# KINETICS OF UNLIGANDED ACETYLCHOLINE RECEPTOR CHANNEL GATING

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ABSTRACT Open- and closed-state lifetimes of unliganded acetylcholine receptor channel activity were analyzed by the method of likelihood maximazation. For both open times and closed times, the best-fitting density is most often a sum of two exponentials. These multiple open states cannot depend on the number of receptor binding sites occupied since they are observed in the absence of ligand. The rate of spontaneous opening and the faster decay constant of closing increased as the membrane was hyperpolarized. The voltage dependence of the rate of spontaneous opening is stronger than that for curare-liganded channels. Evidence that the acetylcholine receptor channel can open spontaneously in the absence of ligand has been presented previously (Sanchez et al, 1983; Brehm et al, 1984; Jackson, 1984). To add to this evidence, $\alpha$-bungarotoxin was added to the patch electrode, causing the frequency of openings to decay with time. The rate constant determined from this decay is similar to rate constants reported for the binding of iodinated $\alpha$-bungarotoxin to the acetylcholine receptor. The frequency of unliganded channel opening has been estimated as $2 \times 10^{-3}$ $s^{-1}$ per receptor. A comparison of carbamylcholine-liganded and spontaneous gating transition rates suggests that ligand binding increases the rate of opening by a factor of $1.4 \times 10^7$. Carbamylcholine binding increases the mean open time by a factor of 5. Thus, a cholinergic agonist activates the acetylcholine receptor by destabilizing the closed state. The liganded and unliganded channel gating rates were used to analyze the energetics of ligand activation of the acetylcholine receptor channel, and to relate the open channel dissociation constant to the closed channel dissociation constant.

## INTRODUCTION

Recently, patch-clamp techniques have revealed spontaneous openings of the acetylcholine receptor (AChR) channel in cultured muscle from rat (Sanchez et al., 1983), mouse (Jackson, 1984), and *Xenopus* (Brehm et al., 1984). Several lines of evidence suggest that the observed spontaneous openings are from the AChR. Spontaneous channel currents were measured as a function of voltage and compared with channel currents activated by the cholinergic ligand suberyldicholine. In solutions with two different concentrations of either NaCl or CsCl, the channel conductances of spontaneous openings and ligand-gated openings were always within 1.6 pS (Jackson, 1984). These ionic manipulations caused the single channel conductance to vary from 23 to 37 pS, with the conductances of spontaneous and ligand-gated channel currents varying in parallel.

Treatment of mouse muscle cultures with 50 nM $\alpha$-bungarotoxin for 1 h reduced the frequency of spontaneous openings by nearly 100-fold (Jackson, 1984). $\alpha$-Bungarotoxin also blocked the spontaneous AChR channel openings in *Xenopus* muscle (Brehm et al., 1984).

The spontaneous openings were not blocked by a chemical modification of a group near the ligand-binding site (Jackson, 1984). This modification entailed the reduction of a labile disulfide bond by dithiothreitol to expose a sulfhydryl group, which was then alkylated by *N*-ethylmaleimide. This treatment is very effective in blocking the response of the AChR to ligands (Karlin, 1980), and reduces the frequency of ligand-gated openings seen in patch-clamp recordings (Jackson, 1984). Thus, the spontaneous openings of the AChR are not caused by a contaminating or endogenous ligand.

Previous analysis of ligand-gated single channel currents has revealed exceedingly complex kinetic behavior in the gating of the AChR channel. Inferring from the number of exponential components in lifetime distributions (Colquhoun and Hawkes, 1981), there appear to be at least two open channel states (Colquhoun and Sakmann, 1981, 1983; Jackson et al., 1982, 1983; Auerbach and Sachs, 1983; Morris et al., 1983; Labarca et al., 1984, 1985; Takeda and Trautmann, 1984; Sine and Steinbach, 1984, 1985*a*, *b*) and as many as five closed states (Colquhoun and Sakmann, 1983; Sine and Steinbach, 1984, 1985*b*). Based on an analysis of correlations in successive open-state lifetimes, at least two closed states can open directly (Jackson et al., 1983; Labarca et al., 1984, 1985).

In many proposed models of AChR channel gating, differences in receptor occupancy are presumed to account for some of the different states detected by single channel

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analysis. Two different open states are hypothesized to be openings of singly and doubly liganded receptors (Colquhoun and Sakmann, 1981; Takeda and Trautmann, 1984). Brief-duration closures are thought to be closures of liganded receptor channels (Colquhoun and Sakmann, 1981; Dionne and Liebowitz, 1982).

The observation of spontaneous openings of the AChR channel offers a new approach to the study of chemically activated channels. The channel-gating process can now be isolated from the ligand-binding process. This makes it possible to ask whether ligand binding is essential to the existence of different channel conformations. The rates of the channel-gating transitions can be measured with and without ligand to assess the effect of ligand binding on the open-closed equilibrium of the channel. In this way, the AChR can be analyzed as an allosteric receptor protein in the context of well-known theories (Monod et al., 1965; Karlin, 1967). In this study, the kinetics of unliganded channel gating have been analyzed and compared with the kinetics of ligand-induced gating of the AChR to provide some insight into the nature of the different channel conductance states and the energetics of ligand activation of the AChR channel. A preliminary account of some of this work has been presented (Jackson, 1985).

## METHODS

Cultured embryonic mouse skeletal muscle was bathed in physiological saline (140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES or 1 mM $Na_2HPO_4$), pH 7.4, at room temperature (21–27°C). Gigohm-seal patch-clamp recordings were made (Hamill et al., 1981) with Q-dope–coated alumina silicate or thick-walled borosilicate (Sakmann and Neher, 1983) electrodes. All recordings were made in the on-cell configuration with either an EPC-5 or an EPC-7 patch-clamp amplifier (List Electronics/Medical Systems, Great Neck, NY). The membrane potential was varied by clamping the voltage inside the patch electrode.

Patch electrodes were filled with saline without glucose and with CsCl replacing NaCl. Since the AChR channel has a higher conductance in Cs, whereas most other channels have a lower conductance in Cs, this procedure has the consequence of increasing the AChR channel current and minimizing the interference of other channel currents. Cholinergic ligands were omitted from the patch electrode for the study of spontaneous openings. For the study of ligand-gated openings, (+)tubocurarine (curare) (10–20 $\mu$M) or carbamylcholine (1–10 $\mu$M) was added. In the study of ligand-gated openings, higher-resistance electrodes allowed the use of relatively high ligand concentrations. This minimized interference from spontaneous openings.

Current was sampled with an analog-to-digital converter (DT2782-A; Data Translation, Marlboro, MA) into an LSI 11/23 computer (Digital Equipment Corporation, Maynard, MA). In most experiments, discrete 1,024-point records were collected with an event triggered program as described previously (Jackson, 1984). Current was sampled at a frequency of 20–50 kHz after filtering at 3–5 kHz with an eight-pole low-pass Bessel filter. The time between separate records was not saved in this mode of data collection.

Continuous sampling of data was necessary to obtain closed-time distributions. A double-buffered sampling program was used to sample continuously at either 20 or 40 kHz (after filtering at 2–5 kHz). Examination of a triangular test signal showed that sampling was continuous except for a 60-$\mu$s gap between buffers that was impossible to eliminate because of the CPU time needed to restart the analog-to-digital converter. Because of the limited storage space, continuous sampling was usually used when spontaneous openings occurred at a high frequency.

Open times, closed times, and current amplitudes were tabulated from the data records. The method of likelihood maximization (Horn and Lange, 1983; Colquhoun and Sigworth, 1983) was used to fit open times and closed times to the following expression:

$$p(t) = \frac{\sum_{i=1}^{N} \lambda_i x_i e^{-\lambda_i t}}{\sum_{i=1}^{N} x_i (e^{-\lambda_i t_L} - e^{-\lambda_i t_H})}.$$

The numerator is the assumed probability density function. The denominator is a correction for the limited range of time intervals (Colquhoun and Sigworth, 1983). $\lambda_i$ and $x_i$ denote the decay constant and the fraction of total density, respectively, of the $i$th component. $t_L$ and $t_H$ defined the range of times used in the curve fit. $i = 1$ was used for the fastest component, with increasing $i$ for more slowly decaying components. $N$, the number of exponentials, was varied from 1 to 4. The number of open times or closed times used in each fit was sometimes >1,000, but was usually 250–600.

The negative logarithm of the likelihood was minimized with a computer program developed by Dr. Kenneth Lange, Dept. of Biomathematics, UCLA. This program uses a variable metric method (Powell, 1978) to search the likelihood surface. The program allowed linear constraints to be imposed; the parameters were constrained to normalize the density function.

A likelihood ratio test (Rao, 1973) was used to compare fits to sums of different numbers of exponentials. Only when the likelihood ratio test gave a probability of <0.05 for the model with one fewer exponential were the parameters obtained from a curve fit taken as significant.

$t_L$ was a short-time cutoff reflecting the fact that brief events cannot be detected in a signal that emerges from a low-pass filter. $t_L$ was of the form $(n - 1/2)s$ (where $n$ is an integer and $s$ is the sampling interval), and was initially estimated from the time at which the density plot was visibly attenuated. Different values of $t_L$ greater than the initial estimate (typically 100–200 $\mu$s) were used in curve fits, and the parameters obtained were compared. In a few instances, fits to a data set gave parameters that varied for different choices of $t_L$. These data sets were not used in further analysis. $t_H$, the long-time cutoff, was usually unimportant because the record size was much longer than all but a few open times.

The frequency of channel openings was measured in two different ways. In one method, the event-driven sampling program was modified to store immediately any event that triggered the computer. The computer logged the time in which a specified number of events was recorded. Events were later examined and the number of channel currents was divided by the time to give the frequency. For a more quantitative measurement, records saved in the event-triggered mode were analyzed with the Poisson distribution, whereby the numbers of records with one, two, three, or more events could be used to estimate the frequency of events. Using a conditional Poisson distribution to account for the lack of knowledge of the frequency of empty records, and applying likelihood maximization, the relation $\rho N = M(1 - e^{\rho})$ was derived, where $N$ is the number of records, $M$ is the number of events, and $\rho$ is the estimated mean number of openings per record. This expression was solved numerically for $\rho$. Because of a small component of brief closures (see Results), events preceded within 0.5 ms by another event were not included in this type of analysis. This omission reduced the contribution of the fast component of closed times to 1% of all closed times, so that the effect of brief closures on the estimate of the frequency of opening was negligible.

## RESULTS

### Open-Time Densities

Open times were determined from channel currents such as the spontaneous channel currents displayed in Fig. 1.

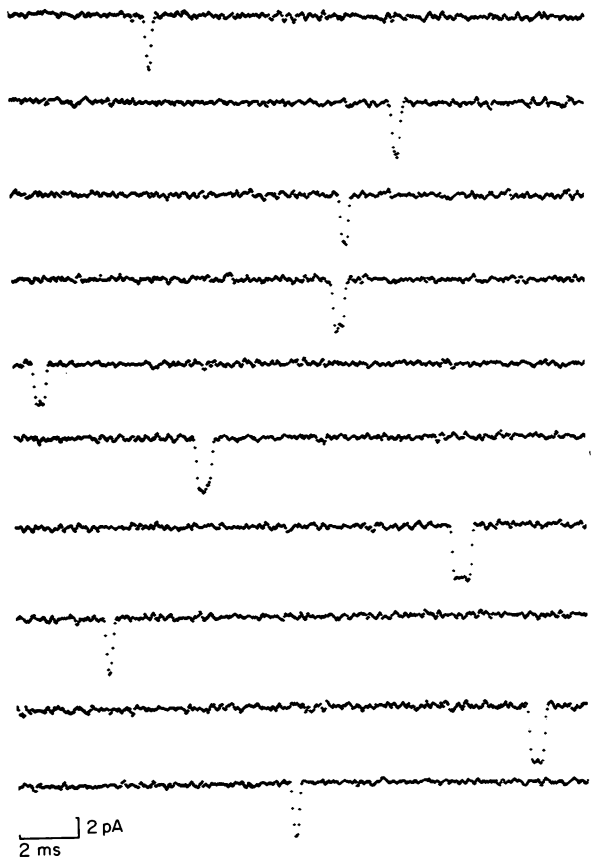



FIGURE 1 Spontaneous AChR channel currents recorded on-cell from cultured muscle with the patch electrode potential held at +50 mV. Current was sampled at 20-μs intervals at a recording bandwidth of 3 kHz. Every other point is displayed.

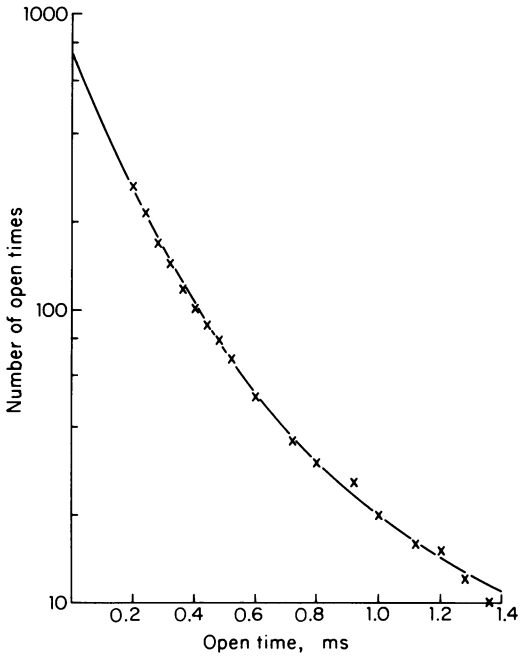



FIGURE 2 The open-time distribution of spontaneous AChR channel currents is plotted semilogarithmically. As in Fig. 1, the patch electrode potential was held at +50 mV. The ×'s denote the number of open times longer than $t$, plotted as a function of $t$. The smooth curve is the integrated best-fitting probability density function (sum of two exponentials). The average values for the decay constants and the fraction of total density are given in Table I.

Most unliganded channel open-time data were better fit by a sum of two exponentials than by a single exponential. A comparison of the two models with the likelihood ratio test indicated that for 81% of the data sets the probability was <0.05 for the single exponential vs. the sum of two exponentials. In most of these cases, the probability was <0.001. The data sets for which fits were not significantly improved by a sum of two exponentials usually had fewer events. Fig. 2 displays a typical set of open times as a tail distribution, with the integrated best-fitting sum of two exponentials drawn through the experimental points.

In a few experiments, unliganded AChR channel open times were best fit by a sum of three exponentials. In these experiments, a population of channel openings with very long open times appeared. This represented a change from the usual spontaneous gating behavior. The nature of these long-duration channel openings and the factors that govern their appearance require further study. Variations in liganded AChR channel gating kinetics have been noted before (Jackson et al., 1982; Sine and Steinbach, 1984; Labarca et al., 1985). The variability in unliganded channel kinetics is most similar to that reported by Labarca et al. (1985), who noted the occasional appearance of a third, slowly decaying component of ligand-gated openings.

Since AChR channels in muscle cultures are known to have open states with conductances that differ by ~30–50% (Hamill and Sakmann, 1981; Trautmann, 1982; Jackson et al., 1983), the single channel current was examined as a function of open time (Fig. 3). Fig. 3 shows that each of the two exponential components usually seen in unliganded openings has essentially the same single channel current. Small differences of ≤2% cannot be ruled out, but if the two open states seen here have different conductances, the differences are much smaller than those

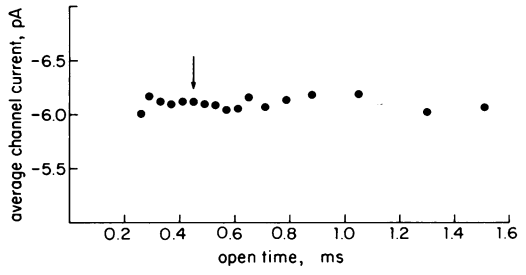



FIGURE 3 Currents of spontaneous AChR channel openings for a small range of open times were averaged and plotted as a function of open time. The patch electrode potential was held at +50 mV. Because channel currents with longer open-state lifetimes occur less frequently, larger time intervals were used to compute the averages. The arrow denotes an inflection in the best-fitting probability density function for which the two exponential terms were equal.

TABLE I
KINETIC PARAMETERS FOR CHANNEL OPEN TIMES

| | $N$ | $\lambda_1$ $\times 10^3\ s^{-1}$ | $\lambda_2$ $\times 10^3\ s^{-1}$ | $x_1$ | $t_o$ *ms* |
|---|---|---|---|---|---|
| Spontaneous | 22 | 11.0 ± 1.4 | 2.03 ± 0.16 | 0.83 ± 0.02 | 0.19 |
| Curare | 6 | 10.8 ± 3.5 | 1.4 ± 0.3 | 0.78 ± 0.03 | 0.30 |
| Carbamylcholine | 3 | 4.0 ± 0.1 | 0.4 ± 0.2 (0.77) | 0.40 ± 0.09 | 2.4 (1.0) |

Kinetic parameters were estimated by fitting open times to a sum of two exponentials as described in the text. $\lambda_1$ denotes the fast decay constant, $\lambda_2$ denotes the slow decay constant, and $x_1$ denotes the fraction of total density in the fast component. In all measurements used to prepare this table, the patch electrode potential was +50 mV. The numbers presented are means ± standard errors. The mean open times, $t_o$, were determined for each data set by integrating the best fitting density functions over $t > 0$. The numbers in parentheses after $\lambda_2$ and $t_o$ for carbamylcholine-gated events were obtained by using the closed-time density to correct for missed brief closures (see text).

already reported between different AChR channel conductance states.

Curare-activated and carbamylcholine-activated channel open times were also best fit by a sum of two exponentials. Kinetic parameters for unliganded, curare-activated, and carbamylcholine-activated channel open times were estimated from fits to a sum of two exponentials. The average values are given in Table I for a patch electrode potential of +50 mV.

With carbamylcholine-activated channels, a significant number of brief closures were missed because of the recording bandwidth. This means that closing rates were faster and mean open times were shorter than they appeared. To correct for this, the number of missed closures was taken as the area under best-fitting closed time density (see below), between 0 and $t_L$. The apparent mean open time was then corrected by multiplying by $N_o/(N_o + N_m)$, where $N_o$ is the number of observed closures and $N_m$ is the number of missed closures, to give the true mean open time. This corrected value is given in parentheses after the apparent mean open time in Table I. The same correction was made for the slow decay constant from carbamylcholine-gated open times. Recent analysis suggests that brief closures are associated with long-duration openings (Sine and Steinbach, 1986*b*; Jackson, 1986). The corrected value of $\lambda_2$ given in parentheses in Table I was obtained by assuming that all brief closures were associated with long-duration openings. It is therefore an upper bound.

The fast decay constant and the fraction of total density of this component are quite similar for unliganded channel openings and curare-activated channel openings. $\lambda_2$, the slower decay constant, is significantly slower for curare. The parameters for carbamylcholine all differ from either curare-gated or spontaneous openings in the direction of longer open times. Mean open times were computed from the parameters by integrating the density functions over $t > 0$ (Table 1). Mean open times for spontaneous openings, curare-gated openings, and carbamylcholine-gated openings are in the ratio 1:1.5:5.

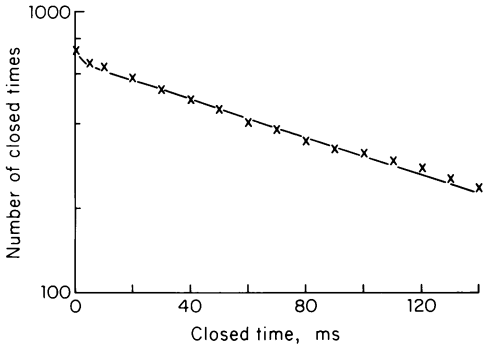



FIGURE 4 The closed-time distribution of spontaneous AChR channel currents is plotted semilogarithmically. The patch electrode potential was held at +50 mV. The ×'s denote the number of closed times longer than $t$, plotted as a function of $t$. The smooth curve is the integrated best-fitting probability density function (sum of two exponentials). The figure shows only one point (near $t = 0$) that deviates from the slowest single exponential, but a likelihood ratio test indicates $P < 0.001$ for a single-exponential probability density. The average parameter values for five curve fits were $\lambda_1 = 4.1 \pm 0.7 \times 10^3\ s^{-1}$, $\lambda_2 = 7 \pm 3\ s^{-1}$, $x_1 = 0.14 \pm 0.05$ (mean ± standard error). In two data sets that were adequately fit by only a single exponential, it was assumed that $x_1 = 0$ (see text). Including these in the average gives $x_1 = 0.10 \pm 0.04$.

## Closed-Time Densities

In continuously sampled data, an analysis of closed times between spontaneous openings was possible. Fig. 4 displays a closed-time distribution and the best-fitting sum of two exponentials. Although the fast component of closed times is barely evident in the distribution, it is reproducible and statistically significant according to the likelihood ratio test. In five of seven continuously sampled records of spontaneous AChR channel openings (two with the patch electrode potential held at +50 mV and three at +100 mV), closed times gave significantly improved fits ($P < 0.05$) to a sum of two exponentials as compared with a single exponential (Fig. 4). For three data sets, $P$ was <0.001. The parameters were averaged for the five experiments and are presented in the legend of Fig. 4.

The fast component accounts for a small fraction ($x_1 = 0.14$ from the legend of Fig. 4) of all closed times. In the two experiments where the sum of two exponentials did not significantly improve the fit to the data, there was no detectable fast component of closed times. Assuming that it was zero in these two data sets, the average value of $x_1$

falls to 0.10 ± 0.04 (mean ± standard error). The larger standard errors in $x_1$ and the slow decay constant, $\lambda_2$, probably reflect the variability in the number of AChR in a patch of membrane from one experiment to the next.

Closed times shorter than 10 ms for carbamylcholine-activated channels were fit to a sum of two exponentials. A fast component of closed times with a decay constant of $4.0 \pm 0.1 \times 10^4\ s^{-1}$ (mean ± standard error) accounted for an average of 71% of all closures. This fast decay constant is ~10 times faster than the fast decay constant obtained from closed times between unliganded channel openings. The average decay constant of the slow component of closed times for carbamylcholine was $0.3 \pm 0.2 \times 10^2\ s^{-1}$ (mean ± standard error).

The rate of opening of a liganded receptor channel, $\beta_L$, and the rate of ligand dissociation, $k_{-2}$, have been estimated from an analysis of closed times by the method of Colquhoun and Sakmann (1981, 1983) (their notation is used). This method proceeds from a sequential model of channel gating in which ligand binding is followed by channel opening. Applying this method to data obtained in this study for carbamylcholine gives $\beta_L = 2.8 \pm 0.3 \times 10^4\ s^{-1}$, and $2k_{-2} = 1.2 \pm 0.3 \times 10^4\ s^{-1}$ (mean ± standard error). These values are within the range of values estimated for other agonists (Colquhoun and Sakmann, 1981, 1983).

## Frequency of Opening

The frequency of spontaneous AChR channel openings was determined by Poisson analysis in 19 experiments to give an average value 4.3 ± 1.3 $s^{-1}$ mean ± standard error). In each of these 19 experiments, enough events were recorded to fit open times to a sum of two exponentials. The parameters from the curve fits were used to correct each measurement of opening frequency for events that were too brief to detect. Typically 20–50% of all openings were detected. The corrected frequencies were multiplied by the patch electrode resistance to normalize all frequencies to equivalent electrode tip sizes. The average of this product was 23 ± 6 $s^{-1}$ MΩ (mean ± standard error). Although this number may seem high, the correction for bandwidth and the standardization to a 1-MΩ patch electrode both increased its value. This number will be used in the Discussion to estimate an absolute rate of opening of an unliganded AChR channel.

Previous work (Brehm et al., 1984; Jackson, 1984) has shown that treatment of cultures with α-bungarotoxin before recording reduces the frequency of spontaneous AChR channel opening. To show this effect in a single patch, some patch electrodes were filled with solutions of α-bungarotoxin in saline. To prevent α-bungarotoxin from sticking to the glass walls of the electrode, the glass capillaries were siliconized with Sigmacote (Sigma Chemical Co., St. Louis, MO) before electrode fabrication. In two experiments with 20 nM α-bungarotoxin and one experiment with 25 nM α-bungarotoxin, the frequency of opening decayed with time (Fig. 5 *A*). The average bimolecular rate constant determined from the three experiments was $4 \pm 2 \times 10^6\ M^{-1}\ min^{-1}$ (mean ± standard error). Rate constants ranging from 1.3 to $5 \times 10^6\ M^{-1}\ min^{-1}$ have been reported previously for the binding of iodinated toxin to rat muscle AChR (Colquhoun and Rang, 1976; Darveniza et al., 1979).

Mean open times and channel currents did not change over a time period during which >80% of the receptors were prevented from opening by the toxin (Fig. 5 *A*). After 40 min, the rate had decayed to 5% of the original rate. In a control experiment without toxin, the frequency of opening fluctuated, but did not decline (Fig. 5 *B*). The mean open time and mean channel current were constant over the course of the control experiment, which lasted for nearly 30 min (Fig. 5 *B*).

## Voltage Dependence

Voltage-dependent gating rates have been reported previously for agonist-induced gating of the AChR channel (Magleby and Stevens, 1972; Neher and Steinbach, 1978). Since cholinergic ligands are charged, it is quite possible that the movement of this charge is a factor in the observed voltage dependence. It is of interest to compare the voltage dependence of spontaneous openings with ligand-gated

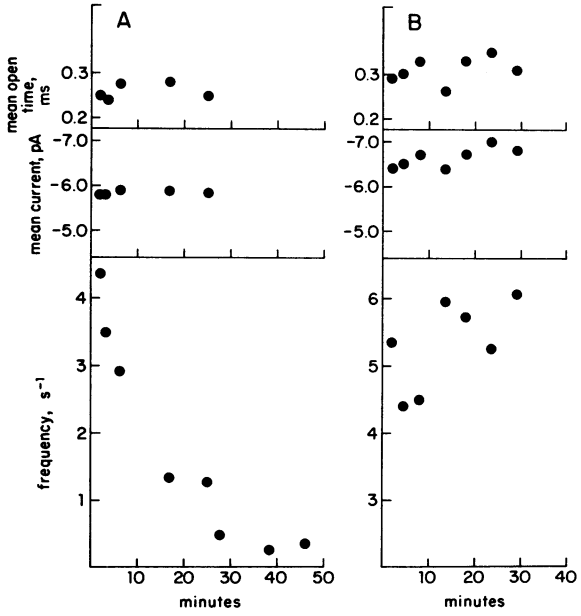



FIGURE 5 The effect of α-bungarotoxin on the frequency of spontaneous channel openings. Channel currents were recorded at various times after the seal was formed. The mean open times (top), mean channel currents (middle), and opening frequencies (bottom) were determined and plotted as a function of time. Each time point typically represents the average of 30–100 channel openings. In the measurement of opening frequency, the less quantitative method described in the Methods was used, and no correction was made for brief events that were missed because of the recording bandwidth. The patch electrode used in *A* contained 20 nM α-bungarotoxin; the patch electrode in *B* contained control saline. The patch electrode potential was held at +50 mV.

openings, in order to test this possibility. However, a direct comparison is complicated by the very brief closures seen with typical agonists (Colquhoun and Sakmann, 1981, 1983; Dionne and Liebowitz, 1982; Sine and Steinbach, 1984). Such fast rate processes are difficult to measure, and they create the need for substantial corrections in other slower rate processes.

The kinetics of unliganded AChR channel gating described above is qualitatively similar to the kinetics of curare-liganded AChR channel gating (Morris et al., 1983; Takeda and Trautmann, 1984). In particular, there are few very brief closures that complicate analysis of gating kinetics for strong agonists (Morris et al., 1983). For this reason, curare has been selected for a comparison of the voltage dependence of channel gating rates. This will allow a direct comparison of the rates of similar kinetic processes.

Fig. 6 shows the kinetic parameters from fits to channel open times as a function of patch electrode potential for spontaneous and curare-gated channel openings. The patch electrode potential was varied from +25 to +100 mV in on-cell recordings. Increasing the patch electrode potential is equivalent to hyperpolarizing the membrane. Below +25 mV, the frequency of spontaneous openings was so low and the single-channel currents so small that it was difficult to collect much data. Above +100 mV, recordings were almost never stable.

$\lambda_1$, the fast decay constant, shows a significant increase with voltage at low potentials for spontaneous openings. Other parameters from spontaneous open times, as well as all of the parameters from curare-gated channel open times, show no significant voltage dependence in the voltage range shown.

The voltage dependence of the rate of opening has also been studied. Measurements of the rate of opening have sources of variation associated with the area of the tip of a patch electrode and the density of AChR channels in the cell membrane. Control of these additional variables can be achieved by comparing measurements from the same patch of membrane at different potentials. A quantitative determination of frequency of opening must also incorporate a bandwidth correction for missed brief events. In seven experiments, channel currents were recorded at three potentials from the same patch of membrane. In each experiment, enough openings were recorded at each potential to permit a Poisson analysis. Open time densities were also analyzed to correct for missed openings. Thus, in each experiment at each potential, a quantitative determination of the rate of unliganded channel opening was made. In five experiments, it was possible to make a similar quantitative determination of the rate of channel opening with patch electrodes containing 10–20 $\mu$M curare.

The results are shown in Fig. 7. These frequencies were not standardized to a 1-MΩ patch electrode, and small-tipped, thick-walled borosilicate electrodes were used for most of the experiments with curare, so the observed frequencies of spontaneous and curare-gated openings fell within a similar range. The average electrode resistance was nearly three times higher in the experiments with curare. The rate of curare-gated opening increased with voltage, as reported previously (Morris et al., 1983). The rate of unliganded channel opening showed a stronger voltage dependence. A small part of the voltage dependence of the frequency of opening seen with curare could

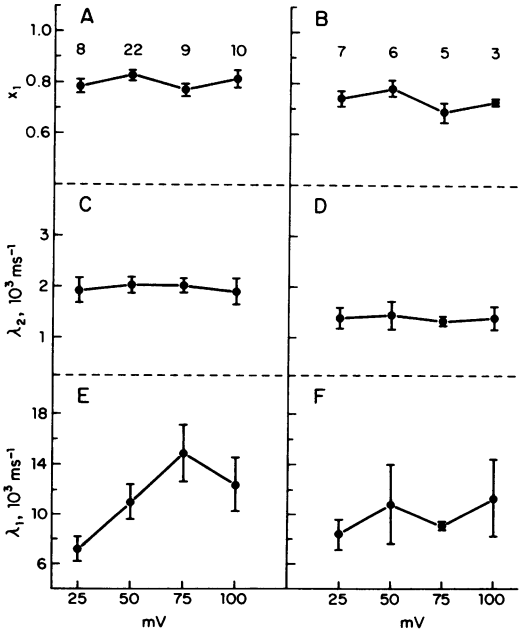



FIGURE 6 The kinetic parameters from open time distributions are plotted as a function of patch electrode holding potential. $\lambda_1$ is the fast exponential decay constant, $\lambda_2$ is the slow exponential decay constant, and $x_1$ is the fraction of total density of the fast component. (*A*) Spontaneous openings. (*B*) Curare-gated openings. The bars indicated standard errors. The number of measurements at each voltage is indicated on the figure.

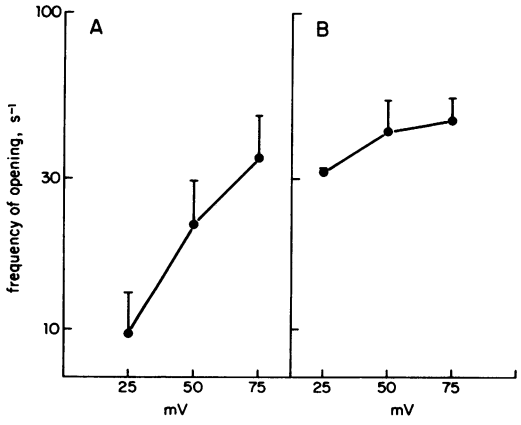



FIGURE 7 The frequency of opening is plotted semilogarithmically as a function of patch electrode holding potential. (*A*) Spontaneous openings were recorded from seven different patches of membrane at each of the three potentials plotted. (*B*) Curare-gated openings were recorded from five different patches of membrane at each of the three potentials plotted. The bars indicate standard errors.

actually have been an increase in the number of spontaneous events. This would increase the difference between the voltage dependences of the spontaneous and curare-gated opening rates. The voltage dependence of the rate of opening showed signs of saturation at hyperpolarized potentials for both unliganded and curare-gated openings.

It should be noted that there are two sources of variability that contribute to the standard errors in the frequency of opening shown in Fig. 7. One source is intrinsic to the measurement and would appear if many measurements were made from the same patch of membrane. The other source arises because, from one patch of membrane to the next, there is a variable number of AChRs. These two sources have not been separated, because it is not practical to make many quantitative measurements from the same patch. Measurements of opening frequency such as those shown in Fig. 5 *B* show variations of the order of 25%, but these measurements were made without performing a Poisson analysis, and they did not include a correction for missed brief openings. The variation in the number of channels in a patch of membrane can be expected to be quite large (Land et al., 1977). Since this source of variation has been controlled for by making measurements at different potentials from the same patch of membrane, the errors relevant to the voltage dependence of the rate of opening are lower than the standard errors displayed in Fig. 7.

## DISCUSSION

The experiments presented here were designed to investigate the phenomenon of spontaneous opening of the AChR channel, in order to learn more about the channel-gating mechanism. An experiment with $\alpha$-bungarotoxin had the additional objective of adding to the existing evidence, summarized in the Introduction, that the AChR channel can open in the absence of ligand. In this experiment, the frequency of spontaneous opening was found to decay with time when $\alpha$-bungarotoxin was included in the patch electrode. It was found that $\alpha$-bungarotoxin inactivates the entity that produces the channel currents seen in the absence of ligand with approximately the same rate that it binds to the AChR (Colquhoun and Rang, 1976; Darveniza et al., 1979).

As the spontaneous opening of more and more channels is blocked by $\alpha$-bungarotoxin, the remaining channel openings have the same mean channel current and mean open time. Thus, if the brief- and long-duration openings are due to different AChR populations, $\alpha$-bungarotoxin does not express any preference. This result is consistent with the finding of Katz and Miledi (1973) that submaximal doses of $\alpha$-bungarotoxin do not alter the corner frequency of acetylcholine-induced noise.

### Multiple States

Two open states are clearly evident in most experiments of unliganded AChR channels, based on the analysis of open-time data presented here. This suggests that occupancy of one or two receptor binding sites by a cholinergic ligand is not a requirement for the appearance of different open states. These results do not rule out the possibility that the two exponential components seen in open times of ligand-induced gating events are associated with different degrees of receptor occupancy, but they do suggest that alternative explanations should be taken seriously. Clearly, two open states with the same conductance occur, which do not depend on a singly and doubly liganded mechanism. Either a single AChR channel has access to two distinct open states, or there are two populations of AChR channels.

Some previous work is consistent with the hypothesis that the two exponential components in the open times of ligand-gated channels arise from singly and doubly liganded receptors (Takeda and Trautmann, 1984). Lester and Chabala (1984) suggested that singly liganded AChR channels open, but that two exponentials can be seen in their open times. Jackson et al. (1982), Morris et al. (1983), Labarca et al. (1985), and Sine and Steinbach (1984) all reported variations in the proportion of rapidly and slowly closing channels for the same agonist concentration. These results are consistent with two receptor populations with the same conductance. In all likelihood, open-time densities that are a sum of two exponentials can be generated by more than one mechanism.

Two unliganded channel closed states are indicated by the two exponential components of closed times. In the case of ligand-induced gating, a population of short-duration closed times has been interpreted as the rapid reopening of the receptor channel with occupied binding sites (Colquhoun and Sakmann, 1981; Dionne and Liebowitz, 1982). In closed-time densities of ligand-gated channels, the fast components are far more prominent and have faster decay rates. One possible explanation for the fast population of closed times in the unliganded channel gating data is that spontaneous opening proceeds by a brief transition through a conformation similar to the closed, liganded conformation. A second explanation is that there is a closed state similar to a blocked state (Neher and Steinbach, 1978) that can only be reached from an open state.

### Frequency of Opening

The product of the frequency of opening and the patch electrode resistance was averaged for 19 measurements to give 23 $s^{-1}$ at a patch electrode potential of +50 mV. The absolute rate of spontaneous opening can be determined if the number of AChR in a patch of membrane sealed off by a 1-M$\Omega$ electrode is known. On the basis of the careful capacitance measurements of Sakmann and Neher (1983), a 1-M$\Omega$ patch electrode pulls in $\sim$12 $\mu m^2$ of cell membrane. Rodent muscle in culture has an average density of $\sim$400 toxin binding sites or 200 AChR channels per square micron (Land et al., 1977), which increases by a factor of 5.1 when treated with tetrodotoxin (Shainberg et al.,

1976). Thus, a patch of membrane formed by a 1-MΩ electrode should contain 12,000 receptors. The absolute rate of spontaneous opening of an individual AChR channel in this preparation is then estimated to be $2 \times 10^{-3}\ s^{-1}$. Despite the uncertainty of many of the numbers used to obtain this estimate, it is probably correct to within an order of magnitude. Multiplying the frequency of opening by the mean open time of spontaneously opening channels gives a value of $3 \times 10^{-7}$ for the fraction of time that an unliganded channel is open. This is well below one earlier estimate of the upper bound to the probability of unliganded AChR channel opening (Dionne et al., 1978), and within experimental error of the upper bound of $10^{-7}$ given by Neubig et al. (1982).

## Voltage Dependence

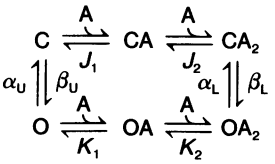
Both $\lambda_1$ and the rate of opening of unliganded receptors increase as the membrane is hyperpolarized. For both rate processes, the voltage dependence saturates. None of the other kinetic parameters associated with unliganded gating have a significant voltage dependence in the voltage range studied.

Previous work has shown that the kinetics of agonist-induced gating of the AChR channel is voltage dependent (Magleby and Stevens, 1972; Neher and Steinbach, 1978; Neher and Stevens, 1979). Saturation of the voltage dependence of AChR channel gating rates has also been observed (Neher and Stevens, 1979; Morris et al., 1983). Morris et al. (1983) have reported a voltage-dependent rate of opening of curare-gated channels similar to that described here. Previous work on curare (Morris et al., 1983) and the curare derivative dimethyl (+)tubocurarine (Sine and Steinbach, 1986*a*) has shown a weak dependence of the closing rates on voltage, but the voltage dependence is most pronounced only at voltages outside the range of voltages studied here.

Molecular interpretations of the voltage dependence of channel gating have been discussed at length elsewhere (Magleby and Stevens, 1972; Ehrenstein et al., 1974; Neher and Stevens, 1979; Steinbach, 1980). The simplest way to explain voltage-dependent channel gating is to invoke charged groups within the AChR that move during the gating conformational transition. The saturation behavior presented here is not accounted for by this simple model. It is worth noting that the voltage dependence of the rate of spontaneous opening is stronger than the voltage dependence of the rate of curare-activated channel opening. This may mean that during a conformational transition of the channel, the ligand moves within the field of the membrane. However, if the saturation of the voltage dependence of the rate of opening is due to a voltage-dependent equilibrium between two closed states, then the rate of curare-activated channel opening could have a weaker voltage dependence because curare shifts this equilibrium toward the closed state that is favored by hyperpolarization.

## A Comparison of Liganded and Unliganded Gating

The following discussion uses the measurements of liganded and unliganded transition rates to analyze the energetics of ligand activation of the AChR. The following scheme is very general, and has been used as a basis for analysis in previous studies (Steinbach, 1980; Karlin, 1980)




Scheme I

C and O denote receptors with open and closed channels, respectively, and A denotes an agonist. $\alpha$ and $\beta$ are the rates for closing and opening, with the subscripts U and L denoting unliganded and liganded receptors. $J$ and $K$ are dissociation constants for ligand from a receptor with a closed and an open channel, respectively. The above scheme does not include desensitization. The model also does not include the two unliganded open states. These omissions have a negligible impact on the conclusions of this analysis. Assuming detailed balance, it follows that

$$J_1 J_2 = \frac{K_1 K_2 \beta_L \alpha_U}{\alpha_L \beta_U}. \quad (1)$$

The fraction of time that the unliganded AChR channel is open, estimated above from the rate of opening and the mean open time, can be used as the unliganded equilibrium constant, $\beta_U/\alpha_U$, giving $3 \times 10^{-7}$.

Taking the slow decay constant of carbamylcholine-gated channel open times (Table I) as the closing rate of doubly liganded AChR channels gives $\alpha_L = 0.77 \times 10^3\ s^{-1}$ (after correcting $\lambda_2$ for brief missed closures). If the reciprocal of the mean open time is used instead, $\alpha_L = 1.0 \times 10^3\ s^{-1}$ (after correction for missed closures). These two values are not sufficiently different to alter the conclusions reached. Using $\alpha_L = 0.77 \times 10^3\ s^{-1}$, and $\beta_L = 2.8 \times 10^4\ s^{-1}$ (calculated from an analysis of closed times in Results), the ratio $\beta_L/\alpha_L$ is 36.

The analysis of carbamylcholine closed times that produced these numbers is based on the assumption that the fast component of closures represents a step in the activation of the receptor by ligand (Colquhoun and Sakmann, 1981). Studies with other methods that are less direct, but do not depend on this assumption, suggest that the ratio $\beta_L/\alpha_L$ is near 1 for carbamylcholine (Dionne et al., 1978; Sakmann and Adams, 1979). This discrepancy reflects a currently unresolved problem in our understanding of ligand-gated channel kinetics, but the comparison of ligand-gated and unliganded channel kinetics

reveals differences that are too large to be affected by this discrepancy.

Using the numbers derived from this data, Eq. 1 gives $J_1J_2 = 1.2 \times 10^8 K_1K_2$. The following points are germane to this result. (*a*) Stronger binding to a receptor with an open channel is expected, since this is generally considered to be the driving force for the conformational change of an allosteric protein (Monod et al., 1965; Karlin, 1967). Other studies have suggested that, compared with the binding of ligand to an AChR with a closed channel, the binding of ligand to an AChR with an open channel should be very strong (Dionne et al., 1978), and that the dissociation should be slow (Adams and Feltz, 1980). (*b*) If the first and second binding steps are taken as equivalent, this relation and the values given for the unliganded and liganded open-closed equilibria allow derivation of the dose-response relation and binding isotherm for the above model with only one free parameter. This may be helpful in the analysis of these kinds of experiments. (*c*) The binding of ligand changes the free energy difference between the open and closed channel by 11 kcal/mol.

It should be noted that a carbamylcholine-liganded AChR channel opens $1.4 \times 10^7$ times faster than an unliganded AChR channel. The rates of closing differ by a factor of $\leq 5$, depending on whether one compares decay constants or mean open time. This comparison indicates that ligand binding exerts its greatest effect by far on the rate of channel opening. The effect of ligand binding is thus to destabilize the closed channel rather than to stabilize the open channel.

This is an important result, because there is no *a priori* reason to expect the binding of ligand to influence the opening rate more than the closing rate. For example, one possibility, which is eliminated by the above results, is that the binding of ligand could activate the receptor by slowing the rate of closing by several orders of magnitude, and change the rate of opening very little. If this were the case, then the rate of spontaneous opening would be quite high, but the open times would be so brief that they would be very difficult to see.

The result presented here permits a strong statement to be made with regard to how ligand binding activates the AChR: interactions within the AChR that stabilize the closed state are weakened by the binding of an agonist. Occupation of both receptor-binding sites is known to produce maximal activation, while the occupation of only one binding site activates much more weakly (Adams, 1975; Dionne et al., 1978; Steinbach, 1980). Thus, each of the two binding sites could be coupled to separate intramolecular contacts within the AChR, each of which accounts for half of the energy difference between the open and closed state. If each contact is weakened independently when the associated binding site is occupied by agonist, when the occupation of only one binding site would leave the closed state energetically favored by 4 or 5 kcal.

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