The Occurrence of Long Openings in the Purified Cholinergic Receptor Channel Increases with Acetylcholine Concentration¹

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Abstract

To establish the relationship between cholinergic ligand occupancy and channel open states, we recorded single-channel currents activated by different acetylcholine (ACh) concentrations from *Torpedo californica* ACh receptors reconstituted in lipid bilayers. Inspection of single-channel records shows that the frequency of occurrence of long openings increases with ACh concentration. Analysis of the probability distribution of open dwell times indicates that the ACh receptor channel has two distinct channel open states, short- and long-lived. The frequency of occurrence of the long openings over the short increased with ACh concentration, whereas the corresponding time constants were virtually unaltered. The extent of agonist occupancy at the ACh-binding sites in the purified cholinergic receptor appears, therefore, to correlate with an increased probability of the long-lived open state. These results are consistent with the notion that the two open-channel states arise from different extents of ligand occupancy at the receptor molecule.

Opening of acetylcholine (ACh) receptor channels requires the binding of cholinergic agonists to specific sites on the α -subunits (Rafferty et al., 1983; Changeux et al., 1984; Kao et al., 1984; Mishina et al., 1985). Relaxation and fluctuation analyses of agonist-activated currents at endplates were found to be consistent with kinetic schemes that considered the doubly liganded, active receptor molecule, undergoing a conformational change to form an open, cation-selective pore (Katz and Thesleff, 1957; Katz and Miledi, 1972; Magleby and Stevens, 1972a, b; Neher and Stevens, 1977; Dionne et al., 1978; cf. Colquhoun and Sakmann, 1983) according to scheme 1:

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where C represents closed-channel states, O represents open states, and L denotes a bound cholinergic ligand. Furthermore, selective occupation of one of the two ACh-binding sites by α -bungarotoxin (Sine and Taylor, 1980) or by the covalent alkylation with the affinity label [4-(N-maleimido)benzyl]trimethylammonium ion (MBTA) (cf. Karlin, 1980; Walker et al., 1984) drastically reduces the agonist-activated permeability response, suggesting that the probability of channel opening is greatest when two agonist molecules are bound per ACh receptor (cf. Dionne et al., 1978). These macroscopic measurements, however, cannot give a unique answer to the question of whether channel opening can proceed after binding a single agonist molecule.

To understand, on a molecular level, the mechanism by which ACh opens the ion channel, it is desirable to determine the time course of single opening events. The technology to record currents flowing through single, open ACh receptor channels (Neher and Sakmann, 1976) allows studies on the ACh receptor gating kinetics with a detail that is not accessible to macroscopic approaches. The initial measurements of single ACh receptor channels, using the patch clamp recording technique, were found to be consistent with equation 1, which determines that the distribution of open-channel durations be single exponential with the predicted time constant (the open state lifetime) (Neher and Sakmann, 1976). More recently, however, histograms of open dwell times were reported to be fitted best with a sum of two exponentials, suggesting the presence of more than one open state (Colquhoun and Sakmann, 1981, 1983; Jackson et al., 1983; Sine and Steinbach, 1984). Similarly, analysis of single-channel records of purified ACh receptor from *Torpedo californica*, reconstituted in planar lipid bilayers, revealed that the ACh receptor channel displays at least two kinetically distinct, open states (Suarez-Isla et al., 1983; Tank et al., 1983; Labarca et al., 1984a, b, 1985). It is clear that equation 1 cannot account for the single-channel results and is, therefore, insufficient to describe the kinetics of ACh receptor activation.

The origin and the nature of the two open-channel states in the ACh receptor are not established. Several kinetic schemes are currently being considered (Colquhoun and Sakmann, 1981, 1983; Jackson et al., 1983; Sine and Steinbach, 1984; Fredkin et al., 1985). A set of plausible models accounts for the occurrence of more than one open state on the basis of multiple ligand occupancy at the receptor. In some models, the open lifetimes vary with agonist concentration:

Others, predict that the frequency of occurrence of each open state is a function of agonist concentration:

Still a third class considers the existence of two doubly liganded open conformations, the durations and frequencies of which are independent of agonist concentration:

To gain more insight into the mechanisms underlying ACh receptor channel gating, we recorded single-channel currents activated by different ACh concentrations from *Torpedo californica* ACh receptors reconstituted in lipid bilayers. The frequency of occurrence of long opening events was found to increase with agonist concentration, whereas the corresponding time constants were not affected. Thus, the probability of occurrence of the two open-channel states correlates with the extent of agonist occupancy. This result is consistent with the notion that the two open-channel states arise from different liganded states of the receptor molecule.

Materials and Methods

ACh receptor preparation. Receptor from the electric organ of *Torpedo californica* (Pacific Bio-Marine Laboratories, Inc., Venice, CA) was solubilized, purified, reconstituted in lipid vesicles, and assayed as described in detail elsewhere (Lindstrom et al., 1980; Anholt et al., 1981, 1982). The reconstituted soybean lipid vesicles (32 mg/ml) were supplemented with cholesterol (8 mg/ml); the ACh receptor concentration ranged between 0.2 and 1.0 μM .

Planar lipid bilayers. Monolayers at the air-water interface were derived from suspensions of reconstituted vesicles (Schindler, 1980) as described previously (Nelson et al., 1980; Labarca et al., 1984a, b). The vesicles were diluted 6-fold in the bilayer chamber. Planar bilayers were assembled from two such monolayers at the tip of patch pipettes (Suarez-Isla et al., 1983; Montal et al., 1984; Labarca et al., 1985). The aqueous medium was 0.5 M NaCl, 5 mM CaCl₂, 2.5 mM HEPES or Tricine (*N*-[tris(hydroxymethyl)methyl]glycine), pH 7.4, and 50 μM DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid). At this DIDS concentration, the *Torpedo* electroplax chloride channel is blocked (Miller and White, 1984). The ACh receptor channel was activated with ACh present inside the pipette at the indicated concentration. All experiments were performed at room temperature.

Electrical recordings. Single-channel currents were recorded at 100 mV as described in detail elsewhere (Suarez-Isla et al., 1983). The List L/M EPC-5 and EPC-7 patch-clamp systems were used (List Medical Electronics, Darmstadt, Federal Republic of Germany) (Hamill et al., 1981). Constant voltage was supplied by a DC source (Omnicol 2001, WPI Instruments, New Haven, CT). Ag/AgCl pellet electrodes were used (E255 from In Vivo Metric Systems, Healdsburg, CA). The *trans*-electrode (opposite to the pipette electrode) was held at virtual ground. The EPC-output was recorded on FM tape (RACAL 4DS, bandwidth DC to 5 kHz; Racal Recorders Ltd., Hythe, Southampton, England).

Data processing. All records were filtered at 2 kHz on an 8-pole Bessel low-pass filter (Frequency Devices, 902LPF, Haverhill, MA). The data were digitized at a sampling rate of 10 kHz in a PDP 11/34A computer (Digital Equipment Corp., Marlboro, MA). Conductance levels were discriminated as described in detail elsewhere (Labarca et al., 1984a). Records in which only one channel was open at any given time were analyzed. Histograms of dwell times in the open state of the ACh receptor channel, normalized to have a total area = 1, were fitted by a probability density function of the form $N(t) = A_S \exp(-t/\tau_S) + A_L \exp(-t/\tau_L)$, giving p values ≥ 0.5 (Labarca et al., 1985). The areas under the fitted probability density function, A_S and A_L , and the time constants of the fit τ_S and τ_L , were calculated by a χ^2 minimization algorithm (Fletcher, 1971; Labarca et al., 1985). S and L refer to the short and long kinetic processes; N is the total number of events analyzed. The results of at least six different experiments at each ACh concentration are presented.

Results and Discussion

Figure 1 shows single-channel currents recorded from planar lipid bilayers containing purified ACh receptor activated by either 0.1 μM ACh (Fig. 1, left) or 10 μM ACh (Fig. 1, right). At high agonist concentrations (10 μM), periods of channel activity are characterized by an increased frequency of channel opening, whereas at lower concentrations (0.1 μM), openings are infrequent. Here, we focus on the effect of ACh concentration on the frequency of occurrence and time constants of the short and long components of the probability distribution of open dwell times. We measured the number of openings with lifetimes shorter than or equal to 1 msec and with lifetimes in the range of $1.1 \leq \Delta t \leq 10$ msec at different ACh concentrations. The results summarized in Figure 2 clearly show that the number of openings per second increases with ACh concentration and that, at 10 μM ACh, this increased probability of channel opening is reflected primarily in the appearance of more long openings. This analysis leads us to conclude that the frequency of occurrence of long openings increases with agonist concentration.

We turn now to describe the dependence of the parameters of the exponential components of the probability distribution of open dwell times on agonist concentration (Labarca et al., 1984a, b). The probability density of open dwell times is well described by a sum of two exponentials at agonist concentrations, in the range $0.01 \mu\text{M} \leq \text{ACh} \leq 0.1 \text{ mM}$. Figure 3 illustrates this analysis for low (0.1 μM) and high (10 μM) ACh. Note that the time constants, τ , for the short (S) and the long (L) components of the distribution at the two ACh concentrations are similar. In contrast, the normalized area A , under the fitted probability density, of the long component increases from 0.1 at 0.1 μM ACh to 0.27 at 10 μM ACh. The results of this study performed at ACh concentrations spanning several orders of mag-

Figure 1. Single ACh receptor channel currents activated by 0.1 μM ACh (left) and 10 μM ACh (right) and recorded at an applied voltage of -100 mV. As indicated, a downward deflection corresponds to a channel-opening event. The single-channel conductance was 45 pS.

Figure 2. ACh concentration dependence of the frequency of channel opening. Single ACh receptor channel currents were activated by ACh at the indicated concentration. Records 10 to 30 sec long were analyzed. To build the histograms, all openings with lifetimes shorter than or equal to 1 msec were distinguished from openings with lifetimes in the range $1.1 \leq \Delta t \leq 10$ msec. The number of openings in these two categories was counted and normalized to unit time to allow comparison among the different experiments. This criterion was adopted because it is simple and appears justified in view of the large difference in time constants of the short and the long kinetic processes involved in channel closure; the accuracy, however, is limited (cf. Jackson et al., 1983). A total of 6733 openings were measured for this analysis. The applied voltage was 100 mV.

Figure 3. Probability density analysis of dwell times in the open state of the ACh receptor channel activated by 0.1 and 10 μM ACh. The data were well fitted by a sum of two exponentials, adopting a 5% significance level ($p \geq 0.05$; see Labarca et al., 1985). The applied voltage was 100 mV. The fitted curve (smooth curve) was superimposed on the histogram of the actual data (noisy curve). The amplitudes of the two histograms were normalized for comparison purposes. The parameters of the fitted curves were at 0.1 μM : $A_S = 0.92$, $\tau_S = 0.42$ msec; $A_L = 0.08$, $\tau_L = 4.30$ msec, and the total number of events analyzed, N , was 967. At 10 μM ACh: $A_S = 0.73$, $\tau_S = 0.63$ msec; $A_L = 0.27$, $\tau_L = 5.16$ msec; N was 3371.

nitude are summarized in Figure 4. As seen, the A_L/A_S ratio increases with the concentration of ACh (Fig. 4A). Figure 4, B and C, shows that the corresponding time constants, τ_S and τ_L , of the short and long kinetic processes involved in channel closure are not altered

Figure 4. Probability density analysis of dwell times in the open state of the ACh receptor channel as a function of ACh concentration. A total of 26,943 openings were analyzed. The applied voltage was 100 mV. The log of the ratio of the areas under the fitted probability densities of the long and short components (A_L/A_S) versus the log of ACh concentration is shown in A. The dependence of the time constants τ_S and τ_L on the concentration of ACh is illustrated in B and C. The dashed lines show the mean values $\pm$ SD of τ_S (0.50 ± 0.06 msec) and τ_L (3.61 ± 0.42 msec).

systematically by agonist concentration. This is in agreement with results obtained using carbamylcholine as the cholinergic ligand (Labarca et al., 1984a). The data, therefore, show that the frequency of occurrence of the long openings over the short increased with ACh, whereas their corresponding lifetimes were unaffected. D. Colquhoun and B. Sakmann (personal communication) have obtained similar results on adult muscle fibers.

These results allow one, in principle, to reject a set of kinetic schemes which predict either that the A_L/A_S ratio is a constant or that the time constants vary with agonist concentration (Jackson et al., 1983; Fredkin et al., 1985). A minimum kinetic scheme that is consistent with the experimental data is

This scheme is compatible with the autocorrelation analysis which showed that there are at least two pathways via which the closed and open states communicate (Labarca et al., 1985), and with the independence of both τ_S and τ_L on agonist concentration (Fig. 4, B and C), implying that liganding is to closed states. Such a model predicts a dependence of the A_L/A_S ratio on agonist concentration, as observed experimentally (Fig. 4A). Furthermore, the predicted form of this functional dependence, when plotted as the $\log A_L/A_S$ versus $\log [ACh]$, should be linear with unitary slope. The function displayed in Figure 4A indeed shows a unitary slope but one

restricted to the range of ACh concentrations between 0.01 and 0.1 μM . At higher concentrations of ACh, the A_L/A_S ratio approaches a constant value. Such behavior does not appear to arise from channel blocking by the agonist (Colquhoun and Sakmann, 1981) since the τ values calculated from the distribution of open times are independent of ACh concentration, within experimental error. Formally, this deviation could be accounted for by introducing an additional, short-lived, doubly liganded open state to the right of CL_2 in equation 5. Models equivalent to equation 5, consisting of multiple open states with higher extents of ligand occupancy or with a more complex closed states structure, could also account for the experimental results. We do not intend to propose here a specific reaction mechanism but to describe the fundamental fact that agonist binding in purified ACh receptors controls the occurrence of different open conformations. The simplest inference derived from our observations is that singly and doubly liganded states of the receptor molecule undergo transitions to the open conformation.

It is worth noting that the ACh concentration dependence of the permeability response, assayed in the same reconstituted vesicles from which the planar bilayers are derived, showed that the fraction of desensitized ACh receptors increased from ~50% at 0.01 μM ACh to 100% at 0.1 μM ACh (Anholt et al., 1982). This is the range of ACh concentrations at which the A_L/A_S ratio is most sensitive to the concentration of ACh. Thus, an equivalent model with a more complex scheme of closed states may be relevant.

What possible physiological significance do the findings of this study have on synaptic transmission? It is known that, in addition to the quantal release of ACh, from mononeurons, there is a continuous leak of ACh from the presynaptic terminal (Katz and Miledi, 1977). This could determine whether ACh receptors at the neuromuscular junction sense an ambient concentration of ACh above or below 0.1 μM . Thus, a nerve could exquisitely regulate the responsiveness of the muscle even to the level of the precise distribution of open channel lifetimes.

Recent biochemical and electrophysiological studies reveal that the mechanism of ACh receptor channel activation is a more complex process than previously realized (Magleby and Stevens, 1972a, b). Such a notion appears valid for other neurotransmitter-activated channels (Gration et al., 1981, 1982; Cull-Candy and Parker, 1982; Cull-Candy, 1983). A selection of findings that complicate the analysis includes: the ACh receptor molecule has two nonequivalent agonist-binding sites (Damle and Karlin, 1978; Kao et al., 1984; Mishina et al., 1985). Blocking of one site precludes the agonist ability to open the receptor channel (Culver et al., 1984; Young et al., 1984). Channel opening can occur spontaneously in the absence of agonist (Jackson, 1984) or can even be induced by a "classic" antagonist such as curare (Trautmann, 1982; Morris et al., 1983). There is evidence for the existence of multiple binding sites for cholinergic ligands and for noncompetitive inhibitors (cf. Raftery et al., 1983; Changeux et al., 1984). The ACh receptor channel has two kinetically distinct, open states (Colquhoun and Sakmann, 1981; Jackson et al., 1983; Suarez-Isla et al., 1983; Tank et al., 1983; Labarca et al., 1984a, b, 1985; Sine and Steinbach, 1984). Here we show that, in purified receptors, these two open states correlate with the extent of agonist occupancy. Knowledge of the molecular anatomy of the ACh receptor combined with biochemical, genetic, and immunological modifications and the electrophysiological measurements in reconstituted receptors should prove advantageous in pursuing the understanding of how ligand binding controls the gating of this channel protein.

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