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THE ACETYLCHOLINE RECEPTOR CHANNEL FROM *TORPEDO CALIFORNICA* HAS TWO OPEN STATES[1]

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Abstract

We recorded single channel currents activated by agonist binding to purified acetylcholine receptors from the electric organ of *Torpedo californica* reconstituted in planar lipid bilayers. Analysis of single channel records indicate that the acetylcholine receptor channel displays two kinetically distinct open states which differ in their mean open times but have similar channel conductances.

The nicotinic acetylcholine (ACh) receptor is currently the best characterized neurotransmitter receptor. The availability of specific neurotoxins that act on the ACh receptor and of a rich tissue source for the biochemical isolation of the receptor protein has facilitated the detailed characterization of the structure of the ACh receptor primarily from *Torpedo* electric organ (for a recent review, see Anholt et al., 1983). Recently, a number of laboratories succeeded in cloning the genes coding for the various receptor subunits, and their entire amino acid sequence has been elucidated (Noda et al., 1982, 1983; Claudio et al., 1983; Devillers-Thiery et al., 1983). On the other hand, detailed electrophysiological information on the channel of the muscle ACh receptor is becoming available through patch recording (Neher and Sakmann, 1976; Sakmann et al., 1980; Colquhoun and Sakmann, 1981; Jackson et al., 1982, 1983); still, little is known about the electrophysiological properties of the electric organ ACh receptor (Moreau and Changeux, 1976; Schindler and Quast, 1980).

In recent years, the technology for the reconstitution of functional ACh receptors in lipid vesicles (Epstein and Racker, 1978; Changeux et al., 1979; Huganir et al., 1979; Lindstrom et al., 1980; Anholt et al., 1981, 1982; Walker et al., 1982) and in planar lipid bilayers (Nelson et al., 1980; Schindler and Quast, 1980; Boheim et al., 1981) has been developed in order to establish systems that allow investigation of structure-function correlates in the receptor molecule. These studies have contributed to our understanding of the molecular basis of postsynaptic transmission, by demonstrating that the $\alpha_2\beta\gamma\delta$ subunit structure of ACh receptor monomers contains the ACh-binding sites and the cation channel it regulates (Anholt et al., 1983). Purified ACh receptors, reconstituted in lipid vesicles and in planar bilayers, display activation and desensitization induced by cholinergic agonists (cf. Anholt et al., 1983). Here, we used the reconstituted membrane to study the gating kinetics of the purified *Torpedo* ACh receptor channel and found that it has two open states. Thus, single channel recordings of purified *Torpedo* ACh receptor provide new facets about the molecular mechanism of channel gating which, in principle, can be correlated with its molecular structure.

Materials and Methods

Experimental procedure

Receptor preparation. Receptor from the electric organ of *Torpedo californica* (Pacific Bio-Marine Laboratories, Inc., Venice, CA) was solubilized, purified, and reconstituted in lipid vesicles as described in detail previously (Lindstrom et al., 1980; Anholt et al., 1981, 1982). The reconstituted soybean lipid vesicles (40 mg/ml) used in

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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-induced $^{22}Na^+$ uptake (Huganir et al., 1979; Lindstrom et al., 1980) before the electrical measurements.

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. (1984). 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% (v/v) hexadecane in hexane or in choloroform: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 ACh receptors. About 30 membranes, obtained from nine different preparations of ACh receptor, were investigated. All experiments shown were performed at room temperature.

Electrical recordings and data processing. Membrane currents under voltage clamp were recorded as previously described (Montal and Mueller, 1972; Labarca et al., 1984). 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- or a 10-gigohm feedback resistor; the amplifier time constants were 120 and 250 μsec. Constant voltage was supplied by a variable DC source, and the *trans* side of the membrane was defined as zero voltage. Accordingly, a negative applied voltage corresponds to a depolarization in the electrophysiological convention. The output of the current to voltage converter was amplified and recorded on FM tape (RACAL 4DS, bandwidth DC to 2.5 or 5 kHz). The recordings were digitized at a sampling interval of 100 μsec for computer analysis. The cumulative open-state lifetime distributions were analyzed using a PDP 11/34 computer (Digital Equipment Corp., Marlboro, MA), as described in detail elsewhere (Labarca et al., 1984).

Results and Discussion

Figure 1*A* shows single channel currents recorded from a planar lipid bilayer containing purified ACh receptor activated by the high affinity agonist suberyldicholine. Single channel current records, where only one channel was open at any given time, were analyzed. Figure 1*B* displays a histogram of the distribution of channel open times derived from the record shown in Figure 1*A*. The distribution of open times does not follow a single exponential function, however, a sum of two exponentials does generate an adequate curve fit to the results. The double exponential distribution of channel lifetimes is a general feature of single channel currents activated by cholinergic agonists in planar lipid bilayers containing the purified ACh receptor, for ACh, suberyldicholine, or carbamylcholine (Fig. 1, *B* and *C*). The single events labeled *L* in Figure 1*A* have open times of ~25 msec. Considering that the time constant of the fast exponential component was 1.0 msec (Fig. 1*B*), it is estimated that 1 of $\sim 10^{11}$ fast events would have lifetimes equal to or longer than 25 msec, while ~1 of 10 of the longer-lived events (the slow component with time constant of 11 msec) would have open times ≥25 msec. The lifetime, τ_L, of the slow kinetic process is ~5 to 10 times longer than τ_S, that of the fast component (Fig. 1, *B* and *C*). The slow component amounts to ~30 to 50% of all openings (Fig. 1, *B* and *C*). However, this is only an apparent fraction since current bandwidth limitations restrict the resolution of events faster than 0.2 msec so that the intrinsic ratio fast/slow is larger than that measured. The conductances of both open states are similar (Fig. 1*A*).

There are at least three simple models which could account for the two exponential components in single ACh receptor channel open time distributions. First, it is conceivable that, in the course of an experiment, the open channel changes from a short to a long open time, or vice versa. Second, there could be two independent populations of ACh receptor. Third, the two components could arise from a homogenous population of receptors displaying two open states.

To test the notion of a time-dependent change in channel open time, experimental records with several hundred fluctuations were divided in three segments. Each period was analyzed and mean open times for each open state were derived. Table I shows the results of this analysis in three different experiments. Clearly, the lifetimes of both open states and their frequency of occurrence were constant throughout the record. Therefore, we conclude that the probability of occurrence of either open state is independent of time.

We turn now to consider whether the two open states are independent or not. Sakmann et al. (1980) observed that, in muscle, in the presence of desensitizing concentrations of agonist, single ACh receptor channel currents display a distinct pattern of activity, characterized by paroxysms of channel openings ("bursts") followed by quiescent periods. Similar results were obtained for the purified ACh receptor from *Torpedo* reconstituted in planar lipid bilayers (Fig. 2). Records displaying paroxysms of channel activity are particularly well suited to study the independence or interdependence of both open states: these contain both, short and long events, as illustrated in Figure 1*A*, and the distribution of open times within bursts is fitted by a double exponential function, as shown in Figure 1, *B* and *C*. During paroxysms of activity where both long and short events coexisted, double openings were not seen (fig. 2). 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. These results suggest that both open states are not independent.

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

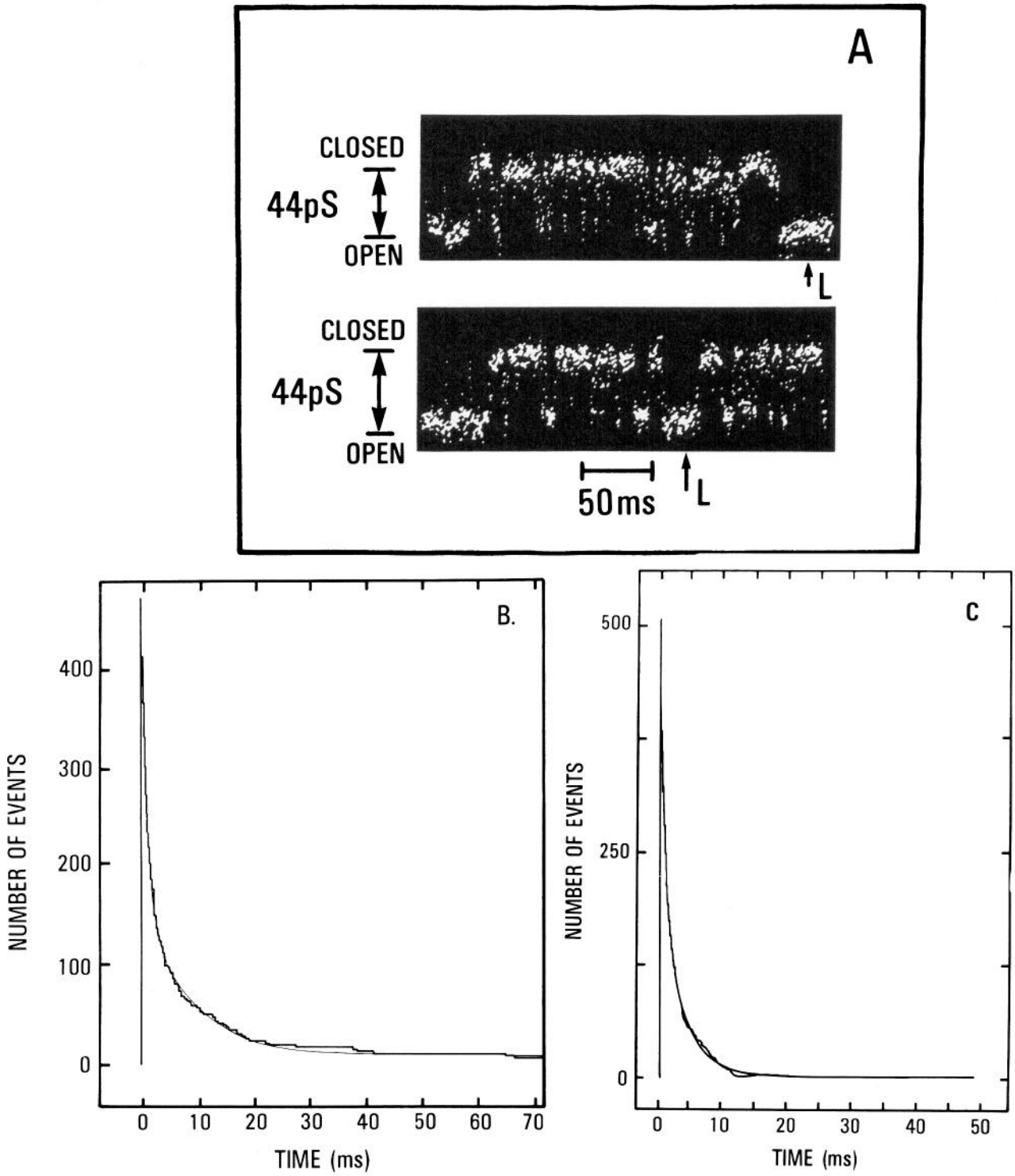

Figure 1. A, Single ACh receptor channel currents activated by 0.1 μM suberyldicholine and recorded at −150 mV. *A* is a computer-digitized signal from the bilayer amplifier sampled at 0.1-msec intervals after filtering at 3 kHz. Two contiguous sections of a record are displayed. The events labeled *L* correspond to long-lived openings. *B*, Frequency histogram of channel open times derived from the record in *A*. Computer-generated signals were used to assist in measuring the bilayer results: position and width of the generated rectangular pulses were checked for fidelity in matching the channel openings and closings, and these parameters were stored. After analysis of the data, cumulative open-state lifetime distributions of the generated rectangular pulses were automatically produced. Time constants were determined by fitting two computer-generated exponentials to the data points displayed on an oscilloscope screen. The fitted curve (smooth curve) was superimposed on the histogram of the actual data (noisy curve). In this experiment, the total number of channel openings that were anlyzed was 468. The zero time amplitude (A) and lifetime (τ) of the fast (S) and slow (L) components were $A_S = 395 \pm 7$ events, $\tau_S = 1.0 \pm 0.02$ msec, $A_L = 73 \pm 3$ events, $\tau_L = 11.2 \pm 0.5$ msec, respectively. Semilogarithmic plots of the data were fitted by similar parameter values (not shown). *C*, Frequency histogram of single ACh receptor channel open times activated by carbamylcholine (10 μM) and recorded at an applied voltage of −70 mV. All other conditions were as for *A* and *B*. The total number of events analyzed, *N*, was 508 and the parameters of the fitted curve were $A_S = 306 \pm 7$; $\tau_S = 0.7 \pm 0.05$ msec, and $A_L = 202 \pm 4$; $\tau_L = 3.5 \pm 0.2$ msec.

TABLE I

Single-channel currents activated by suberyldicholine (0.1 μM) in planar lipid bilayers containing purified ACh receptor

Experimental conditions were as in Figure 1*A* at the indicated applied voltage. Each recording is from a different experiment. The records were divided in three equal periods of 6 sec and the open-state lifetime distributions were analyzed as described in Figure 1*B*.

Recording No.	Period	A_S	τ_S (msec)	A_L	τ_L (msec)
I	1	302 ± 4	0.9 ± 0.02	70 ± 3	11.0 ± 0.6
V = −150 mV	2	278 ± 4	1.0 ± 0.03	95 ± 3	13.0 ± 0.5
	3	353 ± 4	1.1 ± 0.03	114 ± 4	11.4 ± 0.4
II	1	135 ± 2	5.0 ± 0.1	91 ± 4	33.0 ± 1.3
V = 70 mV	2	194 ± 2	4.5 ± 0.1	115 ± 6	35.0 ± 2.0
	3	179 ± 3	4.4 ± 0.1	106 ± 5	27.0 ± 1.0
III	1	72 ± 5	4.3 ± 0.1	60 ± 3	38.0 ± 1.0
V = 100 mV	2	102 ± 2	6.0 ± 0.2	90 ± 5	36.0 ± 2.0
	3	120 ± 4	4.0 ± 0.1	103 ± 4	33.1 ± 1.2

events represent two independent populations of ACh receptor, 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 four different experiments is shown in Figure 3. As seen, the results are in disagreement with the hypothesis of independent populations of ACh receptor: when the number of short openings per trial increases, the number of long openings also increases. This correlation implies that the presence of two open times is not the result of the activity of two independent populations of ACh receptor in planar bilayers. The results can be attributed most simply to the presence of one population of receptors having two distinct open states.

This analysis of single-channel currents of purified ACh receptors demonstrates that there are two kinetically distinct open states. The existence of two kinetic components in the distribution of channel open times has been observed for the ACh receptor in muscle cells (Colquhoun and Sakmann, 1981; Jackson et al., 1982, 1983). Recently, Jackson et al. (1983) analyzed the open-state lifetime distributions of single ACh receptor channel currents in muscle cells in culture in terms of two exponentials and postulated two open states for the receptor *in situ*. The analytical approach described here has been applied to study the muscle ACh receptor yielding results analogous to those described here (B. A. Suarez-Isla, P. Labarca, and M. Montal, unpublished results). The similarities between the results obtained in muscle and in reconstituted membranes strengthens the view that the ACh receptor channel, after purification and reconstitution, displays the features known of the functional receptor in biomembranes. Hence, the reconstituted system emerges as a unique and powerful tool to investigate the *Torpedo* ACh receptor channel in biophysical detail allowing, for the first time, structure-function correlations at the molecular level.

Figure 2. Single channel currents during paroxysms of channel activity. Single channel currents were activated by 0.1 μM suberyldicholine and recorded at a constant applied voltage of −100 mV. This record is displayed at low time resolution (compare time calibration with Fig. 1*A*) to illustrate the occurrence of paroxysms of channel activity in which only one channel was open at any given time.

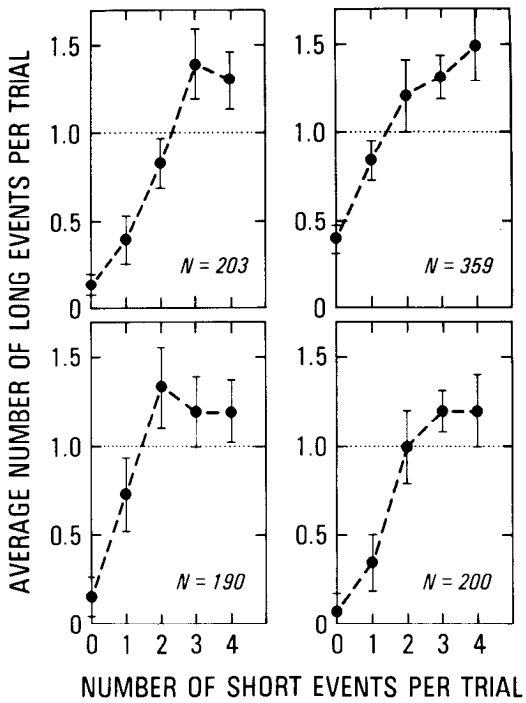

Figure 3. Average number of long events per trial as a function of the number of short events per trial. To distinguish short from long events, a time interval was chosen such that 96% of all short fluctuations would fall within this interval:

$$0.96 = 1 - \exp - T/\tau_S$$

All openings with lifetimes shorter than 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 $\geq T$. This criterion was adopted because it is simple, does not interfere with the independence test, and appears justified in view of the large difference in relaxation times of the fast and slow kinetic processes involved in channel closure. The results of four different experiments recorded at an applied voltage of +100 mV are shown. 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 (± SE) in trials in which 0, 1, 2 . . . short events occurred. The data were normalized to allow comparison among the different experiments. In each experiment 100 trials were analyzed. Trials were taken at random and were defined as time intervals of 200 msec. N denotes the total number of events computed in each experiment. The *dotted line* shows the region where the experimental data should lie if there were two independent populations of channels.

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