Activation and Inactivation Kinetics of *Torpedo californica* Acetylcholine Receptor in Reconstituted Membranes†

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ABSTRACT: By use of a quench-flow technique to measure tracer ion flux rates in a physiologically significant time domain, the kinetics of activation and inactivation of purified reconstituted acetylcholine receptor (AChR) were investigated. After solubilization in sodium cholate, purification by affinity chromatography, and reconstitution into soybean lipids, the AChR from *Torpedo californica* displayed a characteristically fast rate of ion influx measured with $^{86}Rb^+$. At 4 °C 1 mM carbamoylcholine (Carb) stimulated a fast ($t_{1/2}$ = 7 ms) first-order filling of vesicle internal volume that represented a 10^4-fold stimulation of ion flux rate by Carb. The concentration dependence of activation was sigmoidal with a half-maximal value at 3×10^{-4} M Carb. In the presence of Carb, the purified AChR also underwent a two-step inactivation (desensitization) process. Inactivation was measured by preincubating AChR with Carb for various times (milliseconds to minutes) and then measuring the $^{86}Rb^+$ influx rate. The two inactivation processes were each characterized by a distinct maximum rate (5.3 and 0.10 s^{-1}) and by a different dependence on Carb concentration. The slow phase of inactivation gave a half-maximal rate at 2.5×10^{-4} M Carb, and the fast inactivation was half-maximal at 1.3×10^{-3} M Carb. The concentration dependence curves for both inactivation processes were approximately hyperbolic. The results are discussed in terms of models that describe the relationship between ligand binding and the processes of channel activation and desensitization.

The function of nicotinic acetylcholine receptors (AChR)[1] in fish electric organs and at vertebrate neuromuscular junctions can be conveniently divided into four subfunctions: (1) specific ligand *binding*, (2) *coupling* of binding to cation permeability increases, (3) ion *permeation*, and (4) *desensitization*, a process by which ion channels are inactivated in the prolonged presence of activating ligands. Considerable progress has been made in characterizing the biochemical, biophysical, and electrophysiologic aspects of receptor structure and function, and excellent reviews by Karlin (1980), Changeux (1981), and Steinbach (1980) provide a comprehensive analysis of the present state of knowledge. Two recent advances in AChR research now provide a unique opportunity to characterize in detail both the biochemical and ion permeability properties of the receptor in a single, well-defined, membrane environment.

First, reliable methods for reconstitution of AChR ion permeability control properties in lipid vesicles and in planar membranes have been developed (Anholt, 1981; McNamee & Ochoa, 1982). Second, Hess and co-workers have adapted quench-flow techniques to measure the rates of ion fluxes across membrane vesicles in the millisecond time domain (Hess et al., 1979; Cash & Hess, 1980, 1981). Using native membranes from electric eel electroplax, Hess and co-workers measured initial rates of activation and inactivation (desensitization) of cation fluxes over a wide range of concentrations for two agonists (acetylcholine and carbamoylcholine). They developed a minimal mechanism that quantitatively accounted for all the measurable flux properties (Cash & Hess, 1980; Aoshima et al., 1981). A similar approach was used by Neubig & Cohen (1980) and Walker et al. (1981a) to measure initial rates of ion flux in native membranes from *Torpedo californica*. In parallel, Moore & Raftery (1980) developed a stopped-flow technique that detects quenching of a vesicle-trapped fluorophore by thallium ions during agonist-induced thallium influx.

Major limitations of native *Torpedo* membranes are the high surface density of receptor sites and the kinetic heterogeneity of the vesicle population (Moore & Raftery, 1980; Hess et al., 1982). The reconstituted vesicles offer the opportunity to correlate the biophysical and biochemical effects of protein modifications, specific lipids, and other agents (such as anesthetics) directly with functional responses measured by rapid flux techniques.

In this paper, we provide the first quantitative measurements of both activation and inactivation kinetics of receptor-mediated cation flux in reconstituted lipid vesicles containing purified *Torpedo californica* AChR.

Materials and Methods

Chemicals and Other Assays. $^{86}Rb^+$ and [^{125}I]-α-bungarotoxin were obtained from New England Nuclear (Boston, MA), sodium cholate and carbamoylcholine were from Sigma Chemical Co. (St. Louis, MO), and *d*-tubocurarine was from Calbiochem (La Jolla, CA). Iodinated α-bungarotoxin binding was measured by a DEAE filter disk assay in detergent solution as previously described (Walker et al., 1981b).

Acetylcholine Receptor Purification. A crude membrane fraction was prepared from liquid nitrogen frozen *Torpedo californica* electroplax essentially as described by Lindstrom et al. (1980). *Torpedo californica* were obtained from Pacific Biomarine (Venice, CA) or from the Bodega Bay Marine Station of the University of California. From 600 g of tissue a membrane preparation containing on the average 1 g of

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[1] Abbreviations: AChR, acetylcholine receptor; Carb, carbamoylcholine chloride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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protein with a specific binding activity for α-bungarotoxin of 1 nmol/mg of protein was obtained. The crude membranes were suspended in buffer A (100 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, and 0.02% azide, pH 7.4) and frozen in liquid nitrogen.

For a typical purification, one-third of a crude membrane preparation was used, and all steps were carried out at 4 °C. The membranes were diluted to 2 mg of protein/mL with buffer A, and 20% sodium cholate in buffer A was then added to give a final cholate concentration of 1%. The mixture was stirred for 20 min and centrifuged for 40 min at 130000*g* in a type 60 rotor. The supernatant was applied at a flow rate of 1 mL/min to a 15-mL affinity column prepared from Affi-Gel 401 (Bio-Rad, Richmond, CA) and bromoacetylcholine (Reynolds & Karlin, 1978; Huganir et al., 1979). The column was washed with 5–10 column volumes of buffer B [buffer A supplemented with 1% cholate and 2 mg/mL Asolectin (Associated Concentrates, Woodside, NY)]. The protein concentration of the fractions was monitored by the absorbance at 280 nm. Empirically, an absorbance of 1.0 corresponded to a value of 0.6 mg of protein/mL as measured by the procedure of Lowry et al. (1951). AChR was eluted with 10 mM carbamoylcholine (Carb) in buffer B and emerged as a sharp peak just after the void volume of the column. The column could be used at least 5 times without noticeable loss in yield or quantity of receptor if it was washed extensively with buffer B and then with water containing 0.02% azide.

Reconstitution. Ten milligrams of purified AChR in 10 mL of buffer B was taken directly from the affinity column, and 5 mL of a solution containing 80 mg/mL sonicated Asolectin and 4% sodium cholate in buffer A was added to give the following concentrations: 2% cholate, 0.67 mg/mL AChR, 28 mg/mL Asolectin, and ~6.7 mM carbamoylcholine. The mixture was dialyzed for 60 h at 4 °C against 4 L of buffer A with at least three changes of buffer. The membranes were stored on ice until used and retained full activity for at least 3 days.

Ion Flux Assays. Influx of $^{86}Rb^+$ into the vesicles was measured at 0–4 °C by manual or rapid mixing (see Quench-Flow Measurements) of 1 volume of membranes and 1 volume of a 100 μCi/mL solution of $^{86}Rb^+$ in buffer A with or without Carb. The reaction was stopped (quenched) by addition of one volume of quench solution (45 mM *d*-tubocurarine in buffer A). The reaction mixture was then applied to an ice-cold 2-mL disposable column (QSY, Isolab, Inc., Akron, OH) containing Dowex 50W-X8 and eluted with 3 mL of ice-cold 175 mM sucrose. The preparation and use of the ion-exchange resin were essentially as described by Epstein & Racker (1978). The eluate containing membranes and trapped $^{86}Rb^+$ was collected in scintillation vials and counted in a Beckman LS 200 liquid scintillation counter *without* scintillation fluor. In the presence of scintillation fluors the counting efficiency was approximately 3 times greater, but the background counts obtained in the absence of membranes were very high (25000 cpm or 0.5% of the total applied cpm under typical assay conditions). In the absence of fluor the background was very low (200 cpm), and the sensitivity of the assay remained excellent.

Inactivation of flux was measured by premixing 1 volume of membranes with 1 volume of Carb solution for a fixed time prior to addition of the $^{86}Rb^+$ solution (which also contained Carb). In the manual mixing assays the volumes used were typically 50–100 μL for each addition. Larger volumes (225 μL) were required for the rapid mixing experiments.

Quench-Flow Measurements. By use of a quench-flow technique developed by Hess and co-workers (Cash & Hess, 1981), ion flux could be readily measured over a range of 15 ms to several minutes by employing a combination of two modes of operation: continuous and pulse quench flow. In the continuous mode, for reaction times less than 100 ms, ion influx was initiated by forcing the vesicle suspension (0.225 mL) and an $^{86}Rb^+$ solution (100 μCi/mL; 0.225 mL) through a mixing chamber into a reaction tube and then into a second mixing chamber where the influx reaction was quenched with *d*-turbocurarine (45 mM; 0.225 mL). In the pulse mode, for reaction times greater than 100 ms, vesicle suspensions were first mixed with $^{86}Rb^+$ solution and then kept in a thermostated incubation tube. $^{86}Rb^+$ influx proceeded until a second plunger displaced the reaction mixture into a second mixing chamber where it was quenched with the *d*-tubocurarine. For inactivation studies, preincubation was achieved by using the pulse mode. Influx and quenching proceeded in the continuous mode for fast reaction times (<100 ms) or after firing a third plunger for longer times (>100 ms). The apparatus for these experiments was built by the Physics Machine Shop at Cornell University and was assembled at the University of California, Davis.

Results

Flux Properties of Reconstituted Membranes. The procedures used to extract and purify receptor were similar to those used by Huganir et al. (1979). The purified AChR had a specific activity for α-bungarotoxin binding in the range of 6–8 nmol/mg of protein and gave the expected four polypeptide bands on $NaDodSO_4$–polyacrylamide gels (M_r 40K, 50K, 60K, and 65K; Weill et al., 1974). There was no detectable acetylcholinesterase activity in the reconstituted membranes. The AChR was maintained in the presence of lipids at all stages of purification, a requirement shown to be essential for reconstitution of cholate-extracted AChR (Lindstrom et al., 1980; Anholt et al., 1981; Wu & Raftery, 1981; Popot et al., 1981).

The quality of the reconstituted vesicles containing purified *Torpedo californica* AChR and soybean lipids (Asolectin) was initially determined by measuring the 30-s influx of $^{86}Rb^+$ into the vesicles in the presence and absence of 1 mM carbamoylcholine at 0 °C without quenching. Typical influx values were 1000 cpm (no Carb) and 15000 cpm (+Carb) for 50 μL of vesicle suspension added to 50 μL of $^{86}Rb^+$ containing a total of 6×10^6 cpm. The large response was much greater than the response observed in native membranes, and the passive permeability measured by the no Carb sample was low. After a 48-h incubation at 0 °C, the total trapped volume of a 50-μL vesicle suspension was 35000 cpm. These results indicated that 40% of the vesicle internal volume equilibrated with external cations in the presence of Carb within 30 s at 0 °C and that the total internal volume corresponded to 12 μL/mL. On the basis of the immunological results of Lindstrom et al. (1980), the 60% of the vesicles that did not equilibrate in response to Carb probably represented sealed liposomes without receptor.

Quenching of Ion Flux. Effective quenching requires inhibition of the flux response when membranes are exposed to agonist and quench simultaneously. For native membranes 6.7 mM curare and 15 mM curare (final concentrations) were 100% effective in the presence of 10 mM Carb. In reconstituted membranes 6.7 mM curare and 15 mM curare were 71% and 87% effective, respectively. In a variety of quenching experiments using cholinergic antagonists (gallamine and curare) and local anesthetics (procaine and dibucaine), it

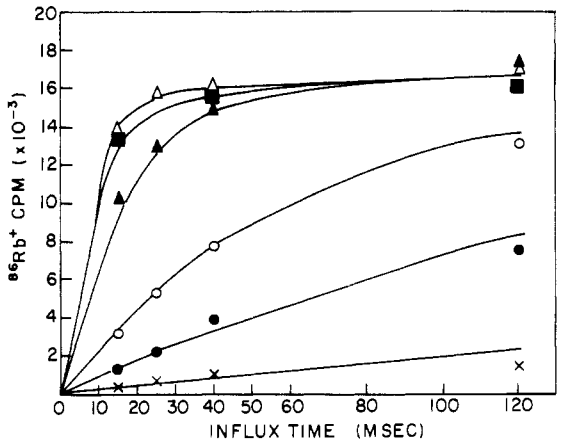

FIGURE 1: Time courses of $^{86}Rb^+$ influx across reconstituted membranes at six Carb concentrations. By use of a rapid mixing quench-flow apparatus, influx was initiated by mixing 0.225 mL of vesicle suspension with an equal volume of $^{86}Rb^+$ solution. Influx was quenched by mixture with 0.225 mL of 45 mM curare. Rates of $^{86}Rb^+$ influx were determined by measuring the $^{86}Rb^+$ content of reconstituted vesicles at four fixed times. Each point represents the average of duplicate determinations. The duplicate values were always within 5% of each other. The data are fit by first-order curves of the form $cpm_t = cpm_\infty(1 - e^{-J_A t})$, where cpm_∞ = 17 000 cpm and J_A represents the first-order flux rate constant for the *active* form of the receptor. (×) 0.02 mM Carb, J_A = 1.2 s^{-1}; (●) 0.05 mM Carb, J_A = 5.6 s^{-1}; (○) 0.1 mM Carb, J_A = 14 s^{-1}; (▲) 0.3 mM Carb, J_A = 58 s^{-1}; (■) 1 mM Carb, J_A = 90 s^{-1}; (△) 3 mM Carb, J_A = 100 s^{-1}.

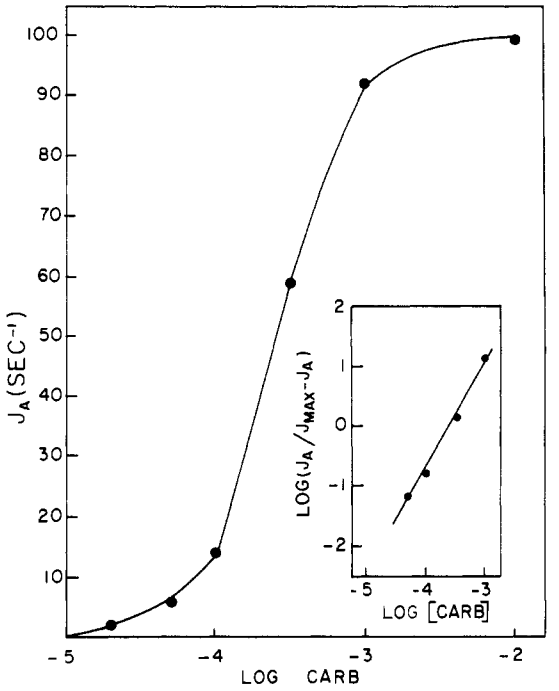

FIGURE 2: Dose–response of $^{86}Rb^+$ influx rates. J_A values obtained from Figure 1 are shown on a semilogarithm plot vs. Carb concentration. The half-maximal flux rate occurred at 3 × 10^{-4} M Carb. The maximum flux rate (J_{max}) was measured in the presence of 3 mM Carb and corroborated by an independent determination at 10 mM Carb after inactivation of a majority of receptor channels (Figure 5). (Insert) A Hill plot of $^{86}Rb^+$ influx rates. Solid line represents a linear least-squares fit with a slope (n_H) of 1.8.

appeared that 10–15% of the flux response was not quenchable. Pretreatment of reconstituted membranes with 100 μM α-bungarotoxin also did not block ~10% of the flux response at 10 mM Carb. This toxin-insensitive flux did not interfere with subsequent measurements or interpretation of flux rates, and the base-line (no Carb) flux response was adjusted to account for it. The reasons for the incomplete quenching are being further investigated.

Carbamoylcholine-Stimulated Ion Flux. The time courses of $^{86}Rb^+$ influx in the presence of various concentrations of Carb are illustrated in Figure 1. With no Carb the initial rate of $^{86}Rb^+$ influx across membranes represented less than 1% of the internal volume per second, indicating well-sealed vesicles. The maximum rate of flux observed at Carb concentrations greater than 3 mM was 100 s^{-1}, representing a 10^4-fold stimulation of the flux rate by Carb. The internal volume that rapidly equilibrated with outside $^{86}Rb^+$ within 120 ms in response to Carb represented about 40% of the total internal volume, the same value obtained after 10–30 s either in the quench flow device or in the manual assay. Thus, the rapid mixing and quenching of reconstituted membranes did not alter the relative volume ratio of receptor-containing vesicles to liposomes in the preparation. On the basis of the observed first-order kinetics, the AChR-containing vesicles were apparently homogeneous in size and receptor density unlike the two kinetically distinct AChR-containing populations observed in the native preparation (Hess et al., 1982). During the time scale of the measurements reported here, the receptor-free liposomes did not contribute to the flux rates.

The ion transport efficiency of reconstituted receptor can be compared to native AChR by normalizing the maximum rate of $^{86}Rb^+$ influx to vesicle internal volume, number of α-bungarotoxin sites, and monovalent cation concentration (Hess et al., 1981). For the reconstituted system these parameters were typically 5.4 μL/mL, 4100 pmol/mL, and 0.1 M, respectively. For a maximum first-order rate coefficient of 100 s^{-1}, the number of ions transported per channel per second becomes 10^4 in agreement with measurements in native *Torpedo* membranes (Hess et al., 1982).

Figure 2 shows the dependence of the rate of ion flux on Carb concentration. The half-maximal rate was achieved at 3 × 10^{-4} Carb (EC_{50}) or about 2 orders of magnitude higher than the EC_{50} obtained from dose–response curves of the 30-s flux amplitude (Lindstrom et al., 1980). A Hill plot of $^{86}Rb^+$ influx rates gave a slope of 1.8, indicating positive cooperativity (Figure 2, insert). If this cooperativity is interpreted to mean that two agonist molecules are required for channel opening, then the K_D for Carb binding can be obtained from eq 1:

$$J_A = J_A(\max)\left(\frac{[A]}{[A] + K_D}\right)^2 \quad (1)$$

where J_A is the flux rate constant for the active form of the receptor and [A] is the agonist concentration. For the data shown in Figure 2, the value for K_D was 1.5 × 10^{-4} M.

Inactivation of Ion Flux. Desensitization rates can be directly measured by preexposure of AChR to agonists followed by a determination of channel activity. In Figure 3A, membrane vesicles were preincubated with different concentrations of Carb for various times (100 ms to minutes), and then $^{86}Rb^+$ influx was measured for 18 ms in the presence of 10 mM Carb. The 18-ms influx value decreased steadily as the preincubation time increased and was described by a first-order rate law for over 90% of the reaction. First-order rate coefficients increased with Carb concentration to a maximum of 5.3 s^{-1} at 10 mM Carb. This fast desensitization did not lead to complete loss of ion flux activity. In experiments outside the quench-flow machine, preincubation with Carb for long times compared to fast inactivation did not abolish a 15-s flux amplitude. However, longer preincubation with Carb caused a first-order decrease in the 15-s flux amplitude (Figure 3B). The rate of this slow inactivation process increased with Carb concen-

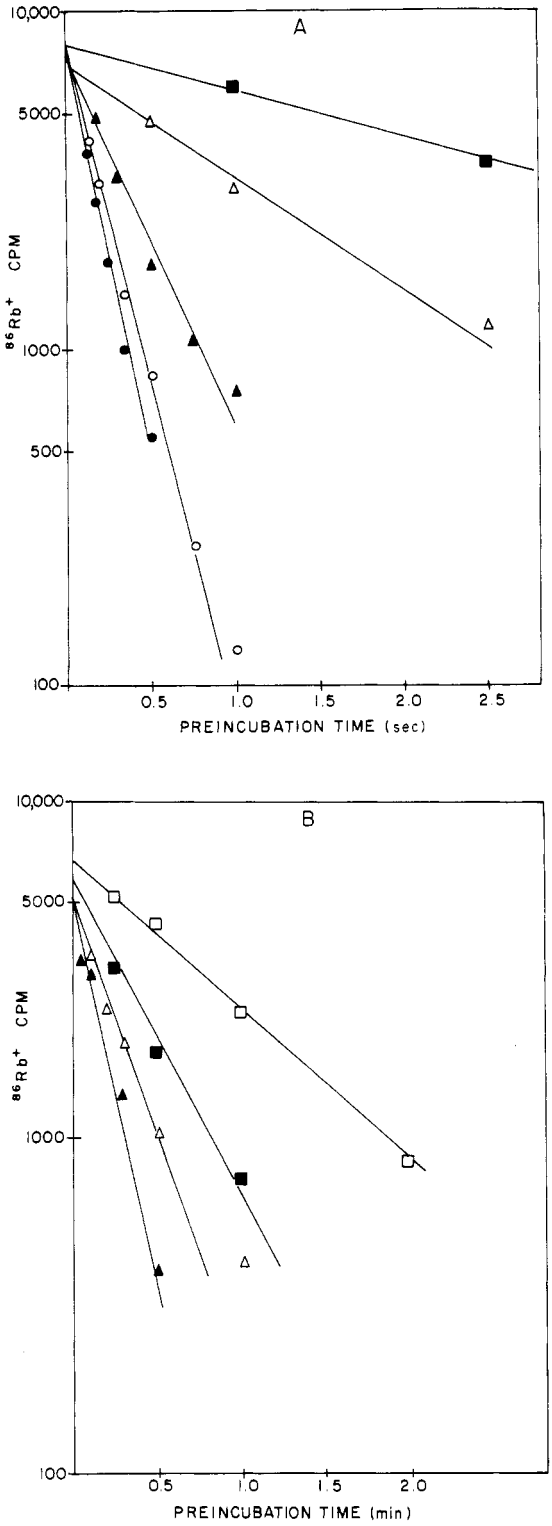

FIGURE 3: Rates of inactivation of receptor-controlled flux. Vesicles were preincubated with Carb for times given on the abscissa followed by a $^{86}Rb^+$ influx measurement. (A) After preincubation with Carb in the quench-flow apparatus, influx was allowed to proceed for 18 ms before quenching with curare. With no preincubation the $^{86}Rb^+$ content of vesicles was 9767 cpm after 18 ms of flux. (B) Preincubation and flux were performed with a manual assay. Influx was allowed to proceed for 15 s followed by quenching with curare. Inactivation of the 15-s amplitude is dominated by the slower process as the flux that remains after the fast process has gone to completion is enough to completely fill the vesicles in 15 s. With no preincubation the 15-s flux amplitude was 7231 cpm. The Carb concentration during influx in all cases was 10 mM and during preincubation as follows: (□) 0.03, (■) 0.1, (△) 0.3, (▲) 1, (○) 3, and (●) 10 mM.

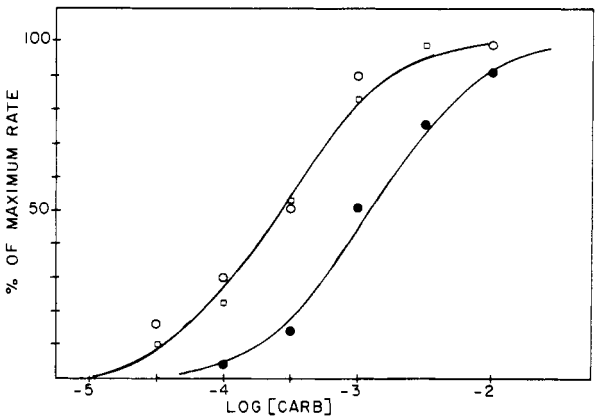

FIGURE 4: Dependence of fast and slow inactivation rates on Carb concentration. Rate coefficients for each inactivation process at each Carb concentration were determined by linear regression analysis of data obtained as shown (Figure 3). (○ and □) Slow inactivation measured in two different preparations: maximum rate 0.10 s^{-1}, K_{app} = 2.5 × 10^{-4} M, n = 1.1. (●) Fast inactivation: maximum rate 5.3 s^{-1}, K_{app} = 1.3 × 10^{-3} M, n = 1.1. Solid lines represent theoretical curves obtained from $v/V_{max} = [A]^n/(K^n + [A]^n)$ where [A] is agonist concentration, n is the Hill coefficient, and $K = K_{app}$ given above.

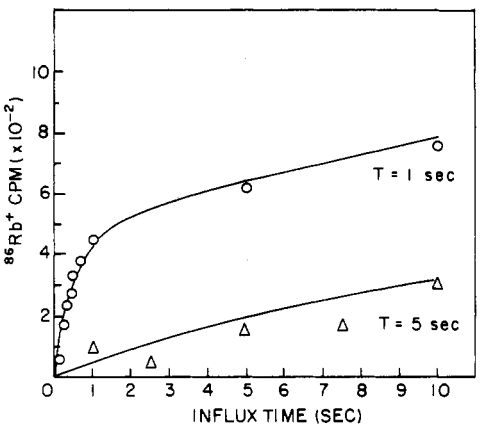

FIGURE 5: Ion flux rates after receptor inactivation. By use of a pulse–pulse setup of the quench-flow apparatus (Cash & Hess, 1981), reconstituted membranes were preincubated for a time (T) in 10 mM Carb before measuring the $^{86}Rb^+$ influx rate in the presence of 10 mM Carb. (○) One-second preincubation with 10 mM Carb; (△) 5-s preincubation with 10 mM Carb. For T = 0, equilibration was complete within 100 ms and gave a value of 1100 cpm. Solid lines represent curves obtained from the integrated rate equation that includes two inactivation processes (eq 2). The best fit of the curves gave the following values for the kinetic parameters: α = 4.0 s^{-1} and β = 0.10 s^{-1}; (1-s preincubation) J_A = 1.5 s^{-1} and J_I = 0.08 s^{-1}; (5-s preincubation) J_A = 0 and J_I = 0.04 s^{-1}. The fast rate of inactivation was measured directly for this membrane preparation and gave α = 3.9 s^{-1}.

tration to a maximum of 0.10 s^{-1} at 10 mM Carb.

Each desensitization process was characterized by a different dependence on Carb concentration (Figure 4). Rate coefficients for each process appeared to fit a nearly rectangular hyperbolic dependence on Carb concentration as shown by the solid lines in Figure 4. The half-maximal inactivation rate for the slow process was achieved at 2.5 × 10^{-4} M, while a 5-fold higher concentration (1.3 × 10^{-3} M) was required to give the half-maximal rate for the fast inactivation process.

Influx Rates after Preincubation. The existence of two distinct inactivation processes was demonstrated by a different approach. As shown in Figure 5, preincubation of vesicles with 10 mM Carb for 5 s (which is 25 times longer than the half-time for fast inactivation) allowed the measurement of ion flux that remained after the fast process was complete. The flux rate continued to decrease to an undetectable level as

preincubation time increased to 30 s. Preincubation for 1 s brought the rate of influx into an easily measured time range, though influx was no longer a simple first-order process. When the data were fit to the integrated rate equation derived by Hess et al. (1982):

$$\text{cpm}_t = \text{cpm}_\infty\left[1 - \exp\left[-J_A\left(\frac{1-e^{-\alpha t}}{\alpha}\right) - J_I\left(\frac{1-e^{-\beta t}}{\beta}\right)\right]\right] \quad (2)$$

the constants for two flux processes, J_A and J_I, as well as two inactivation processes, α and β, could be determined. Such a curve-fitting procedure provided estimates of α (4.0 s^{-1}) and β (0.1 s^{-1}) that agreed with direct measurements of maximum desensitization rates (α = 5.3 s^{-1}; β = 0.1 s^{-1}).

The use of the partial inactivation by Carb allowed an independent estimate of the maximum ion flux rate before the onset of inactivation. By use of the value of 1.5 s^{-1} for J_A after 1-s preincubation, α = 4.0 s^{-1}, and

$$J_A = J_A(\text{max})e^{-\alpha t} \quad (3)$$

the calculated value of 82 s^{-1} for J_A(max) agrees well with the directly measured value of 100 s^{-1} at 3 mM Carb (Figure 1). After 5 s of preincubation, J_A = 0 and only terms containing J_I and β remain in eq 2. Curve fitting again provides estimates of J_I (0.04 s^{-1}) and β (0.1 s^{-1}) that are consistent with the other measurements. The J_I value obtained at 1-s preincubation (0.07 s^{-1}) decreased to 0.04 s^{-1} after 5 s of preincubation, providing a third consistent estimate for β (0.14 s^{-1}).

Discussion

The ion flux data presented here show that the purified AChR protein from *Torpedo californica* electroplax in reconstituted lipid vesicles retains three characteristics of AChR in its native membrane environment: (i) a high efficiency of agonist-stimulated cation flux, (ii) a fast inactivation process in the millisecond time region, and (iii) a slower inactivation process in the second time region that leads to a complete loss of ion channel activity.

AChR Activation. The maximum flux rate constant obtained for Carb-stimulated $^{86}Rb^+$ flux across the reconstituted membranes (100 s^{-1}) is consistent with the movement of $\sim 10^4$ ions/s through each AChR-associated ion channel. Similar values were obtained with native *Torpedo* membranes (Hess et al., 1982) and are consistent with the expected functional properties of AChR channels as measured by electrophysiologic techniques (Steinbach, 1980; Adams, 1981). The results indicate that a high proportion of the reconstituted receptors must be functional and that the rapid mixing and quenching procedures developed for native membranes are applicable to reconstituted membranes. The influx rate is sufficiently fast that the maximum flux amplitude is limited by the internal volume of the active vesicles. Wu et al. (1981) also found that AChR activation is recovered with good efficiency after reconstitution.

The sigmoidal nature of the Carb dependence of flux activation is consistent with electrophysiologic data [reviewed in Steinbach (1980) and Adams (1981)] and with initial rate measurements of ion flux in eel vesicles (Cash & Hess, 1980), native *Torpedo* membranes (Neubig & Cohen, 1980; Wu et al., 1981), and BC3H1 muscle cells (Sine & Taylor, 1980). A requirement for binding two agonists prior to channel opening could account for the observed positive cooperativity in ion flux rates, and the existence of two agonist binding sites is compatible with the known structural and biochemical properties of the receptor (Karlin, 1980).

Table I: Summary of Kinetic Parameters Based on $^{86}Rb^+$ Flux Rates in *Torpedo californica* Membranes[a]

parameter[b]	native	reconstituted
activation		
maximum rate (J_{max})	310 ± 66 s^{-1} [c]	92 ± 9 s^{-1} (n = 3)
half-maximal Carb concentration (C_{50})	8 × 10^{-4} M	3 × 10^{-4} M
K_D (two ligand model)	4 × 10^{-4}	1.5 × 10^{-4} M
Hill coefficient (n_H)	1.6	1.8
slow inactivation		
maximum rate (β)	0.07 ± 0.02^{-1} (n = 3)	0.10 ± 0.03 s^{-1} (n = 6)
half-maximal Carb concentration (K_{app})	4.5 × 10^{-4} M	2.5 × 10^{-4} M
Hill coefficient (n_H)	1.1	1.1
fast inactivation		
maximum rate (α)	2.2 s^{-1} [c]	4.9 ± 1.3 s^{-1} (n = 6)
half-maximal Carb concentration (K_{app})	3 × 10^{-3} M[c]	1.3 × 10^{-3} M
Hill coefficient (n_H)		1.1

[a] By use of a quench-flow technique, the concentration dependence of three distinct processes (channel activation and two inactivations) was measured. The data revealed three characteristic kinetic constants for each process. Native membranes were assayed by the Millipore filtration technique [see Delegeane & McNamee (1980)] while for reconstituted membranes the ion-exchange column assay was used (Materials and Methods). The design of experiments was otherwise identical for both preparations. [b] Maximum rates of $^{86}Rb^+$ influx were determined in the reconstituted membranes by two approaches (see Figures 1 and 5). Maximum rates of inactivation (α and β) were measured at 10 mM Carb (Figure 3) and are presented as mean ± standard deviation. Half-maximal values are midpoints of the concentration dependence curves from Figures 2 and 4 which show typical results for a single membrane preparation. Similar results were obtained by using a different membrane preparation. Hill coefficients were obtained from slopes of double log plots as shown in Figure 2, insert. [c] Data from Hess et al. (1982).

AChR Inactivation. The ion flux data provide a new direct measure of agonist-induced flux inactivation. The loss of channel activity in reconstituted membranes occurred in two distinct time domains as observed for native *Torpedo* membranes (Walker et al., 1981a,b; Hess et al., 1982). In both native and reconstituted membranes, the fast inactivation decreased the ion flux rates by a factor of 250, and the slower inactivation decreased ion flux to an undetectable level.

Analysis of AChR-mediated single channel events in frog muscle also revealed two rates of AChR desensitization within time ranges comparable to those measured here (Sakmann et al., 1980). Neubig & Cohen (1980) originally proposed a two-step desensitization for *Torpedo* membranes to account for differences between EC_{50} values for flux activation rates measured in the millisecond time domain and apparent agonist binding constants measured in the second to minute time domain (Boyd & Cohen, 1980). They postulated an agonist-induced decay of the active state (K_{app} = 600 μM for Carb) on the millisecond time scale to an intermediate state (K_{app} = 30 μM) that further decays on the second to minute time scale to a high-affinity binding state (K_{app} = 0.02 μM) that is fully desensitized. The postulated very low affinity state has not yet been directly resolved by binding assays, but Heidmann & Changeux (1980) have detected a rapid fluorescence change with DNS-C6-choline that is much faster than the slow binding change but too slow to be attributable to channel opening. There is not yet sufficient data to directly link the postulated binding changes with the direct measurements of flux inactivation reported here.

Concentration Dependence of Flux Inactivation. Both the fast and slow inactivation rates showed nearly hyperbolic

saturation curves (Figure 4 and Table I), suggesting that cooperative interactions were not involved in flux inactivation. In eel vesicles, Hess and co-workers observed sigmoidal activation curves and hyperbolic inactivation curves (Aoshima et al., 1981), although only a single fast inactivation process was observed. In the Hess model, inactivation occurs when agonist is bound to one or both agonist binding sites.

The fast inactivation rate observed in both native and reconsituted *Torpedo* receptor membranes saturated at a 5–7-fold higher Carb concentration (K_{app} = 1.3 mM) than the slow inactivation process (Table I). The K_{app} for the fast inactivation is especially striking since it is 4.5-fold larger than the measured EC_{50} for flux activation and 9-fold larger than the calculated K_D for activation assuming two ligand binding sites. Thus, the rate of fast inactivation continued to increase with increasing agonist concentration even after both agonist binding sites were presumably saturated. By contrast, the slow inactivation rate had a K_{app} nearly identical with the calculated K_D for activation.

The shift in the fast inactivation concentration is difficult to explain by a mechanism in which both activation and desensitization are regulated through only one or both of the two agonist sites. Therefore, we have examined models that include a third agonist binding site. Proposals for a third site of action for agonists are not unique (Adams, 1981), since some agonists are known to induce both channel opening and channel blockade. Decamethonium, for example, activates AChR channels at low concentrations (<20 μM) and then blocks open channels at high concentrations (K_{app} = 60 μM) in a manner similar to local anesthetic action (Adams & Sakmann, 1978). Such agonists give bell-shaped dose–response curves with maximum responses observed at intermediate ligand concentrations.

The Carb concentration dependence of 30-s influx amplitudes in both native and reconstituted *Torpedo* membranes give bell-shaped curves (Lindstrom et al., 1980; J. W. Walker and M. G. McNamee, unpublished observations), and the flux amplitudes fall off in the concentration region where the fast inactivation process saturates. The existence of a third agonist site provides the simplest explanation for the falloff in flux amplitudes at high Carb concentrations and for the concentration dependence of the fast inactivation process measured directly with the quench-flow technique.

The nature of this third site is not known. However, it is probably not associated with a direct blockade of open ion channels. Fluctuation analysis of open channel blocking and unblocking gives time constants in the 100–600-μs region (Nehr & Steinbach, 1978). Even in the quench-flow device, such fast events would be detected as an instantaneous decrease in the number of channels rather than as a time-dependent transition from active to inactive channels over several hundred milliseconds as observed. Furthermore, Sakmann et al. (1980) observed no direct channel plugging by acetylcholine under conditions where two inactivation rates were observed. The postulated third site may be identical with or interact with sites on the receptor that bind allosteric inactivators of AChR flux, such as histrionicotoxin and some local anesthetics (Stallcup & Patrick, 1980; Changeux, 1981). The interactions between agonists and local anesthetics and the effects of temperature, divalent cations, and membrane potential on activation and inactivation are now under investigation.

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