CHANNEL PROPERTIES OF THE PURIFIED ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA* RECONSTITUTED IN PLANAR LIPID BILAYER MEMBRANES

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ABSTRACT The electrophysiological properties of the cation channel of the purified nicotinic acetylcholine receptor (AChR) reconstituted in planar lipid bilayers were characterized. Single-channel currents were activated by acetylcholine, carbamylcholine and suberyldicholine. The single channel conductance (28 pS in 0.3 M NaCl) was ohmic and independent of the agonist. Single channel currents increased with Na^+ concentration to a maximum conductance of 95 pS and showed a half-saturation point of 395 mM. The apparent ion selectivity sequence, derived from single-channel current recordings, is: $NH_4^+ > Cs^+ > Rb^+ \geq Na^+$ Cl^-, F^-, SO_4^{2-}. The distribution of channel open times was fit by a sum of two exponentials, reflecting the existence of at least two distinct open states. The time constants depend on the choice of agonist, being consistently longer for suberyldicholine than for carbamylcholine. Similar channel properties were recorded in bilayers formed from monolayers at the tip of patch pipets. Single-channel currents occur in paroxysms of channel activity followed by quiescent periods. This pattern is more pronounced as the agonist concentration increases, and is reflected in histograms of channel-opening frequencies. Computer simulations with a three-state model, consisting of two closed (unliganded and liganded) and one open state, do not resemble the recorded pattern of channel activity, especially at high agonist concentration. Inclusion of a desensitized liganded state reproduces the qualitative features of channel recordings. The occurrence of paroxysms of channel activity thus seems to result from the transit of AChR through its active conformation, from which it can open several times before desensitizing.

INTRODUCTION

Although detailed electrophysiological information on the channel of the muscle acetylcholine receptor (AChR) is becoming available through patch recording (Neher and Sakmann, 1976; Sakmann et al., 1980; Hamill and Sakmann, 1981; Colquhoun and Sakmann, 1981) and the structure of AChR from electric organs is being characterized in detail, (cf. Anholt et al., 1983), very little is known about the electrophysiological properties of electric organ AChR (Moreau and Changeux, 1976; Schindler and Quast, 1980). Reconstitution of the purified AChR in planar lipid bilayers (Nelson et al., 1980; Boheim et al., 1981) provides a strategy for combining the biophysical analysis of the AChR channel with the biochemical knowledge about the AChR macromolecule and permits for the first time the study of structure/function correlates at the molecular level.

The nicotinic acetylcholine receptor (AChR) from *Torpedo californica* electric organ has four kinds of homologous glycopeptide subunits. AChR monomers (mol wt ~270,000) consist of two α-subunits (protein mol wt ~50,116) and one each of β-(protein mol wt ~53,681), γ-(protein mol wt ~56,601), and δ-(protein mol wt ~57,565) subunits (Reynolds and Karlin, 1978; Lindstrom et al., 1979; Raftery et al. 1980; Noda et al., 1982, 1983; Claudio et al., 1983). The two α-subunits are responsible for the binding of acetylcholine (ACh) (Weill et al., 1974; Wolosin et al., 1980; Karlin et al., 1975). Local anesthetics label the δ-subunit (Oswald and Changeux, 1981; Oswald et al., 1980). The cation channel is an integral component of the monomer (Anholt et al., 1980; Popot et al., 1981), but it is not known which subunits form the channel.

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Abbreviations used in this paper: ACh, acetylcholine; AChR, acetylcholine receptor; CCh, carbamylcholine; γ, single channel conductance; HEPES *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; SubCh, suberyldicholine; τ_o, channel open time; *V*, applied voltage.

Electron microscopy suggests that the AChR molecule is shaped like a mushroom 85 Å in diameter and 110 Å long with a roughly peaked asymmetric cap on the external surface of the membrane and a stem extending across the membrane. The five subunits are arranged as the staves of a barrel around an ion channel in the center (Kistler et al., 1982; Zingsheim et al., 1982).

The reconstitution of the purified AChR in lipid vesicles (Changeux et al., 1979; Huganir et al., 1979; Lindstrom et al., 1980; Anholt et al., 1980, 1981, 1982; Wu et al., 1981) and in planar lipid bilayers (Nelson et al., 1980; Boheim et al., 1981) established that the $\alpha_2\beta\gamma\delta$-subunit structure of the receptor contains both the agonist binding site and the ion channel that it regulates. The reconstituted purified AChR showed two prominent features of the postsynaptic membrane, namely activation and desensitization induced by cholinergic agonists (Anholt et al., 1980, 1981, 1982; Walker et al., 1982; Popot et al., 1981; Epstein and Racker, 1978; Huganir et al., 1979; Schindler and Quast, 1980; Nelson et al., 1980; Boheim et al., 1981; cf. Anholt et al., 1983).

These advances invite studies on the molecular mechanism of channel function. Here, we describe single channel properties of the purified AChR reconstituted in planar lipid bilayers. The contribution of AChR desensitization is evaluated from the pattern of single channel activity.

MATERIALS AND METHODS

Receptor Preparation

Receptor from the electric organ of *Torpedo californica* (Pacific Biomarine, Venice, CA) was solubilized, purified, and reconstituted in lipid vesicles as described in detail previously (Lindstrom et al., 1980; Anholt et al., 1980, 1981, 1982). The reconstituted soybean lipid vesicles (40 mg/ml) used in this study were supplemented with cholesterol (8 mg/ml) prior to cholate dialysis and were subjected to a freeze-thaw cycle (Anholt et al., 1982). The functional integrity of the receptor in the reconstituted vesicles was assayed by carbamylcholine (CCh)-induced $^{22}Na^+$-uptake (Huganir et al. 1979; Lindstrom et al. 1980) before the electrical measurements were taken.

Planar Lipid Bilayers

Monolayers were derived from the reconstituted vesicles (Schindler, 1980; Schindler and Quast, 1980) as described by Nelson, et al. (1980) and Labarca, et al. (1983).[1] Planar lipid bilayers were assembled from two monolayers across a ~200-μm diameter hole in a 12-μm thick Teflon partition separating two 1-ml capacity Teflon chambers, as described elsewhere (Montal, 1974). The hole was treated with 2 μl of 0.5% (vol/vol) hexadecane in hexane or in chloroform:methanol (2:1). Each chamber was filled with a buffer composed of 0.5 M NaCl, 5 mM $CaCl_2$, 5 mM HEPES, pH 7.4. In one chamber (the *cis* chamber) a monolayer was assembled from reconstituted vesicles. In the opposite (*trans*) chamber, a monolayer was derived from soybean lipid vesicles devoid of AChR. All experiments shown were performed at room temperature.

[1]Labarca, P., J. Lindstrom, and M. Montal. 1983. Acetylcholine receptor in planar lipid bilayers: Characterization of the channel properties of the purified actylcholine receptor from *Torpedo californica* reconstituted in planar lipid bilayer membranes. Submitted for publication.

Patch Pipettes

Pipets were produced as described in detail by Hamill et al. (1981) using hematocrit capillaries made of flint glass (BLU-TIP, plain, I.D. 1.1–1.2-mm, Lancer, St. Louis, MO). A commercial vertical pipet puller (David Kopf, Model 700*C*, Tujunga, CA) was used. The pipet tip was fire-polished in a microforge to an opening of $\leq$ 1 μm and coated with Sylgard (Dow Corning Corp., Midland, MI) within 40 μm from the tip.

Electrical Recordings and Data Processing

Membrane currents under voltage clamp were recorded as previously described (Montal and Mueller, 1972; Labarca et al., 1983).[1] Voltage was applied and current was measured by using Ag/AgCl electrodes with a current-to-voltage converter (National Semiconductor LF357AH) having either a 1-GΩ or a 10-GΩ feedback resistor; the amplifier time constants were 120 and 250 μs. Constant voltage was supplied by a variable DC source and the *trans* side of the membrane was defined as zero voltage. For the recordings with patch pipets, a commercially available extracellular patch clamp system was used (Hamill et al., 1981) (LIST L/M EPC-5, List Electronic, Darmstadt, Federal Republic of Germany) set at a gain of 10 mV/pA and a filter setting of 10 KHz. The signal coming from the current amplifier was amplified and recorded on FM tape (RACAL 4DS, bandwidth DC to 2.5 or 5 KHz) and later digitized at a sampling interval of 100 μs for computer analysis. Distributions of dwell times in the open or closed states were analyzed using a PDP 11/34 computer (Digital Equipment Corp., Marlboro, MA), as described in detail elsewhere (Labarca et al., 1983).[1]

Computer Simulation

Rate equations corresponding to kinetic models were solved numerically on a VAX 11/780 computer (Digital Equipment Corp.). The Runge-Kutta-Fehlberg fourth-fifth order method (Forsythe et al., 1977) was used to obtain a matrix of transition probabilities among receptor states from one sampling time to the next. The corresponding Markov chain was then simulated using the high-quality portable random-number generator *urand* (Forsythe et al., 1977). Gaussian noise was added to the resulting simulated conductance record using the same random-number generator.

RESULTS AND DISCUSSION

Single Channel Properties

Lipid bilayers containing purified AChR respond to the addition of cholinergic agonists with a transient increase in conductance that spontaneously relaxes to a steady-state value. The agonist-activated membrane conductance increases with agonist concentration and is inhibited by *d*-tubocurarine and hexamethonium (Nelson et al., 1980; Labarca et al., 1983).[1]

At high-current resolution, the opening and closing of individual AChR channels activated by cholinergic ligands can be monitored. Both the single-channel conductance, γ, and the channel open times can be readily measured.

Single Channel Conductance, γ. The currents flowing through individual AChR channels appear as discrete transient steps that fluctuate between two identifiable levels. These levels are associated with the closed and open state of the channel. The channel remains open for a few milliseconds before closing. This is illustrated in Fig. 1. A downward deflection corresponds to a channel opening

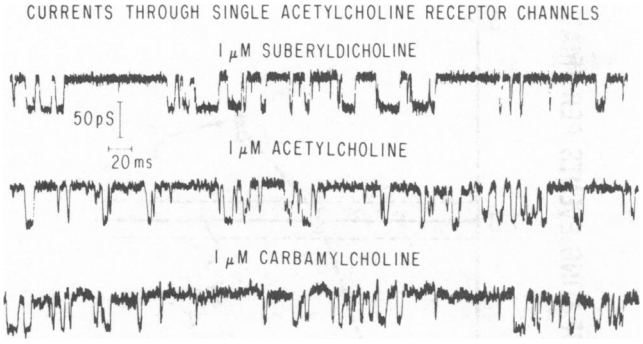

FIGURE 1 Single-AChR–channel currents activated by different cholinergic agonists. Planar lipid bilayers were formed in 0.5 M NaCl, 5 mM $CaCl_2$, 2. 5 mM HEPES, pH 7. 4. SubCh (*upper record*), ACh (*middle record*) and CCh (*lower record*) were added to the *cis*-side of the membrane. $V = -70$ mV, negative in the compartment containing the agonist. The open channel conductance, $\gamma = 48$ pS for all three agonists.

event; the reverse is true for the closing event. The single channel conductance, γ, is ohmic and is independent of the agonist used to activate it, for ACh, CCh or suberyldicholine (SubCh). In contrast, the single channel currents vary nonlinearly with Na^+ activity. This is shown in Fig. 2. The results can be fit with a simple saturation curve with an extrapolated value for the maximum γ of 95 pS and a Na^+ activity at which γ equals 50% of γ_{max}, of 395 mM. Fig. 2 shows that at 0.3 M monovalent salt concentrations, $\gamma = 28$ pS for Na^+, $\gamma = 30$ pS for Rb^+, $\gamma = 38$ pS for Cs^+ and $\gamma = 50$ pS for NH_4^+. The selectivity and saturation characteristics of the AChR open channel agree with those determined in muscle cells (Adams et al., 1980; Horn and Patlak, 1981).

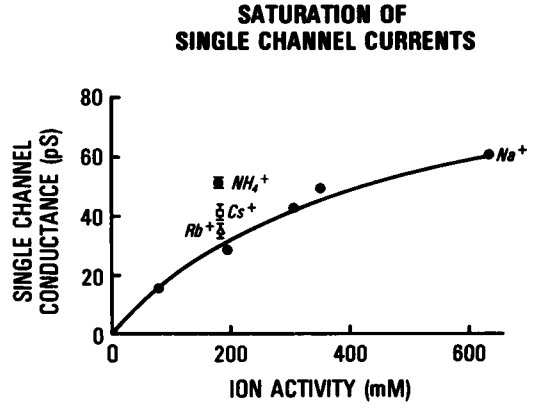

FIGURE 2 Saturation of single-AChR–channel currents. Single-channel currents were activated by SubCh (0.1 μM) and values for the open channel conductance were measured at different activities of salt and in different salts, at negative applied voltages (-70 mV $\leq V \leq -100$ mV). The closed circles (•) correspond to the open channel conductance (± SEM) as a function of the NaCl activity. The solid line (—) is the best fit of a first-order saturation function to the experimental results (Horn and Patlak, 1980): $\gamma = \gamma_{max} \times [NaCl]/K_{1/2} + [NaCl]$, where γ_{max} is the maximal value of the open channel conductance, [NaCl] is the activity of salt in the aqueous buffer, and $K_{1/2}$ is the salt activity at which $\gamma = 1/2\ \gamma_{max}$. The best fit was obtained with $\gamma_{max} = 95$ pS and $K_{1/2} = 395$ mM. The open channel conductance (± SEM) measured for Rb^+, Cs^+ and NH_4^+, at the indicated activities, are also illustrated.

Channel Open Times. At room temperature, the channel open times are of the order of milliseconds and depend on the agonist used according to the following sequence: SubCh > ACh > CCh. This is also illustrated in Fig. 1.

Single-channel current records, where only one channel was open at any given time, were analyzed to measure the channel dwell times in the open or the closed states. The results of such analysis for SubCh at an applied voltage, $V = -150$ mV, are shown in Fig. 3. The distribution of open times does not follow a single exponential function but is best fitted with the sum of two exponentials yielding values for the fast component, (τ_{o1}), of 1.0 ± 0.02 ms, and for the slow component, (τ_{o2}), of 11.2 ± 0.5 ms.

The distribution of open times can be expressed as

$$N_{\geq}(t) = A_1 \exp(-t/\tau_{o1}) + A_2 \exp(-t/\tau_{o2}). \quad (1)$$

$N_{\geq}(t)$ represents the number of events with a channel open time equal to or longer than time t and $\tau_{o1} < \tau_{o2}$. Eq. 1 describes a general feature of the distribution of open times

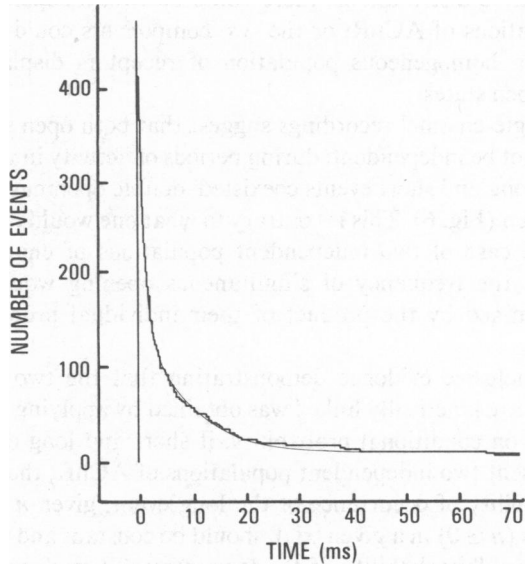

FIGURE 3 Frequency histograms of single AChR channel open times activated by SubCh. Single-channel currents were activated by SubCh (0.1 μM) at $V = -150$ mV. All other conditions as for Fig. 1. Computer-generated signals were used to assist in measuring the channel parameters. The position and the width of the computer-generated signals corresponding to the channel openings and closings were stored in a PDP 11/34 computer. After analysis of the data, cumulative open-state lifetime distributions of the generated rectangular pulses were automatically produced. Time constants were determined by fitting one or two computer-generated exponentials to the data points displayed simultaneously on an oscilloscope screen. The fitted curves (smooth curve) were superimposed on the histograms of the actual data (noisy curve). The distribution of open times could not be fitted with a single exponential (not shown). As illustrated, a sum of two exponentials does generate an adequate fit to the results. The total number of channel openings that were analyzed, N, was 468. The zero time amplitude (A) and lifetime (τ) of the two components were $A_1 = 395 \pm 7$; $\tau_{o1} = 1.0 \pm 0.02$ ms and $A_2 = 73 \pm 3$; $\tau_{o2} = 11.2 \pm 0.5$ ms. Semilogarithmic plots of the data were fitted by similar parameter values (not shown).

of single-channel currents activated by agonists in planar lipid bilayers containing the purified AChR.

τ_{o1} and τ_{o2}, the fast and slow components of the distribution of open times, are independent of the agonist concentration. For CCh this has been verified in the range of 10^{-6} M < C < 10^{-3} M. τ_{o1} and τ_{o2} are ~3 times longer for SubCh than for CCh. τ_{o1} and τ_{o2} are moderately voltage-dependent, increasing as the applied voltage in the compartment-containing agonist is made more positive with respect to the other.

The existence of two kinetic components of the distribution of channel open times has been observed for the AChR in muscle cells (Colquhoun and Sakmann, 1981; Jackson et al., 1983).

The distribution of closed times is best fitted with the sum of three exponentials. This analysis will be described elsewhere. Here, we focus on the channel openings.

AChR Channel Has Two Open States. There are at least two simple models that could account for the two exponential components in single-AChR–channel open time distributions: there could be two, independent, populations of AChR, or the two components could arise from a homogeneous population of receptors displaying two open states.

Single-channel recordings suggest that both open states may not be independent: during periods of activity in which both long and short events coexisted, double openings were not seen (Fig. 6). This is contrary to what one would expect in the case of two independent populations of channels, where the frequency of simultaneous opening would be determined by the product of their individual probabilities.

Conclusive evidence demonstrating that the two open states are kinetically linked was obtained by applying a test based on conditional probability: if short and long events represent two independent populations of AChR, then the probability of occurrence of the long event, given n short events ($n \geq 0$) in a given trial, should be constant and equal the overall probability of the long event. To perform this simple test, records of single channel currents were divided into periods of equal length (trials) . We computed the average number of long events over all trials, as well as the number of long events per trial containing n ($0 \leq n \leq 4$) short events. A summary of these studies in three different experiments is shown in Fig. 4. As seen, the results are in disagreement with the hypothesis of independent populations of AChR: when the number of short openings per trial decreases, the number of long openings also decreases. Thus, it appears that the presence of two open times is not the result of the activity of two independent populations of AChR in planar bilayers. The results can be attributed most simply to the presence of one population of AChR having two distinct open states (Labarca et al., 1983). Recently, Jackson et al. (1983) analyzed the open-state lifetime distributions of single AChR currents in muscle

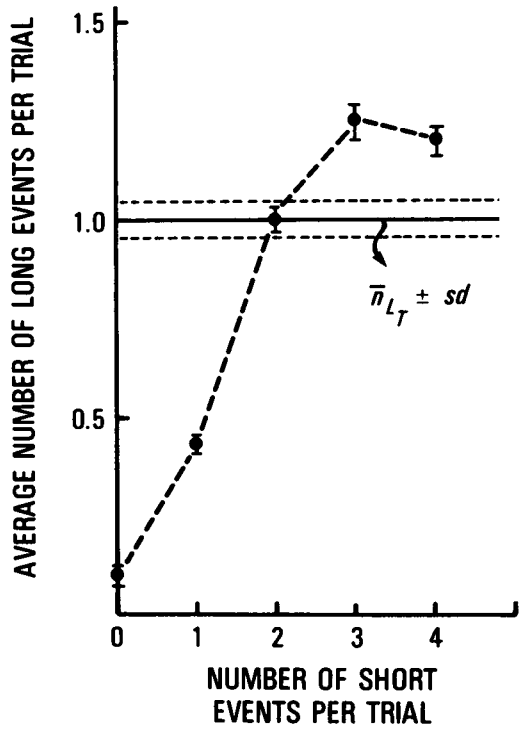

FIGURE 4 Average number of long events per trial as a function of the number of short events per trial. Single channel currents were activated by 0.1 μM SubCh and recorded at V = 100 mV. To distinguish short from long events, a time interval was chosen such that 96% of all short fluctuations would fall within the interval $0.96 = 1 - \exp - T/\tau_S$. All openings with lifetimes shorter or equal to T were classified as "short events" and all remaining ones as "long events. " Using the amplitudes, A_S and A_L, and the time constants, τ_S and τ_L, it is estimated that ~15% of all events labeled "short" would be "long" events with open times $\leq T$, and ~3% of all events classified as "long" must be short events with open times $\geq T$. The subscripts S and L correspond to the first and second components of the distribution of open times (Eq. 1). This criterion was adopted because it is simple, does not interfere with the independence test, and appears justified based on the large difference in relaxation times of the fast and slow kinetic processes involved in channel closure. The results of three different experiments are pooled. An average of 60% of all openings correspond to "short" events, whereas 40% correspond to "long" events, as determined from the zero-time amplitudes of the distribution of open times (A_S and A_L). Each point in the figure represents the average number of long events (± SD) in trials in which 0, 1, 2 ... short events occurred. In each case 30–100 trials were analyzed. Trials were defined as time intervals of 150 ms. A total of 348 short and 153 long events was computed. The continuous line represents the overall average of long events ($\bar{n}_{LT}$), ± one standard deviation (SD), showing the region where the experimental data should lie if there were two independent populations of channels.

cells in culture in terms of two exponentials, and postulated two open states for the AChR in situ. The similarities between the results obtained in muscle and in reconstituted membranes strengthens the view that the AChR channel, after purification and reconstitution, displays the features known of the functional AChR in biomembranes.

Recordings from Bilayers Formed at the Tip of Patch Pipets. To improve the sensitivity and time resolution of the single-channel recordings from purified

receptor reconstituted in vesicles, we have turned to the use of patch pipets. Thus far, similar-channel properties have been recorded using patch pipets either after formation of high-resistance seals (gigaohm range) by gentle suction of the vesicles into the pipet (Tank et al., 1982) and by the formation of a reconstituted bilayer from monolayers at the tip of the pipet (Wilmsen et al., 1982; Suarez-Isla et al., 1983; Schuerholz and Schindler, 1983; Coronado and Latorre, 1983).

Single channels were activated by the cholinergic ligands ACh, CCh and SubCh (Fig. 5). The single-channel conductance, γ, was 40 ± 5 pS for the three agonists in 0.45 M NaCl, 5 mM $CaCl_2$, 2.5 mM HEPES, pH 7.0. The distributions of channel open times were fit by a sum of two exponentials irrespective of the choice of agonist. At desensitizing concentrations of agonists the single events appeared in clusters of channel openings followed by quiescent periods. Thus, purified AChR in reconstituted bilayers formed at the tip of patch pipets displays channel activity associated with activation and desensitization in the presence of cholinergic agonists (Suarez-Isla et al., 1983).

Desensitization of the Purified AChR. Single-channel current records, activated by SubCh at concentrations ≥ 0.1 μM or by CCh ≥ 10 μM, revealed a characteristic pattern of occurrence of single-channel activity (Fig. 6). Paroxysms of activity were followed by quiescent periods during which little or no activity was observed. This behavior resembles that of single-AChR–channel currents in muscle at desensitizing concentrations of ACh (Sakmann et al., 1980). Paroxysms were composed of many single-channel openings. Each opening was often interrupted by brief closings.

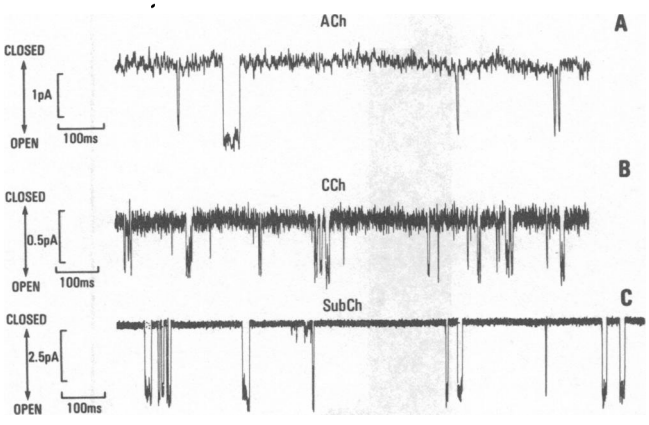

FIGURE 5 Single-channel currents activated by different cholinergic agonists and recorded in bilayers formed at the tip of patch pipets. ACh (10 μM) (*upper record*): $V = -51$ mV. CCh (10 μM) (*middle record*): $V = -10$ mV. SubCh, (10 nM) (*lower record*): $V = -100$ mV. The single-channel conductance $\gamma = 40 \pm 5$ pS for all three agonists. The seal resistances were 17 GΩ, 5 GΩ and 10 GΩ, respectively. The records were low-pass filtered at 1 KHz. Other conditions as for Fig. 1 (from Suarez-Isla et al., 1983).

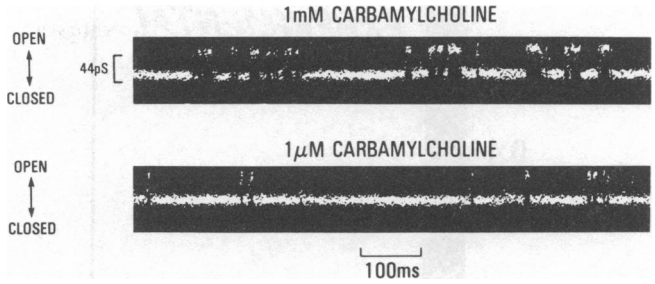

FIGURE 6 Frequency of channel opening in the presence of CCh. Records of single AChR channel currents activated by CCh and recorded at $V = 70$ mV. *Upper panel*: 1 mM CCh. *Lower panel*: 1 μM CCh. Other conditions as for Fig. 1.

To study the paroxysm phenomenon quantitatively, a long record was divided into many 100-ms periods (trials) and, for each period, the number of channel openings was counted. Histograms were constructed showing how many 100 ms trials had 0, 1, 2 etc. openings. A typical histogram for 1-mM CCh is shown in Fig. 7. There is a big peak at zero openings, corresponding to the quiescent periods, and there is a peak at ~6 openings/100 ms trial, corresponding to the extent of a typical paroxysm of openings. The results of a similar analysis for 1-μM CCh are shown in Fig. 8. No peak is seen at this low agonist concentration.

The simplest model of AChR function (Fig. 9, *upper panel*) has three states consisting of an unliganded closed state (*C*), a liganded closed state (*CL*), and a liganded state with an open channel (*OL*) (cf. Adams, 1981). Such a model is, of course, too simple to account for all the available information about the AChR, such as multiple ligand occupancy, two open states, and the brief closing gaps within an opening. However, the simplicity of the model and its focus on channel opening, which our experiments measure, are appealing. The three-state model accounts for our low-agonist concentration data satisfactorily, but it fails to explain the long quiescent periods at high concentrations. Therefore, we made the model slightly more complicated by including a functionally desensitized state (*DL*) (Fig. 9, *lower panel*) (Katz and Thesleff, 1957). This state need not be identified with the high-affinity state of binding studies (cf. Changeux, 1981). Its main properties are that it is extremely stable, but is entered infrequently.

A comparison of the experimental histogram with Monte Carlo simulations based on the two models just described is shown in Fig. 7. All of these histograms are constructed from 100 trials, and show noticeable statistical variation. The agonist concentration was 1 mM. Notice that the three-state model does not generate trials with no channel openings. In contrast, the four-state model exhibits the same general characteristics as the real data. Low-agonist concentration (1 μM) histograms (Fig. 8) do not distinguish the two models. The Monte Carlo simulations show that, in this case, the receptor is usually unliganded during the quiescent periods.

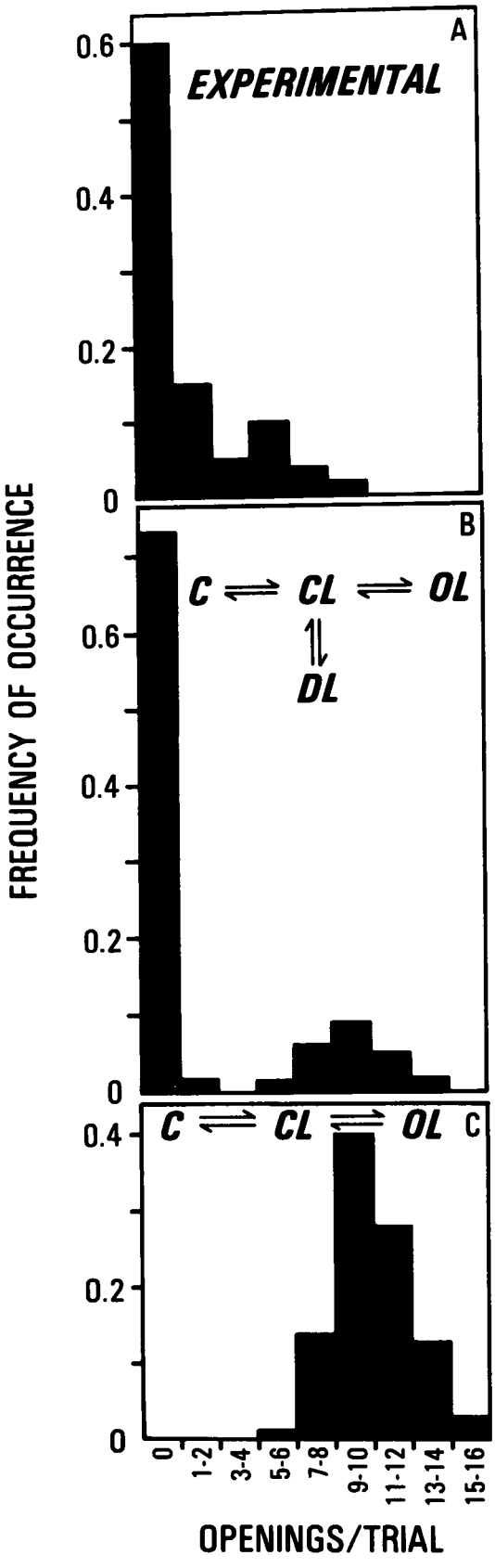

FIGURE 7 Frequency of channel opening in the presence of 1 mM CCh. To build the histograms, the frequencies corresponding to 0, 1–2, 3–4, 5–6 etc. openings were pooled together. V = 70 mV. *A*, experimental data (Fig. 6); *B*, Monte Carlo simulation with the four-state model (Fig. 9); *C*, Monte Carlo simulation with the three-state model (Fig. 9).

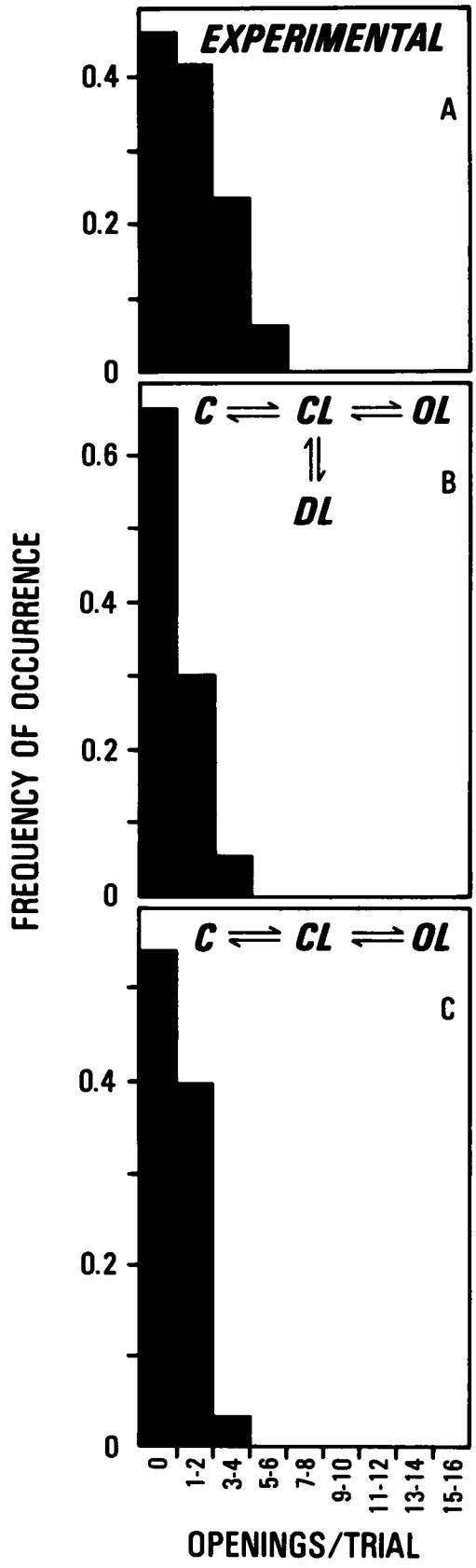

FIGURE 8 Frequency of channel opening in the presence of 1 μM CCh. Conditions were analogous to Fig. 7.

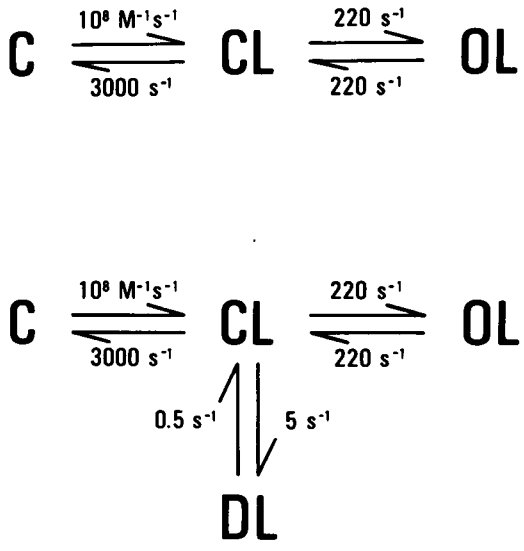

FIGURE 9 Models of AChR channel kinetics. *Upper panel*: A three-state model consisting of two closed states (unliganded [*C*] and liganded [*CL*]) and one open-liganded state [*OL*]. *Lower panel*: A four-state model including a functionally desensitized (liganded) state [*DL*]. The rates shown consider agonist binding to be diffusion-limited with a K_D of 30 μM. Channel opening and closing rates are measured results, and rates for desensitization and for recovery from desensitization are taken from Sakmann et al. (1980) channel data and Walker et al. (1982) flux measurements.

Fig. 10 shows the results of a Monte Carlo simulation, displayed in the same format used to display the real data illustrated in Fig. 6. At high agonist concentration, paroxysms of channel openings with an occasional isolated opening are observed. In contrast, at low agonist concentration, predominantly single openings are observed.

Therefore, the paroxysms of channel openings are associated with desensitization (Sakmann et al., 1980). Rapid transitions occur between open and closed states with lengthy interruptions due to desensitization. No novel ideas are needed to understand the paroxysm phenomenon.

CONCLUSIONS

This study demonstrates that the purified AChR, consisting only of its $\alpha_2\beta\gamma\delta$-subunits reconstituted in planar lipid bilayers, exhibits the kinetic, pharmacological, and ion-conduction properties that are associated with the postsynaptic membrane. The full cycle of solubilization, purification, and reconstitution of AChR can be achieved without impairment of channel function. Furthermore, the channel activity can be assayed with an electrophysiological method of high resolution and sensitivity under conditions amenable to biochemical manipulations. The channel properties of the purified *Torpedo* AChR in the reconstituted bilayer are in remarkable agreement with those recorded in amphibian muscle or rodent cultured-muscle cells, both qualitatively and quantitatively. This is the most compelling validation for using the biochemically characterized *Torpedo* AChR in reconstituted membranes as a model of AChR at the neuromuscular junction. Thus, planar lipid bilayers in conjunction with the reconstituted receptor vesicles provide a powerful model system to investigate the molecular physiology of postsynaptic transduction and to establish structure/function correlates in the AChR molecule.

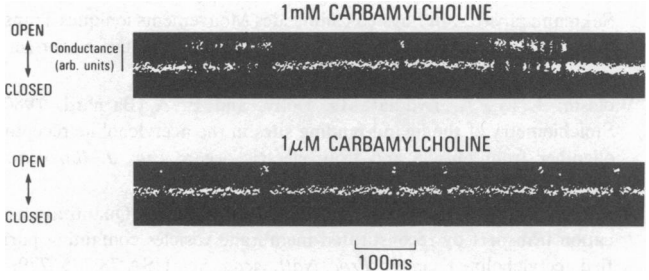

FIGURE 10 Frequency of channel opening in the presence of CCh. Monte Carlo simulations using the four-state model (Fig. 9) are displayed in the format of Fig. 6. *Upper panel*: 1 mM CCh. *Lower panel*: 1 μM CCh.

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DISCUSSION

Session Chairman: Harold Lecar *Scribes*: Thomas N. Earnest and Sarah S. Garber

LECAR: In the prototypical scheme for AChR kinetics, an empty receptor becomes singly liganded, remaining in a closed state, then becomes doubly liganded and undergoes a conformational transition which leads to an open state. One consequence of this scheme is that whatever else becomes complicated, the histogram of open state lifetimes obeys a single exponential. All the channels that close are closing by a Poisson process.

In this paper and in work done in tissue culture cells, there is evidence for histograms of open state lifetimes that do not seem to obey a single exponential. This is not just a question of multiple pathways from the open state, but rather two exponentials that would mean two distinct open states. Two pathways from an open state would merely give a single exponential with an altered rate constant. So there is a disparity in single channel work at the present; maybe it has to do with the statistical data processing or maybe alternative open states of the AChR molecule are expressed in some environments. It is striking that the bilayer kinetics do resemble the kinetics obtained from tissue culture cells.

MONTAL: In 1980, when we began to get longer single channel recordings, we found that distribution of open times could not be fit by a single exponential, and we were quite concerned. We later found out that this was also observed in tissue cultured muscle cells and in native muscle preparations. David Tank has reported that this occurs in patch clamped liposomes, and Alfred Maelicke has also found two open states of AChR in reconstituted systems.

LEIBOWITZ: It is clear that many people are seeing multiple components of the open-time data. Comparison of many different preparations does not tell us more about the function of the neuromuscular junction (NMJ). This is not the important consideration. It is the physiological function of the multiple conduction states that we want to understand.

MAZET: Given the single channel activity, the macroscopic current should be smoothly decreasing. Does the large fluctuation of the macroscopic current that you see reflect the same working mode of the receptor as the single channel activity or does it reflect the cooperative activity observed by Hans Georg Schindler?

MONTAL: The transient overshoot in the macroscopic response can be accounted for quantitatively by desensitization. Fredkin has simulated the macroscopic response by addition of single channel records.

TANK: There is evidence that the phosphorylation state of the *Torpedo* AChR changes during development. Have you compared the single channel properties of the receptor isolated from *Torpedo* neonates with those of the adult?

MONTAL: No. But we intend to test whether covalent modification of the protein could account for changes in the conductance states. It is known that the receptor can be phosphorylated or methylated. These covalent changes could account for changes during maturation of the receptor.

SACHS: The presence of two conductance states does complicate the kinetic model, but they are found at end plates and they show up in the isolated receptor as well. There is a suggestion of a fast component to the open time decay in Fig. 4 *b* of Leibowitz and Dionne. Whether or not the fast component is seen may depend on the value of the time constant of the particular channel. If the time constant is 200 μs or more, it should be seen; if the time constant is 100 μs, it could be lost in the noise. It may be that the lack of two open times is an artifact, rather than an experimental observation.

LECAR: An obvious interpretation of the other open state is the existence of a mono-liganded open state (AR*). This interpretation may be inconsistent with the data, but it is a simple, satisfying interpretation that provides a tangible explanation for two time constants.

FINKELSTEIN: The picture you have of the bursting behavior involves movement of the channel from AAR to AAR*. How does one preclude that bursting is not due to the AAR* state going into another closed state, distinct from the inactivated state, and that this is what you are seeing as flickering of the channel?

MONTAL: Nothing precludes that possibility. We tried to be more simplistic by using a singly liganded state rather then a doubly liganded state. But there is an additional state of desensitization. The desensitization acts like a sink to which the receptor can go and recover into the active state. This was proposed by Sakmann et al. a few years ago (Sakmann, E., J. Patlak, and E. Neher. 1980. *Nature* (*Lond.*). 286:71–73), and it can account quantitatively for the bursting.

DIONNE: We have used the simplest model to fit our data

$$2A + R \rightleftharpoons AR + A \rightleftharpoons A_2R \rightleftharpoons A_2R^*$$

at the lower limit of agonist concentration. Of course more complicated models can be used, introducing more closed and open states. This introduces parameters in the curve-fitting schemes but does not provide more information, since these new parameters are freely adjustable. Therefore one uses the simplest model to fit the data, and tries to make inferences from this.

Leibowitz and I have also seen open time distributions with two components, but not always. Have you manipulated any conditions such as lipid composition, and observed changes in the distribution of open time durations?

MONTAL: No. This is one of our next steps.

GARDNER: Concerning the two populations of open states, you have used the principle of conditional probabilities and find a correlation between the probability of long openings and the probability of short openings. You use this to argue against two populations of channels. Is it possible that some other parameter may vary, such as the concentration of ACh, and that could be driving two independent populations of channels?

MONTAL: In principle there could be some other parameter. But we have applied the conditional probability argument over two orders of magnitude of agonist concentrations, and the situation still prevails.

EISENMAN: Does the receptor stick out far enough from the membrane so that it is insensitive to the surface charge of the lipids, or is it possible the channel properties could be modified by the surface charge?

LABARCA: We don't have a precise answer to your question. The receptor does stick out of the *cis*-side of the membrane (against-binding side) by some 55Å and by ~15Å from the opposite side. The channel might be modified by surface charge in the lipid or more probably, by charge residues in the protein itself. In our hands the apparent K_d of saturation is probably affected by the concentration of calcium ions. There is also evidence that calcium modifies the receptor properties at end plates.

MONTAL: There are technical problems in using pure lipid compositions for reconstitution. Thus far, mixed lipid compositions must be used for optimal reconstitution activity.

DONOVAN: There are two classes of models we have discussed. One is derived from the physiology, where we talk of ligand binding and the subsequent opening of the channel; the other is derived from formal kinetic schemes, in which we try to fit parameters from electrical measurements. It is important not to blur the two. For example, using ligand binding rates, you stated that the double exponential closed times cannot be fit by a three state model; but if approached strictly from a formal kinetic model, a double exponential can easily be fit using a three state model. Similarly, the k_{-2} found by Liebowitz and Dionne may not necessarily be a ligand unbinding step.

DEFELICE: You can think of the flickering in one of two ways: returning to the closed state or going to a special closed state, as comes up often in voltage dependent channels. It is not clear what one means when one speaks of the simplest interpretation for fitting the data. Are there experiments that one could do which could distinguish between a closed-open-closed and a closed-closed-open model?

DIONNE: The formalism demands three states. We assume the initial binding states to be at equilibrium; in the low concentration limit this gives a C-C-O model which makes only slightly different predictions from a C-O-C model. There are some experiments that could distinguish between these schemes but they haven't been done.

MANNELLA: There is evidence that the receptor can exist in dimer form. Is there any correlation between the number of lifetimes that one sees in different systems and the concentration of receptors that you would expect there to be in the membrane, so that the difference in conductance might correlate with different aggregation states of the single channel that you're looking at?

MONTAL: We have carried out flux measurements on reconstituted vesicles in which we have incorporated exclusively monomers or exclusively dimers, and we see no differences (Anholt, R., J. Lindstrom, and M. Montal. 1980. *Eur. J. Biochem.* 109:481–487). We have not pursued the studies at the level of the single channel, but Hans Georg Schindler has done these measurements and found some differences between monomers and dimers.

MOCZYDLOWSKI: You mentioned that you've made measurements over several orders of magnitude of agonist concentration. Do you expect that the singly liganded closed state can open and could this be an explanation of the two-exponential open state distribution? Is there a change in the amplitude of the exponentials with a change in agonist concentration? What about the distribution of closed state lifetimes?

MONTAL: We are studying these questions systematically. We have not found any amplitude changes so far with a change in agonist concentration; the ratio of the amplitudes of the fast and slow components seems to be constant.