Curves

agonist	α-BTX block (%)	flux time	fitted parameters[a] K_A (μM)	N_1	K_B (mM)	N_2	K_B/K_A
ACh	0	10 s	1.1 ± 0.1	1.9 ± 0.1			
ACh	85	10 s	6.0 ± 0.3	1.4 ± 0.1	104 ± 23	0.9 ± 0.1	1.7×10^4
ACh	75	15 ms	49 ± 3	1.7 ± 0.1	110 ± 12	0.9 ± 0.1	2.2×10^3
ACh	80	31 ms	38 ± 5	1.8 ± 0.2	92 ± 7	1.2 ± 0.2	2.4×10^3
CCh	0	10 s	13 ± 4	1.9 ± 0.1			
CCh	85	10 s	130 ± 7	1.6 ± 0.1	244 ± 28	1.0 ± 0.1	1.9×10^3
CCh	75	15 ms	1000 ± 100	1.9 ± 0.2	211 ± 44	1.0 ± 0.2	2.1×10^2
SubCh	0	10 s	0.35 ± 0.03	2.1 ± 0.1	100 ± 10	1.2 ± 0.2	2.9×10^5
SubCh	80	10 s	3.5 ± 0.2	1.3 ± 0.1	3.0 ± 0.2	0.9 ± 0.1	8.6×10^2
SubCh	0	15 ms	11 ± 2	1.3 ± 0.1	12 ± 2	1.3 ± 0.1	1.1×10^3
SubCh	65	80 ms	22 ± 4	1.0 ± 0.2	14 ± 2	1.2 ± 0.2	6.4×10^2
PTA	0	10 s	125 ± 5	1.6 ± 0.1	39 ± 3	1.0 ± 0.1	3.1×10^2
nicotine	0	10 s	135 ± 12	1.3 ± 0.1	8.9 ± 0.7	1.5 ± 0.2	6.6 × 10

[a] Parameters were determined from nonlinear least-squares fits of data to eq 2 with F_A(max) fixed at the maximum value observed in each experiment.

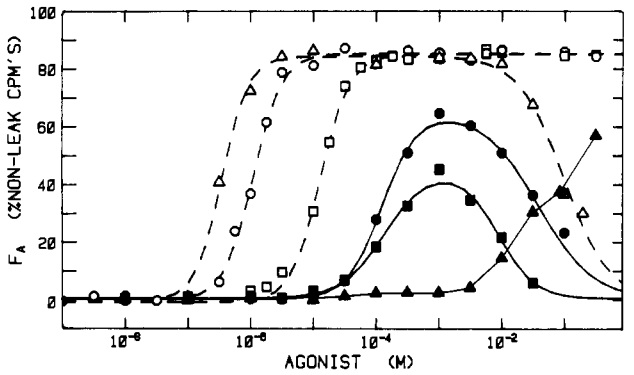

FIGURE 1: Ten-second agonist concentration–response curves in untreated vesicles. F_A values were calculated from measurements of 10-s net $^{86}Rb^+$ efflux from *Torpedo* vesicles as described under Materials and Methods and in eq 1. Data are shown for ACh (○), CCh (□), SubCh (△), PTA (●), (−)-nicotine (■), and decamethonium (▲). Curves drawn through PTA, nicotine, and SubCh represent nonlinear least-squares fits of data to eq 2, with F_A(max) values fixed at 65.0%, 45.0%, and 84.3%, respectively. Curves drawn through ACh and CCh data represent nonlinear least-squares fits to a single-phase logistic eq (eq 2 with K_B = infinity), with F_A(max) values fixed at 85.0%. The fitted parameters are reported in Table I.

minutes, and potentials produced in *Torpedo* vesicles are transitory on this time scale. Thus, it was essential to make both flux and electron spin resonance measurements in the absence of a transmembrane voltage.

Integrated Agonist Concentration–Response Curves. Initial studies were carried out with a convenient 10-s manual assay (see Materials and Methods) and untreated vesicles. Flux responses were measured for ACh, CCh, SubCh, PTA, (−)-nicotine, and decamethonium over 10^8-fold concentration ranges (Figure 1). Under these conditions, ACh, CCh, and SubCh (dashed lines in Figure 1) all elicited the same maximal flux response [F_A(max) = 85%], while PTA, (−)-nicotine, and decamethonium elicited lower F_A(max) values (65%, 45%, and 57%, respectively). SubCh, PTA, and (−)-nicotine concentration–response curves were bell-shaped, and the data were fitted by nonlinear least-squares to a biphasic logistic equation:

$$F_A = F_A(\max)\frac{[A]^{N_1}}{[A]^{N_1} + K_A{}^{N_1}}\left(1 - \frac{[A]^{N_2}}{[A]^{N_2} + K_B{}^{N_2}}\right) \quad (2)$$

where K_A is the agonist concentration producing 50% of maximum response, N_1 is the Hill coefficient of activation, K_B is the agonist concentration that inhibits 50% of maximum flux, and N_2 is the Hill coefficient of the self-inhibitory action. ACh and CCh responses reached plateaus but were not biphasic. Data for these drugs were fitted to an equation similar to eq 2, but with the self-inhibition terms (K_B and N_2) omitted. Data for decamethonium did not display a plateau at up to 320 mM and were not analyzed further. Fitted paramters from nonlinear least-squares analyses are summarized in Table I.

Because *Torpedo* vesicles have small internal volumes (300–600-nm diameter) and high AChR content, activation of only a fraction of the receptors causes complete emptying of entrapped $^{86}Rb^+$ in less than 1 s. The F_A(max) observed above with the potent agonists, ACh, CCh and SubCh, represents complete emptying of $^{86}Rb^+$ from vesicles containing active AChR in the correct orientation for channel activation; the remaining 15% of entrapped $^{86}Rb^+$ could be released in the presence of valinomycin but not by repetitive agonist stimulation. When complete emptying occurs, agonist and antagonist concentration–response curves vary, depending on the AChR surface density and internal volume characteristics of a given AChR preparation, a problem analogous to that of spare receptors in pharmacology (Furchgott & Burstyn, 1967). For example, under the conditions of the experiment in Figure 1, K_A values for CCh determined with 11 different vesicle preparations ranged nearly 5-fold from 12 to 57 μM.

In order to establish conditions where flux response was proportional to agonist occupation of receptor sites, vesicles were treated so that *less than complete emptying of $^{86}Rb^+$ occurred over the full range of agonist concentrations.* Removal of excess or "spare" AChR-mediated flux activity was accomplished by irreversibly blocking between 75% and 90% of the ACh sites on the vesicles with α-BTX (Moore et al., 1979; Neubig & Cohen, 1980), as assayed by specific [^{3}H]-ACh binding. CCh concentration–response curves measured with the 10-s flux assay in α-BTX treated vesicles were very reproducible, with K_A values from 110 to 140 μM (mean ± SD = 120 ± 10 μM for eight different vesicle preparations). Typical concentration–response curves for ACh, CCh, and SubCh, measured in α-BTX-treated vesicles, are shown in Figure 2. Under these conditions, all three agonists elicited bell-shaped response curves, with K_B values of 104, 244, and 3.0 mM, respectively (Table I). We established that *Torpedo* vesicles were not osmotically disrupted by up to 500 mM ACh and CCh in the flux medium by using vesicles that were blocked with excess α-BTX. Relative F_A(max) values for ACh, CCh, SubCh, PTA, and (−)-nicotine were 1.00 ± 0.02, 0.94 ± 0.03, 0.84 ± 0.04, 0.12 ± 0.01, and 0.07 ± 0.01, respectively, established by measurement of F_A in triplicate from a single batch of α-BTX-treated vesicles, at 1 mM ACh, 5 mM CCh, 100 μM SubCh, 1 mM PTA, and 1 mM (−)-nicotine.

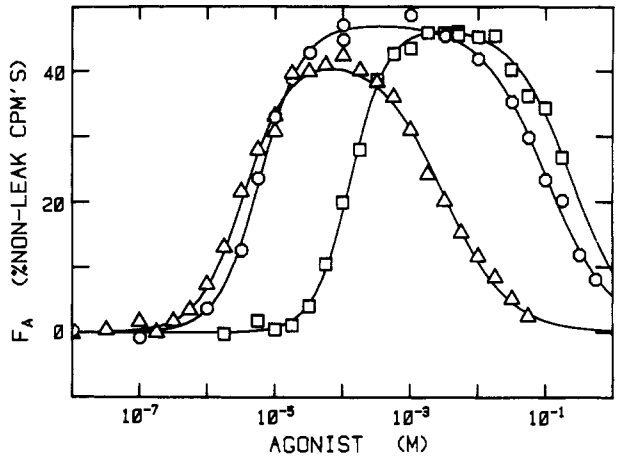

FIGURE 2: Ten-second agonist concentration–responses in vesicles partially blocked with α-BTX. F_A values were calculated from 10-s $^{86}Rb^+$ efflux measurements from three different vesicle preparations that were incubated overnight with α-BTX to block ACh sites. Data are shown for ACh (○), CCh (□), and SubCh (△) and have been normalized to the relative F_A(max) values reported under Results. Curves drawn through data represent nonlinear least-squares fits to eq 2 with F_A(max) values fixed at 47.4%, 47.0%, and 45.4% for ACh, CCh, and SubCh, respectively. Fitted parameters and α-BTX block conditions are reported in Table I.

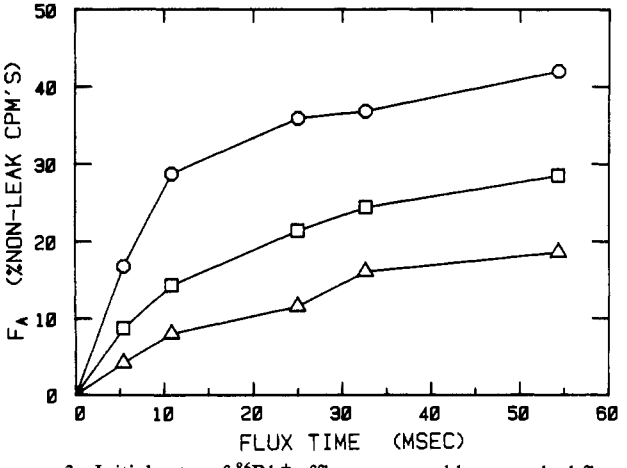

FIGURE 3: Initial rates of $^{86}Rb^+$ efflux measured by quenched flow. F_A values were calculated from quenched-flow $^{86}Rb^+$ efflux measurements from a single *Torpedo* vesicle preparation that was incubated with α-BTX to block 65% of [^{3}H]ACh sites. Measurements were made in triplicate with five different delay tubes, and flux times were calculated as described under Materials and *Methods*. Average F_A values are plotted for 1 mM ACh (○), 5 mM CCh (□), and 320 μM SubCh (△).

Initial Flux Rate Agonist Concentration–Response Curves. Although 10-s ion flux data in α-BTX-treated vesicles are reproducible, they do not account for the concentration-dependent inactivation of AChR (desensitization) that develops during prolonged agonist exposure. Therefore, measurements of net tracer ion flux in millisecond time periods were used to provide initial $^{86}Rb^+$ efflux rate data. Measurement of initial ion flux rates by quenched flow requires both an effective "quench" solution and an adequate time resolution. We established that 50 mM procaine did not alter the passive leak of $^{86}Rb^+$ from vesicles, while it totally blocked $^{86}Rb^+$ efflux for 10 s in untreated vesicles with ACh concentrations up to 320 mM.

Maximum activating concentrations of ACh and CCh caused complete emptying of $^{86}Rb^+$ from untreated *Torpedo* vesicles even within 10 ms. Figure 3 shows initial efflux kinetics in the presence of maximum activating concentrations of ACh (1 mM), CCh (5 mM), and SubCh (320 μM), measured in a single vesicle preparation after initial flux rates were reduced by irreversibly blocking 65% of [^{3}H]ACh sites with α-BTX. Even under these conditions of maximal stimulation, SubCh-induced efflux increased linearly with time up to 33 ms, while CCh and ACh gave linear efflux kinetics up to 10 ms. The relative initial ion flux rates for ACh, CCh, and SubCh in Figure 3 were estimated from the initial slopes to be 1.00 ± 0.08, 0.52 ± 0.08, and 0.25 ± 0.05, respectively. PTA and (−)-nicotine elicited little efflux within 100 ms even in unblocked vesicles and were not studied further with quenched-flow techniques.

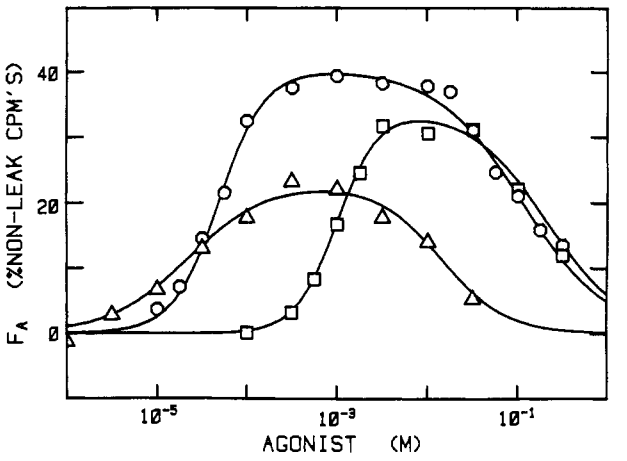

FIGURE 4: Agonist concentration–responses measured with quenched flow. F_A values were calculated from quenched-flow $^{86}Rb^+$ efflux measurements from three vesicle preparations that were partially blocked with α-BTX. Flux time and α-BTX block conditions for experiments were as follows: for ACh (○) and CCh (□), 15 ms and 75% block; for SubCh (△), 80 ms and 65% block. Curves drawn through data represent nonlinear least-squares fits to eq 2, with F_A(max) values fixed at 40.5% for ACh, 34.3% for CCh, and 23.0% for SubCh. Fitted parameters are reported in Table I.

The dependence of F_A on the concentrations of ACh, CCh, or SubCh were determined in α-BTX-treated vesicles at fixed times in the millisecond range. An example for each drug is shown in Figure 4. The fixed time and α-BTX block level for each agonist was chosen in order to provide adequate signal over the whole concentration range while remaining close to the period for linear kinetics. All three agonists display biphasic effects, and the data were fitted by nonlinear least squares to the biphasic logistic equation (eq 2). The resulting fitted parameters are reported in Table I. K_A values for each drug were essentially independent of flux time in the millisecond range when spare receptors were removed with α-BTX. These values for K_A are in good agreement with those found in other rapid kinetic studies (Takeyasu et al., 1986; Neubig et al., 1982; Walker et al., 1982). More importantly for this study, K_B values from quenched-flow (15 ms) studies in α-BTX-treated vesicles were essentially the same as the values derived from 10-s integrated flux experiments (Table I).

Others (Takeyasu et al., 1986) have studied ACh self-inhibition in *Torpedo* vesicles using influx measurements. To enable comparison of Takeyasu's et al. results with ours and to establish that there were no asymmetrical forces on cation flux across vesicle membranes, we measured ACh concentration–response curves in α-BTX-treated vesicles by measuring influx of $^{86}Rb^+$ (see Materials and Methods). K_B values were determined in both the 10-s assay (95 ± 15 mM) and 20-ms assays (100 ± 20 mM) and were found to be identical with K_B values measured by efflux.

Antagonist Concentration–Response Curves. Ion flux inhibition and membrane perturbation by a membrane perturbing drug (butanol) and an amine local anesthetic (pro-

Table II: Effects of Drugs on Membrane Order Parameter

drug	concn (mM)	change in order parameter[a]	drug	concn (mM)	change in order parameter[a]
butanol	33	−0.016	CCh	700	−0.002
butanol	67	−0.022	SubCh	7.5	−0.002
butanol	100	−0.032	SubCh	25	−0.009
procaine	0.60	−0.006	PTA	90	0.000
procaine	2.0	−0.002	(−)-nicotine	11	−0.002
ACh	310	−0.006			

[a] Order parameters were calculated from ESR spectra of 12-DS incorporated into *Torpedo* membranes at 4 °C as described under Materials and Methods. In the absence of drugs, the order parameter is 0.670 ± 0.005 (N = 4). Standard deviations for reported changes in order parameter are all less than 0.01.

caine) were studied in order to provide models for comparison with self-inhibition by agonists. Inhibition of agonist-induced $^{86}Rb^+$ efflux by these two drugs was measured in α-BTX-treated vesicles with the maximal stimulating concentration of CCh (5 mM) in both 10-s and quenched-flow assays. Data (not shown) were fitted by nonlinear least squares to a logistic equation:

$$F_A = F_A(\max)\left(1 - \frac{[I]^N}{[I]^N + K_I^N}\right) \quad (3)$$

where K_I is the concentration at which the inhibitor (I) blocks 50% of the observed ion flux. Procaine's K_I was 210 ± 20 μM in the 10-s assay and 220 ± 50 μM in a 20-ms quenched-flow experiment. Hill coefficients (N) in the two experiments were both 1.2 ± 0.2. Butanol gave K_I values of 22 ± 2 mM at 10 s and 26 ± 3 mM at 13 ms; Hill coefficients were 1.8 ± 0.2 in both experiments.

Membrane Disordering Studies. The control order parameter reported by the spin-label 12-DS incorporated into *Torpedo* AChR-rich membranes was 0.670 ± 0.005 at 4 °C. Changes from control order parameter measured in the presence of drugs are reported in Table II. No agonist effects were found at any concentrations examined, up to 3 times their K_B. Even at 8 times K_B, SubCh caused no significant change (less than 0.01) in the order parameter. In addition, neither 600 μM nor 2 mM procaine (3 and 10 times K_I) induced a detectable decrease in the order parameter. In contrast, butanol produced large decreases in the membrane order parameter at concentrations that were comparable to the K_I for butanol. At 33 mM butanol (1.3K_I), a decrease in the order parameter of 0.016 is observed, which is larger than any change observed with procaine or agonists. The change from control order parameter was a linear function of butanol concentration with a slope of −0.31 ± 0.04 M^{-1} (r = 0.96).

Discussion

Removing "Spare Receptors" To Determine K_A and K_B. Our ion flux studies demonstrate that self-inhibition by agonists can be observed in the absence of a transmembrane voltage for all the drugs listed in Table I. However, determining the full concentration–response relationship for different agonists required different experimental conditions. PTA, (−)-nicotine, and decamethonium were such weak agonists that they did not induce complete emptying of tracer ions from untreated *Torpedo* vesicles in 10 s. Concentration–response curves for these partial agonists were reproducible under these conditions. In the 10-s assay with α-BTX-treated vesicles or in millisecond flux assays, the partial agonists elicited such low F_A values that they could not be analyzed reliably. In contrast, ACh and CCh both induced complete emptying of untreated vesicles in 10 ms, while SubCh completely emptied vesicles in the manual assay. In order to prevent complete emptying of vesicles and achieve reproducible concentration–response relationships with these full agonists, flux rates were reduced by irreversibly blocking most of the AChRs with α-BTX. This increased apparent K_A values for all three drugs, decreased the apparent K_B for SubCh, and brought K_B values for ACh and CCh into the measureable concentration range (Table I). Quenched-flow studies on vesicles that were partially blocked with α-BTX revealed further information about the actions of the strong agonists.

Even in the absence of spare receptors, millisecond flux measurements give an apparent 7–8-fold increase in K_A values for ACh, CCh, and SubCh, when compared to values derived from 10–s flux assays (Table I). Neubig and Cohen (1980) showed that K_A values determined from integrated flux (20 s) data are lower than those determined from quenched-flow data, because flux kinetics are not linear over 10 s. In addition, the range of linear flux kinetics is limited by rapid agonist-induced desensitization, which accelerates with increasing agonist concentration. Full desensitization, which at low agonist concentrations takes minutes to develop, is reached in increasingly shorter time periods as concentration increases, cutting off efflux in several hundred milliseconds at the highest agonist concentrations used (Aoshima et al., 1981; Neubig et al., 1982; Walker et al., 1982).

Unlike K_A values for ACh and CCh, K_B values determined from quenched-flow data were the same as those from 10-s flux measurements in partially blocked vesicles (Table I). The lack of K_B dependence on flux period indicates that the AChR desensitization rate is unchanged over the concentration range where self-inhibition is observed for these agonists. This conclusion is supported by other reports (Pasquale et al., 1983; Takeyasu et al., 1983, 1986) that the fast desensitization process reaches a maximum rate at agonist concentrations lower than those causing self-inhibition. The fitted K_B value for SubCh increases when determined from quenched-flow measurements, but this may be due to uncertainty in the determination of F_A(max) and hence of K_B (see below).

Values reported here (Table I) for agonist concentrations that block 50% of AChR function at V_M = 0 [K_B(0 mV)] are in agreement with most previously reported measurements. K_B(0 mV) values have been estimated from electrophysiological measurements of voltage-dependent self-inhibition of AChR in cultured BC3H-1 cells (Sine & Steinbach, 1984). For ACh, CCh, and SubCh, they reported K_B(0 mV) values of 50, 110, and 5.2 mM, respectively, at 11 °C. At the frog neuromuscular junction, these three agonists also self-inhibit, but at lower concentrations than in BC3H-1 cells and apparently with a lower voltage sensitivity (Ogden & Colquhoun, 1985). In *Electrophorus* vesicles, K_B(0 mV) values have been reported for SubCh (500 μM; Pasquale et al., 1983) and for ACh (50 mM; Takeyasu et al., 1986). K_B(0 mV) in *Torpedo* vesicles has been previously reported only for ACh (Takeyasu et al., 1986) and was found to be 1 mM, much lower than the above values (50 mM) and ours (110 mM; Table I). In Takeyasu's et al. study, initial rates were extrapolated from quenched-flow influx data measured after agonist-induced desensitization for several hundred milliseconds. In the present study, initial flux rates were reduced by pretreatment with α-BTX to levels that were directly measureable by quenched flow, and data from both influx and efflux measurements gave identical K_B values. Significantly, our K_B(0 mV) for ACh is close to the value reported for *Electrophorus* vesicles (Tak-

eyasu et al., 1986), which have a lower surface density of receptors than *Torpedo* vesicles.

We are unaware of previous reports of AChR inhibition by PTA or (−)-nicotine, but there are reports describing biphasic responses with decamethonium [e.g., Adams and Sakmann (1978)]. The lack of *Torpedo* vesicle responsiveness to submillimolar decamethonium observed in this study (Figure 1) has also been reported by others using *Torpedo nobiliana* (Neubig, 1980) and *Torpedo marmorata* (Moreaux & Changeux, 1976).

Shapes of Biphasic Concentration–Response Curves. The shapes of the agonist concentration–response curves reveal both similarities and differences between agonists. Hill coefficients for activation (Table I) by all of the agonists reveal cooperativity (average $N_1 = 1.6 \pm 0.3$), in agreement with previously reported values (Dionne et al., 1978; Neubig & Cohen, 1980; Sine & Taylor, 1980). In addition, Hill coefficients for self-inhibition (N_2), which have not been previously determined from *Torpedo* or *Electrophorus* vesicle flux studies, are all close to 1.0 (average $N_2 = 1.1 \pm 0.2$). Our values are in agreement with those derived from single-channel measurements in frog muscle (Ogden & Colquhoun, 1985).

Significant differences between angonists are revealed by the K_B/K_A ratio (Table I). The two strongest agonists, ACh and CCh, both display high K_B/K_A ratios, as seen by the width of the bell-shape curves from 10-s data (Figure 2). The plateau in the middle of these curves allows determination of F_A(max) at agonist concentrations that saturate the activation site but cause negligible self-inhibition. In contrast, K_B/K_A ratios for SubCh, PTA, and (−)-nicotine are smaller (Figure 1, Table I, 10-s data). With these drugs, F_A(max) is measured at agonist concentrations that both activate *and* inhibit, leading to an underestimate of the true F_A(max) value (i.e., the value in the absence of inhibition). Indeed, the relative F_A(max) values measured in 10-s assays for ACh, CCh, SubCh, PTA, and (−)-nicotine (see Results) correlate strongly with their K_B/K_A ratios (Table I).

The variation in F_A(max) between agonists may be *caused* by different K_B/K_A ratios. If we assume that all agonists, in the absence of self-inhibition, would open channels of the same conductance with equal probability, then all agonists should produce the same F_A(max). Making this assumption, integrated flux data for PTA and (−)-nicotine (Figure 1) were fitted to eq 2 with F_A(max) fixed at the level projected for ACh in untreated vesicles (600%) and N_1 and N_2 fixed at 2.0 and 1.0, respectively. With these constraints the least-squares parameters obtained are as follows: for PTA, $K_A = 1.2 \pm 0.2$ mM and $K_B = 470 \pm 120$ μM ($K_B/K_A = 0.39$); for (−)-nicotine, $K_A = 790 \pm 100$ μM and $K_B = 170 \pm 30$ μM ($K_B/K_A = 0.22$). Note that this model leads to the conclusion that for these two partial agonists K_A is larger than K_B. Thus, the K_A of nicotine, for example, shifts 6-fold to the right (from 135 to 790 μM) and its K_B 50-fold to the left (from 8.9 mM to 170 μM). This means that at the *experimentally observed* K_A (135 μM) about half of the inhibitory sites are occupied. These calculations emphasize the interdependence of K_A and K_B and F_A(max) for those agonists that do not produce a wide enough plateau in their concentration–response curves. Since F_A(max) values were constrained to the measured values when data were fit to eq 2 (Figures 1, 2, and 4), values reported in Table I for partial agonists may well underestimate K_A and overestimate K_B.

In α-BTX-treated vesicles the dependence of SubCh's fitted K_B on the time over which flux was integrated contrasts with the independence shown by K_B values for ACh and CCh. This is again probably due to an underestimate for SubCh's F_A(max) in quenched-flow experiments (see above discussion of partial agonists) relative to the integrated flux experiments (Table I, Figures 2 and 4). Since underestimating F_A(max) causes overestimation of K_B (see above), SubCh's K_B derived from 10-s flux data (3 mM) is probably a better estimate that the value from quenched-flow data. Clearly, large K_B/K_A ratios are desirable for studies on self-inhibition, because they enable independent determination of activation and self-inhibition concentration dependence. With this principle, ACh is the best agonist for studying self-inhibition, and hyperpolarization of the membrane should be avoided, because it reduces K_B, while K_A is only weakly affected. The high K_B/K_A ratio of ACh is also noteworthy, because it suggests that the AChR synapse mechanism evolved in a way that minimized self-inhibition.

Specific vs. Nonspecific Action. ESR spectra of spin-labeled probes localized deep in membrane bilayers are very sensitive to changes in the short-term (10^8 s^{-1}) order of phospholipid acyl chains (Hubbell & McConnell, 1971). Reconstitution experiments have shown that AChR is sensitive to its membrane environment (Dalziel et al., 1980; Criado et al., 1984) and suggest that an optimal range of membrane "fluidity" may be required for AChR function (Fong & McNamee, 1986). Lipophilic molecules, such as alkanols and volatile anesthetics, inhibit AChR function (Gage & Hamill, 1976), increase the rate of receptor desensitization (Young & Sigman, 1983; El-Fakahany et al., 1983), and decrease biomembrane order in *Torpedo* (Firestone et al., 1983) and other systems (Goldstein, 1984) with potencies that are proportional to their membrane/buffer partition coefficients.

The K_B(0 mV) values for the agonists tested in this study range from 3 to 200 mM (Table I). This range may be due to variations in the membrane disordering efficacy for these drugs or variations in the affinity for a protein site that inhibits channel activity. Our ESR studies indicate that agonists at up to 8 times their K_B do not alter order parameters of a fatty acid spin probe in *Torpedo* membranes. Thus, we find no evidence for bulk lipid perturbation associated with flux inhibition by agonists, although we cannot rule out perturbations of "boundary" lipids intimately associated with the receptor protein. On the other hand, our observations are consistent with the hypothesis that a single site (or a number of independent sites with equal affinity) mediating agonist inhibition exists on the acetylcholine receptor protein, because the Hill coefficient for agonist self-inhibition is 1.

The location of the self-inhibition site is probably within the voltage gradient of the membrane, but is otherwise undetermined. It has been proposed that both agonists and noncompetitive inhibitors such as procaine reduce AChR function by binding to the open channel, where ion movement is sterically hindered. Such a sequential block model, shown in eq 4, also

$$R + 2A \rightleftharpoons RA_2^{OPEN} \underset{I}{\rightleftharpoons} RA_2I^{BLOCKED} \qquad (4)$$

requires that the blocking drug dissociates before channel closing can occur. This mechanism would lead to a low level of flux, accumulating over time, as the inhibitor (I) dissociated from the open channel (Neher, 1983). Single-channel measurements (Sine & Steinbach, 1984) indicate that SubCh "unblocks" with a rate constant of over 10^4 s^{-1} at $V_M = 0$. Our observation that high doses of procaine, SubCh, and (−)-nicotine all reduce 10-s net $^{86}Rb^+$ efflux to passive leak levels (Figures 1 and 2) indicates both that initial flux rates are reduced and that no slowly accumulating flux occurs. Thus,

either rapid desensitization occurs from the proposed blocked state, or these inhibitors must act on AChR whether the channel is open or closed (Adams, 1977).

Binding studies using radiolabeled agonists (Neubig & Cohen, 1979; Conti-Troconi & Raftery, 1982) reveal only two high-affinity agonist sites per AChR monomer. Unfortunately, low-affinity sites are impossible to detect above 10^{-3} M because of nondisplaceable binding (Strnad & Cohen, 1985). Recent site-directed mutagenesis experiments (Mishina et al., 1985) also identify only two functionally significant agonist sites on the two α subunits of the AChR pentamer, but these studies did not include tests of self-inhibition. Indeed, all five subunits display striking primary structural homology (Raftery et al., 1980; Noda et al., 1983), and the possible existence of additional low-affinity agonist sites has been raised by studies using fluorescent agonist analogues (Dunn et al., 1983).

Other Inhibitory Sites. This study shows that self-inhibition is most likely the result of low-affinity binding to a specific site on AChR, which may be identifiable by its pharmacological interactions with other drugs. Of the two inhibitors we tested, the effects of high agonist concentrations more closely resemble the actions of procaine than those of butanol. Butanol inhibits AChR-mediated ion flux with a larger Hill coefficient than the agonists or procaine and at concentrations that decrease bulk lipid order in *Torpedo* membranes (Table II). Alcohols also induce desensitization of AChR by a different mechanism than do site-directed noncompetitve inhibitors Heidmann et al., 1983; Boyd & Cohen, 1984). These data support the hypothesis that the site at which alcohols act is separate from those at which other noncompetitive blockers act, perhaps within the membrane lipids.

In contrast, procaine, a drug that has been shown to bind to AChR (Cohen et al., 1986), blocks flux with a Hill coefficient close to 1.0 and produces no observable change in membrane order at up to 10 times its K_i for flux blockade (Table II). Two studies (Shiono et al., 1984; Takeyasu et al., 1986) have reported that procaine and ACh inhibit AChR-mediated ion flux at separate sites. Thus, available data suggest that procaine's inhibition is site-directed but not competitive with agonist self-inhibition. However, it is probable that not all noncompetitive blockers (Heidmann et al., 1983; Karpen & Hess, 1986) bind to the same site within the AChR molecule, indicating the need for further studies on the interactions of drugs with the agonist self-inhibition site.

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Registry No. Acetylcholine, 51-84-3; carbamylcholine chloride, 51-83-2; suberyldicholine dichloride, 100930-12-9; phenyltrimethylammonium chloride, 138-24-9; (−)-nicotine, 54-11-5; rubidium, 7440-17-7.

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Regulation of Bilayer Stability in *Clostridium butyricum*: Studies on the Polymorphic Phase Behavior of the Ether Lipids†

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ABSTRACT: Three of the major phospholipids of the cell membrane of *Clostridium butyricum* are phosphatidylethanolamine (PE), plasmenylethanolamine (PlaE), and the glycerol acetal of plasmenylethanolamine. When cultured in the absence of biotin in media supplemented with a cis-unsaturated fatty acid, the cellular lipids become highly enriched with the fed fatty acid. Under these conditions, the ratio of the glycerol acetal of PlaE to the sum of PE plus PlaE increases markedly over that seen in cells containing mixtures of saturated and unsaturated fatty acids [Johnston, N. C., & Goldfine, H. (1985) *Biochim. Biophys. Acta 813*, 10–18]. We have studied the polymorphic phase behavior of the phospholipids from *C. butyricum* grown on oleic acid using differential scanning calorimetry, ^{31}P nuclear magnetic resonance, and X-ray diffraction. The mixed PE plus PlaE fraction undergoes a transition from the gel to liquid-crystalline state at −1.9 °C and a lamellar to reversed hexagonal (L → H) transition at or near 0 °C. The glycerol acetal of PlaE melts at 16.1 °C, and as predicted from lipid packing theory, the lamellar phase is stabilized, up to 50 °C. Addition of the oleate-enriched glycerol acetal of PlaE to dioleoylphosphatidylethanolamine, or the PE plus PlaE fraction from oleate-grown cells, stabilized the lamellar arrangement of the mixtures. A ratio of glycerol acetal of PlaE to total PE (PE plus PlaE) of 0.5, which is close to that found in cells grown on palmitic plus oleic acid, 0.6–0.7, did not produce a lamellar phase at 37 °C when the lipids enriched with oleic acid were tested, but a 1:1 mixture of these lipids was sufficient to produce the lamellar arrangement. In cells grown on oleic acid, the ratio is close to 2.0. It appears that these cells are capable of regulating the stability of the bilayer arrangement of the cell membrane by altering the ratio of the glycerol acetal of PlaE to the total PE fraction in response to changes in membrane lipid unsaturation.

Biological membranes contain mixtures of lipids which individually do and do not aggregate to form bilayers at physiological temperatures upon hydration. Examples of the former include phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, and diglycosyldiacylglycerols. Among the latter group are unsaturated species of phosphatidylethanolamine, plasmenylethanolamine, and monoglycosyldiacylglycerols [for general references, see Shipley (1973), Cullis and Hope (1985), and Lohner et al. (1984)]. To form bilayers, the mixtures of lipids in biological membranes appear to be carefully balanced to include appropriate proportions of lipids from the two groups

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