ACETYLCHOLINE RECEPTOR KINETICS

A Description from Single-Channel Currents at Snake Neuromuscular Junctions

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ABSTRACT Single-channel currents from acetylcholine receptor channels of garter snake neuromuscular junctions were recorded using the patch-clamp technique. Low concentrations of acetylcholine or carbamylcholine induced populations of single current events whose amplitudes and durations had unimodal distributions. The probability with which channel opening transitions occurred was time dependent, so that it was more probable for channels to open during the several hundred microseconds following a closing transition than during any later equivalent interval. The time-dependent distributions of duration and opening-transition probability were fitted by a sequential, reversible kinetic model in which the agonist binding steps occur before, and separately from, channel activation. This description allowed estimates to be obtained of both the opening ($\sim 750\ s^{-1}$) and closing ($\sim 500\ s^{-1}$) transition rates of these channels and of the mean lifetimes of the open- (~ 2 ms) and the closed-channel state ($\sim 200\ \mu s$) to which the open state was reversibly related.

INTRODUCTION

The analysis of acetylcholine receptor kinetics over the past decade has provided useful insights into the mechanisms of membrane channels. The work (reviewed by Adams, 1981 and Steinbach, 1980) has been derived from studies of the transient behavior of large populations of receptors, which were activated either synchronously, producing evoked, miniature, and voltage-jump end-plate currents (Magleby and Stevens, 1972 and Adams, 1974), or asynchronously producing end-plate current noise (Katz and Miledi, 1970 and Anderson and Stevens, 1973). In principle, the rates of agonist binding and channel activation can be inferred from such measurements (Colquhoun and Hawkes, 1977). Nevertheless, in practice the data most often reveal only a single kinetic component which has been interpreted as the result of channel closing (however, see Dionne, 1981). In such cases only the closing-transition rate of open channels has been estimated. The closing-transition rate obtained in this manner is actually an apparent rate which is necessarily contaminated to an unknown degree by other coupled steps of the receptor population's activation kinetics.

To address this problem we have recorded trains of single-channel activity from patches of receptor-rich junctional membrane. Such records arise from the same fundamental kinetic processes as the population responses listed above, but more extensive evaluation of receptor activation kinetics is possible because the temporal relations among individual channel opening and closing transitions can be measured (Colquhoun and Sakmann, 1981).

Single acetylcholine receptor currents have been treated analytically by Colquhoun and Hawkes (1981). Their approach was to predict the "burst behavior" of single-channel activity, that is, the number of openings accompanying each receptor occupancy and the distribution of closed intervals between these openings. In principle, their treatment was suited only to data recorded from patches containing one active receptor or a few receptors whose activity could be unambiguously separated in the data records. However, that requirement is impossible to verify in most experimental situations, and it clearly does not apply here where our membrane patches were taken from muscle end-plate membrane, richly populated with receptors.

Our approach has been to examine the stochastic properties of the time intervals between successive opening and closing transitions in these records, making no assumptions about bursting behavior, and to fit them with predictions derived from kinetic models of receptor activation. The treatment is appropriate when there are large numbers of channels capable of activity; it requires only that the activation rate be low so that usually only one channel is open at any time. For the acetylcholine receptor (AChR) both the rate of channel opening and the lifetime of the specific closed receptor state, from which opening transitions occur, can be estimated, as well as the rate of channel closing. These fitted rates can then be used to calculate the apparent rates, which should be found from population studies such as noise analysis. Noise studies appear to underestimate the rate at which single channels close by 10–20%.

A preliminary account of some of this work has been presented (Leibowitz and Dionne, 1982).

METHODS

Experiments were performed on the AChR of garter snake (sp. *Thamnophis*) neuromuscular junctions in twitch muscle fibers of *m. costocutaneous*. Whole muscles consisting of 50–100 fibers were removed together with portions of the rib and scale and placed in a saline bath (NaCl 159, KCl 2.15, $CaCl_2$ 1.0, $MgCl_2$ 4.2 mM, HEPES [*N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulphonic acid] 5.0 mM; pH 7.2) containing 100 nM tetrodotoxin. The cells were held in position by two small stainless steel spring clips placed near the tendinous ends. The membrane surface was prepared for recording by enzymatic digestion of the collagenous matrix and basement membrane between the nerve terminal and the receptor-rich junctional membrane. This allowed removal of the nerve terminal and formation of high impedance seals between the membrane and recording electrode. Our normal enzyme treatment consisted of a 2 h exposure at room temperature to 2 mg/ml collagenase (Advance Biofactures Corp. Lynwood, NY, form TD) followed by a 20–40 min period in 0.02 mg/ml protease (from *Streptomyces griseus*, Sigma Chemical Co., St. Louis, MO, type XIV) together with the collagenase. The cells were then rinsed with saline and could be used for several hours. Successful treatment usually resulted in a significant amount of cell lysis (30–50%). The exposed junctional regions of treated fibers were visually located with a compound microscope equipped with Nomarski optics. All recordings were made at room temperature, ~20°C.

The methodology and circuitry for recording single-channel currents using patch electrodes have been described by others (Neher and Sakmann, 1976; Hamill et al., 1981). The method allows the patch transmembrane voltage to be controlled while transmembrane currents on the order of 1 pA can be resolved to several kHz. With a Burr-Brown model 3523 operational amplifier (Burr-Brown Research Corp, Tuscon, AZ) used as the current-to-voltage converter and a frequency compensation stage, the residual noise in our equipment was less than the Johnson noise of a 1 GΩ resistor for frequencies below ~2.5 kHz. The current signal was low-pass filtered at 2.5 kHz (4-pole, Butterworth response), and digitally recorded at 100 μs intervals using a laboratory computer (Digital Equipment Corp, Maynard, MA, model PDP 11/34). Entire current-time records consisted of a sequence of many 0.92 s segments taken at 2 s intervals, the dead time being used by the computer for data transfer.

Patch electrodes were prepared following the descriptions of Hamill et al., 1981; they were uncoated and, after polishing, had a nominal resistance of 2–5 MΩ when filled with 90% saline. The formation of gigohm seals between the patch electrodes and the treated junctional membrane was precipitated by lowering the pressure within the pipette. Seal resistances of 1–3 GΩ were routinely obtained and remained stable during recording. Agonist was applied to the AChR by dissolving it to the desired concentration in the pipette saline. Recordings were made from membrane patches on the intact cell.

The amplitude, duration, and relative timing of single-channel events were measured from the current-time records using a computer-assisted search procedure. Each event was detected and evaluated off line with the computer and visually confirmed. The search was implemented in such a manner that events with a measured duration less than three sample intervals (here, 300 μs) were neglected as were events whose amplitude was below a detection threshold. In addition, closed times less than two sample intervals (200 μs) were also neglected. Because such procedures could bias the results, their effect will be considered in detail in the Results section.

RESULTS

Digital records of transmembrane current vs. time were obtained from small portions of junctional membrane using the patch recording technique. Within each record many events identified as single-channel currents could be resolved together with less frequent multiple or overlapping events (Fig. 1). Presumably, the patches of junctional membrane from which single-channel currents were recorded contained large numbers of AChR, so that the event activity was derived from many different channels, not just one. Events occurred at a near-steady rate, the specific value of which varied among patches and with agonist concentration. Conditions were adjusted to keep the average rate of channel activation low, so that multiple or overlapping events were rare. The great majority of events were from single channels that opened briefly and then closed before another channel became active. The occurrence and timing of multiple events was noted during data processing and accounted for during analysis. All of the data illustrated in the figures were recorded from a single end plate with 500 nM ACh as the agonist. At the agonist concentrations we have tried (carbamycholine, 1–2.5 μM; acetylcholine [ACh], 250–500 nM) the single-channel activity was sustained during the entire recording period. On some occasions we have noted a progressive decline in the frequency of events over several minutes, which we believe was due to receptor desensitization. For this analysis we have omitted recordings in which desensitization appeared to be a concern, and we have not considered its potential influence on receptor kinetics.

Four measureable parameters were obtained from the current-time records of channel activity in each patch. Two parameters characterize the single-channel event, the mean current and the open duration. Two other parameters specify the temporal relations among events; both of these are probability functions that depend on time because they reflect the history of prior channel activity. These parameters will now be described in detail and

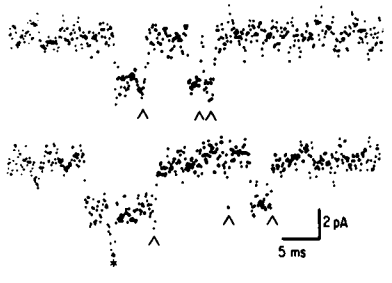

FIGURE 1 Examples of junctional acetylcholine receptor currents elicited with 500 nM acetylcholine. Two noncontiguous 51.1 ms records were selected to show both single channels and an example of an infrequent multiple event. Single-channel currents at the recording voltage occurred as pulse-like downward displacements from the base line and were caused by increased membrane conductance. These records and all subsequent figures were derived from 125 recordings each 0.92 s long obtained with two successive patches from the same end plate under the same conditions. Separate analysis of the data from each patch revealed no significant differences. The resting transmembrane potential was hyperpolarized by 74 mV to improve the signal-to-noise ratio, and the signal was low-pass filtered at 2.5 KHz before digitization at 100 μs intervals. Carets indicate closing transitions at which $t = 0$ as described in the text, while the asterisk marks a channel closing at which $t \neq 0$.

illustrated using the data recorded from a single patch at a fixed voltage and temperature.

For each patch, a histogram of the current amplitudes of all the single events was compiled (Fig. 2 *a*). These amplitude distributions had only one peak with a near-Gaussian shape. Note, however, that the amplitude distribution accurately represents only that population of single events whose amplitudes exceeded a threshold value; small events were systematically excluded. We determined to what extent the amplitudes of the briefest events that we accepted for analysis (300 μs) were distorted by the bandwidth limitations of the recording equipment. That is, events with a sufficiently short open duration must be attenuated by the electronics and would go undetected in our analysis if their amplitudes fell below threshold. A plot of event amplitude against open channel duration (Fig. 2 *b*) indicated that errors from this source, although they must arise, did not affect the amplitude distribution. Although it would be desirable to make a similar examination of very brief closed durations, that was not possible because the distribution of closed durations is not known a priori. Nevertheless, the briefest closed duration that we accepted in our analysis (200 μs) should have been resolved with good accuracy because the time constant of the recording system was <100 μs and there was no evidence of amplitude attenuation of currents with durations as short as 300 μs.

The measured durations of the individual single events were assembled into a histogram containing bins of 100 μs

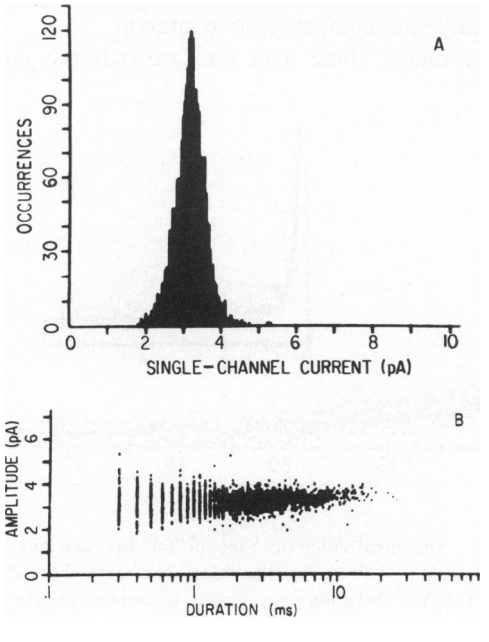

FIGURE 2 Amplitude Characteristics. *A*, single-channel amplitude distribution for 2,305 events. Amplitude threshold = 1.8 pA. The distribution had a mean and standard deviation of 3.1 ± .3 pA. *B*, single-channel amplitude vs. duration. Brief events as short as 300 μs were not systematically attenuated.

width (Fig. 3), and the mean single-event duration, τ_{open}, was estimated as the time constant of a fitted exponential curve. The exponential was fitted using a nonlinear least-squares method in which each datum value was given equal statistical weight and in which all values including the zeros were used through 25 ms. This procedure was implemented with only one free parameter, the decay rate ($= 1/\tau_{open}$) of the exponential theory; the amplitude was determined by the number of observations multiplied by $\exp(0.0003/\tau_{open})$ to correct for the 300 μs resolution limit.

In principle, there are two reasons why this unweighted least-squares procedure is inappropriate. First, the expected number of observations in each bin has a binomial distribution with a probability that changes exponentially (presumably) with increasing bin number (time). Thus, the expected variance in each bin is largest for the early intervals in the histogram and falls almost exponentially to asymptotically approach zero at later intervals. Because the variance differs from bin to bin, the data values should not be given equal statistical weight. Second, statisticians caution against using bins containing few observations because undue value may be given to them. However, any decision on how to truncate the data set must be essentially arbitrary despite a reliance on statistical considerations.

The decision to estimate τ_{open} using a nonlinear, unweighted, least-squares fit to a data set that spanned $>10\tau_{open}$ was made after careful evaluation of several alternative methods. These will be discussed later. Aside from its faults in principle, the selected approach has certain favorable properties. Notable was a marked insensitivity to significant levels of truncation of the data set. Furthermore, this procedure gave estimates of the open

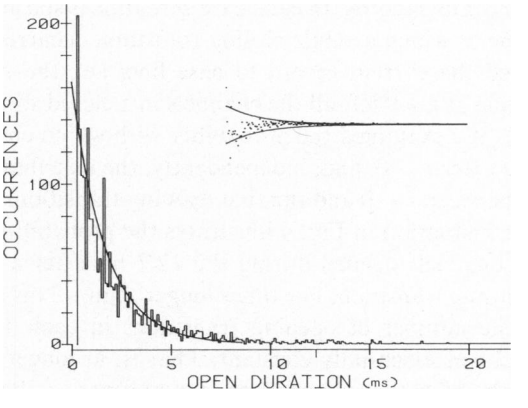

FIGURE 3 Distribution of single-channel open durations. Using a nonlinear, unweighted least-squares method, the distribution of open durations was fitted by an exponential curve with a time constant equal to the mean open duration (here 1.69 ms). From Eq. 1 the channel closing rate α was 593 s^{-1}. Note that both axes have been displaced from zero so as not to obscure the data. The *inset*, shows the signed differences between data and theory as points for the entire histogram, together with two curves that are ±3 times the expected standard deviation (see text).

duration distribution which, together with the variance distribution, accurately described the measured values. This is illustrated in the inset of Fig. 3 where the signed difference between measurement and theory is plotted together with smooth curves that are $\pm 3\times$ the expected standard deviation. In this figure two trends are evident: at short times the data appear to deviate systematically below theorical values; at longer times the deviation is above. At least in part this is a property of (*a*) a one-sided distribution with skewed variance and (*b*) a fitting procedure that does not take *a* into account. Furthermore, we estimate that as many as ~5% of the single events may have been corrupted by unresolved brief closing episodes (see Discussion). This would tend to overpopulate the longer duration bins at the expense of the shorter, and so skew the data set as shown here. Finally there is the possibility that the data actually require more than one exponential term to describe the distribution (Colquhoun and Sakmann, 1981; Cull-Candy and Parker, 1982); however, given all the factors, there is not sufficient evidence to support this proposal for our data.

The distribution of the mean single-channel current amplitudes had a single mode, and the distribution of open-channel durations was well described by a single exponential curve. These observations suggest that the single-channel events that we have studied were produced by a homogeneous population of channels. This becomes an important point, because, as we will show below, the probability with which channels open is not independent of time. Although one explanation of that time dependence might be that there are several types of channels operating simultaneously, the results that we have just presented do not support that idea.

The two parameters that will be described next are time-dependent probability functions that can be evaluated from the data records. In each case zero time is specified as the time at which a single closing transition occurred that returned the current record to base line; i.e., the closing transition at $t = 0$ left all the channels in a closed state (see Fig. 1). We examined the probability with which channels opened after $t = 0$, and, independently, the distribution of times between $t = 0$ and the first opening transition.

The histogram in Fig. 4 illustrates the probability with which channels opened during the 12.7 ms after a $t = 0$ type closing transition. For times longer than ~2 ms after $t = 0$, the number of opening transitions in each 100 μs interval was essentially constant. That is, at longer times the probability that channels opened appeared to be independent of prior channel activity. However, during the first milliseconds after a channel closed, the probability that an opening occurred decayed from an intially enhanced value to the average, time-independent value that characterized longer times. Thus, these AChR channels were more likely to open soon after a channel closed than later. The probability of opening was time dependent, indicating that

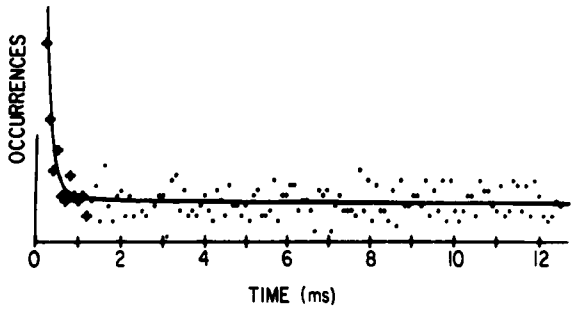

FIGURE 4 The distribution in time of opening transitions. The times at which opening-transitions occurred during 12.7 ms following any $t = 0$ closing transition (see text) were compiled into a histogram to estimate the probability of opening transitions, $P(t, \Delta t)$. Each point is the count in a bin 100 μs wide. For clarity the first ten data points have been plotted as crosses. The data indicate that $P(t, \Delta t)$ was time independent except during the first millisecond or so following a closing transition when $P(t, \Delta t)$ was greater than its time independent value. Here Eq. 2 has been fitted to the data with $\bar{n} = 0.039$, $\alpha = 593\ s^{-1}$, $\beta = 750\ s^{-1}$, and $k_{-2} = 6{,}000\ s^{-1}$. 2,262 time segments.

the single events were not completely independent of one another.

If channels were entirely independent the time between events should have an exponential distribution, similar to the open duration. Fig. 5 is a histogram of the intervals between events; it indicates that their distribution is not a single exponential. This distribution shows a prominent and very rapid decline during the first few hundred microseconds followed by a slower decay toward zero. Thus, it was much more probable for opening transitions to occur within several hundred microseconds of a closing transition than in any later equivalent time interval.

In summary, these data indicate that the junctional

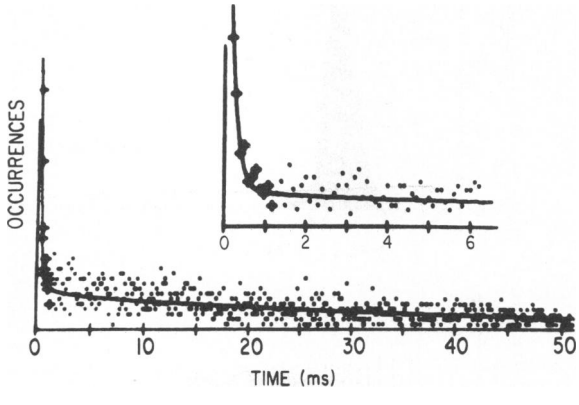

FIGURE 5 The distribution in time of the first opening transition following each $t = 0$ closing transition (see text). This distribution indicates that the probability with which a channel opening transition occurs subsequent to a closing transition has both rapid and slow time-dependent phases. For clarity the first ten data points have been plotted as crosses. Data plotted in this way were fitted with Eq. 4; the values for $\bar{n}$, α, β, and k_{-2} used here were the same as those in Fig. 4. 2,402 entries including 543 at times >51.0 ms. The inset shows the first 6.3 ms of the histogram using an expanded time scale.

AChR channels from which we recorded comprised a homogenous population in terms of their open channel conductance and duration. However, the channel opening probability depended in some way upon the history of channel activation, because it was enhanced just after a channel closed. We will show below that these observations are consistent with a simple model for receptor kinetics.

KINETIC ANALYSIS

Envisioned at its simplest, the AChR is an entity that combines two chemical binding sites with a channel through which cations cross the cell membrane. In its resting state the channel is closed and both binding sites are vacant. When two agonist molecules bind to the receptor, the probability that the channel will open is dramatically increased, although opening is by no means obligatory. If it is supposed that the agonist molecules bind sequentially to the receptor, the kinetics of binding and activation can be represented schematically as

$$2A + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} A + (AR) \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} (A_2R) \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^*. \quad (1)$$

Here the A_2R^* state represents the only open-channel (conducting) conformation, and the other states depict the closed receptor-channel complex R with either 0, 1, or 2 bound agonist molecules A. The subscripted transition rates are the unidirectional macroscopic rates for agonist association and dissociation while α and β are the transition rates for channel closing and opening, respectively. In this scheme the equilibrium dissociation constants $K_1 = k_{-1}/k_{+1}$ and $K_2 = k_{-2}/k_{+2}$ need not be equal.

This kinetic scheme may be treated quantitatively to predict the conditional probability with which channel open-transitions occur after a $t = 0$-type closing transition. In Fig. 4 it is this probability that we have experimentally evaluated. Define it as follows:

$P(t, \Delta t) \equiv$ Probability (An opening transition occurs in the time interval τ, $t < \tau \leq t + \Delta t$, conditional upon a closing transition at $t = 0$.).

Now, the practical experimental result of using low agonist concentration as we have is that only one channel is active at any time. Then with N AChR in a patch of membrane from which records are collected,

$P(t, \Delta t) =$ Probability (Channel 1, or channel 2, or ..., or channel N opens during the interval t, Δt.).

Because the simultaneous opening of two channels was not seen, the probability of two or more channels opening during the same Δt can be set to zero. This serves to ensure that the magnitude of $P(t, \Delta t)$ never exceeds 1. It follows that

$$P(t, \Delta t) = \sum_{k=1}^{N} \text{Probability (Channel } k \text{ opens during the interval } t, \Delta t.).$$

Because the channels have been assumed to be independent and to abide by the same activation kinetics, the probability term under the sum above is independent of k; call it $p(t, \Delta t)$ and write

$$P(t, \Delta t) = Np(t, \Delta t).$$

A partial expression for $P(t, \Delta t)$ can be written directly, without regard to any specific kinetic description of AChR activation. To do this, define $f(t)$ as the expectation that each AChR will reside at time t in a kinetic state from which it can open immediately. Then, neglecting second-order processes,

$$P(t, \Delta t) = N \cdot \text{opening-transition rate} \cdot f(t) \cdot \Delta t.$$

Referring to Eq. 1, one sees that $f(t)$ would be the expectation of occupying A_2R, and β would be the opening-transition rate: $P(t, \Delta t) = N\beta f(t)\,\Delta t$. This expression can be made conceptually simpler by observing that the product $Nf(t)$ is the expected occupany of A_2R by the N AChR in the membrane patch. We will call this occupation number $n(t)$ and write

$$P(t, \Delta t) = \beta\, n(t)\, \Delta t.$$

A specific expression for $n(t)$ appropriate for the experimental conditions must now be derived from Eq. 1. To do this, divide the AChR into two groups. The first group will contain just one member; it is that specific AChR whose channel closes at $t = 0$, so this receptor must begin its evolution in the A_2R state. All the remaining AChR will be members of the second group; presumably at $t = 0$ these receptors are distributed at equilibrium among the three closed-channel states. For each group we can compute the probability that its members will reside in A_2R as time passes; this probability times the number in each group gives the occupation number of the state. For the first group, the probability that its single AChR will reside in A_2R, $n_1(t)$, may be obtained in general by solution of the set of differential equations that relate the time-dependent populations of the states in Eq. 1. This problem can be simplified with a modified version of Eq. 1 which holds in the low concentration limit. Because low concentration here implies that for any AChR the likelihood of binding agonist molecules is $<< 1$ we approximate the vanishingly small binding likelihood with a fixed value, zero, and considerably simplify the mathematical complexity of the problem. Solving the differential equations of this reduced kinetic model gives

$$n_1(t) \doteq \frac{\lambda_1 - \alpha}{\lambda_1 - \lambda_2} e^{-\lambda_1 t} + \frac{\alpha - \lambda_2}{\lambda_1 - \lambda_2} e^{-\lambda_2 t}$$

The parameters λ_1, λ_2 are the characteristic or observed rates of Eq. 1 in this low concentration limit and are given below. The most apparent inaccuracy of this approximation is the equilibrium value, which should be $c^2/(K_1K_2)$, not zero; but at low concentration this correction is negligi-

ble. For the second group which contains N-1 receptors, equilibrium conditions determine the average number of receptors in A_2R at low concentration as $(N\text{-}1)c^2/(K_1K_2)$. Assuming N is large in any patch, $n_2(t) \doteq (Nc^2)/(K_1K_2)$. Then, because $n(t) = n_1(t) + n_2(t)$, we have the following expression for $P(t, \Delta t)$.

$$P(t, \Delta t) = \beta\left(\bar{n} + \frac{\lambda_1 - \alpha}{\lambda_1 - \lambda_2} e^{-\lambda_1 t} + \frac{\alpha - \lambda_2}{\lambda_1 - \lambda_2} e^{-\lambda_2 t}\right)\Delta t \quad (2)$$

where

$$\lambda_{1/2} = \frac{1}{2}\{(\alpha + \beta + k_{-2}) \pm [(\alpha + \beta + k_{-2})^2 - 4\alpha k_{-2}]^{1/2}\};$$

$$\bar{n} = (Nc^2)/(K_1K_2).$$

It is Eq. 2 that should describe the data plotted in Fig. 4. This expression for $P(t, \Delta t)$ contains four variables, $\bar{n}$, α, β, and k_{-2}, where $\bar{n}$ gives the population of A_2R at equilibrium. Two of these (α and $\bar{n}$) may be evaluated independently of the time-dependent part of $P(t, \Delta t)$, so that when the theory is fitted to the data only β and k_{-2} are adjustable parameters.

A second probability function that characterizes the data has been illustrated in Fig. 5; it describes the likelihood with which the first opening transition after any closing transition will occur in the time interval $t < \tau \leq t + \Delta t$. It is a joint conditional probability defined as

$P(t, \Delta t; t) \equiv$ Probability (An opening transition occurs in the time interval τ, $t < \tau \leq t + \Delta t$, and no openings have occurred in the interval 0, t, conditioned upon a closing transition at $t = 0$.).

This is just the earlier probability $P(t, \Delta t)$ times the probability that no opening transitions occur before the interval specified. Note, however, that in deriving an expression for the joint probability $P(t, \Delta t; t)$, we do not need to consider the possibility that channels might open and close repeatedly, although such behavior necessarily affects Eq. 2 for $P(t, \Delta t)$. This simplification arises because the measurement begins at zero time when all channels are closed and ends at time t when the first opening transition is observed. Let us write a modified expression $n'(t)$ for the population of A_2R which takes this into account. Following the same approach that led to the equation for $n(t)$ above, we obtain in the low concentration limit,

$$n'(t) = \bar{n} + \exp[-(\beta + k_{-2})t].$$

This expression results in a modified opening probability term $P'(t, \Delta t)$ which is analogous to Eq. 2:

$$P'(t, \Delta t) = \beta\{\bar{n} + \exp[-(\beta + k_{-2})t]\}\Delta t.$$

Now, we can derive an expression for $P(t, \Delta t; t)$ by multiplying this modified opening probability $P'(t, \Delta t)$ with a term that specifies the probability that no opening transitions have occurred between times zero and t. Define $Z(0, t)$ as

$Z(0, t) \equiv$ Probability (No channels open in the interval τ, $0 < \tau \leq t$.)

Then the joint probability above becomes

$$P(t, \Delta t; t) = P'(t, \Delta t) \cdot Z(0, t). \quad (3)$$

A more explicit development of Eq. 3 now depends on evaluating $Z(0, t)$. Following the approach used to derive the Poisson distribution (Colquhoun, 1971), one can obtain a differential equation for $Z(0, t)$:

$$dZ(0, t)/dt = -\beta n'(t) \cdot Z(0, t).$$

Unlike the equivalent expression for a Poisson probability, however, the coefficient $n'(t)$ is explicitly dependent upon time. Nevertheless, the variables can be separated and the equation rewritten as

$$dZ(0, t)/Z(0, t) = -\beta n'(t)dt$$

and integrated to obtain the solution

$$Z(0, t) = \exp\left(-\beta\bar{n}t - \frac{\beta}{\beta + k_{-2}}\{1 - \exp[-(\beta + k_{-2})t]\}\right).$$

Combine this with the expression for $P'(t, \Delta t)$ in Eq. 3 to express $P(t, \Delta t; t)$, the probability that channels open after the waiting time t:

$$P(t, \Delta t; t) = \beta\{\bar{n} + \exp[-(\beta + k_{-2})t]\} \cdot \exp\left(-\beta\bar{n}t - \frac{\beta}{\beta + k_{-2}}\{1 - \exp[-(\beta + k_{-2})t]\}\right)\Delta t. \quad (4)$$

Before Eq. 2 and 4 were fitted to the measured results, the variables α and $\bar{n}$ were evaluated. The closing rate α was obtained from the distribution of open-channel durations as $\alpha = 1/\tau_{open}$, a relation predicted from the kinetic scheme (Colquhoun and Hawkes, 1977). The equilibrium number of AChR in A_2R, $\bar{n}$, was obtained by fitting the time-independent part of Eq. 2 to the time-invariant part of the data plotted as in Fig. 4. That is, because

$$\lim_{t \to \text{large}} P(t, \Delta t) = \beta\,\bar{n}\,\Delta t$$

we expect to find an average value $Q\beta\bar{n}$ in each time interval Δt in a histogram like Fig. 4, where the factor Q gives the number of "trials," or time segments, of which the histogram is comprised. Entries in the Fig. 4-type histogram occurring after the first two milliseconds were averaged to estimate the product $Q\beta\bar{n}$. Then, given Q for any particular experiment, the product $\beta\bar{n}$ was determined.

Next, Eq. 2 and 4 were fitted to the data plotted as in Figs. 4 and 5, respectively, to evaluate the remaining variables β and k_{-2}. Although both β and k_{-2} determine

the time constants in each probability expression, β alone controls the amplitude because it appears as a multiplier in both equations. Therefore β and k_{-2} were independently varied to optimize the quality of fit between data and theory using the same rates for both experimental assessments. The quality of fit was judged by eye and is illustrated in the figures.

In seven patches from six different cells, sufficient single-channel events were obtained to produce useful estimates of the variables $\bar{n}$, α, β, and k_{-2}. Acetylcholine was the agonist in three cases, carbamylcholine in the other four. Because we have no measure of the true transmembrane voltage, which is known to affect the kinetic rates (Magleby and Stevens, 1972), we cannot make a definitive comparison of the rates obtained with the two agonists. Nevertheless, in these examples the fitted variables were quite similar, independent of the agonist. The estimates for $\bar{n}$, the average population of the state A_2R, varied between 0.01–0.05 receptors. The directly measured closing rate, α, varied from 520–740 s^{-1}. The opening transition rate β showed much less variation: 750–825 s^{-1}, while the unbinding rate k_{-2} ranged between 3400–6000 s^{-1}. In terms of these rates, estimates of the mean open-channel duration, $\tau_{open} = 1/\alpha$, ranged from 1.35 to 1.92 ms, and the mean lifetime of the doubly liganded but closed state A_2R, $\tau_c = 1/(\beta + k_{-2})$, ranged from 150–280 μs.

DISCUSSION

Independent of the analysis, these results indicate that the probability with which an end-plate channel opens is transiently increased just after a closing transition occurs. This might happen by several entirely distinct mechanisms. It is possible that the enhanced opening probability reflects just an increased likelihood that the channel that just closed will reopen (Nelson and Sachs, 1979, Sakmann et al., 1980; Colquhoun and Sakmann, 1981; Cull-Candy and Parker, 1982); this is the explanation that was developed in the analysis section. We have portrayed the AChR as having separate but sequentially related capacities to (*a*) bind two agonist molecules and (*b*) briefly open its channel. When the channel closes, the AChR still retains the agonist molecules, and its channel has some likelihood of reopening until at least one of those molecules dissociates. Alternatively, it is plausible that channels, upon closing, cause a local perturbation which is sensed by adjacent channels and which increases their opening probability. We have not developed this idea in detail, but it surely could be formulated in a way that would accurately describe the data. Indeed, many models would fit the formalism necessitated by our data: at least three kinetic states, one of which represents the open channel. Thus, although we interpret k_{-2} as the unbinding rate of the first of two agonist molecules from the AChR, that picture is not unique. It could also represent, for example, an apparent rate for unbinding both agonist molecules. Likewise, τ_c, the lifetime of the closed state from which the AChR channel opens, cannot be uniquely depicted. Nevertheless, our description is consistent with most existing data on the binding and activation properties of the AChR, and it is similar to the kinetics of AChR in slow muscle fibers of these same snakes (Dionne and Parsons, 1981; Dionne, 1981).

This analysis of AChR kinetics has produced a measure of α, the rate of channel closing, and of two apparent rates, λ_1 and λ_2. These apparent rates are the quantities that would be evaluated in population studies of AChR kinetics. For example, current noise studies should produce spectral density measurements that exhibited corner frequencies given by $\lambda_1/2\pi$ and $\lambda_2/2\pi$. In addition the relative amplitude of the higher frequency component would be less than that of the lower. For the set of data that we have used to illustrate this paper the apparent rates are $\lambda_1 = 6821$ s^{-1} and $\lambda_2 = 522$ s^{-1}. In most AChR noise studies the highest frequency examined has been 1,000 Hz, so that presumably only λ_2 would have been resolved if such an experiment had been performed here. It has become commonplace to interpret the apparent rate λ_2 obtained from noise studies as α, because no methods have been available to describe it more precisely. However, in these data α was 14% $>\lambda_2$. Using either acetylcholine or carbamylcholine we found α to be 10–20% greater than the estimates that should presumably have been obtained from noise studies.

There are other statistical paradigms that might provide more reliable estimates of α than the nonlinear, unweighted least-squares approach that we used. Several of these were tested, including a group of nonlinear, weighted least-squares techniques and the maximum likelihood method; in each case the effect of truncating the data set was assessed. The weights that were used with the least-squares methods were approximately proportional to the expected variance. With no truncation of the data set, all of the weighted least-squares methods yielded values for α that were as much as 20% less than the comparable unweighted value. Truncating the data set reduced the discrepancy because the shift brought about by truncation was always markedly greater for the weighted estimates than that produced on the unweighted. Thus the weighted least-squares fits yielded a smaller α than that given in the text, and the value was more sensitive to arbitrary amounts of truncation. The maximum likelihood method was also examined. This approach was most efficient in terms of computational time at fitting the open duration histogram, largely because the data set has a multinomial distribution that depends only upon a single parameter, α. The distribution can be maximized with respect to α with a single computation involving the data set. For the entire data set used to illustrate this paper the maximum likelihood estimate of α was 555 s^{-1}, a value giving a nearly indistinguishable fit from that shown in Fig. 3. However, the maximum likelihood method was most sensitive to truncation. For example, when the longest 0.4% of the data values

(10 out of 2,305) were not included, the estimate of α increased to 577 s^{-1}, and when 4% of the set was deleted α rose to >700 s^{-1}. By comparison, this same amount of truncation caused a change in the unweighted least-squares estimate of <1 s^{-1}! Because of this extreme sensitivity to those entries that are most likely to be contaminated by unresolved brief closures, the maximum likelihood method was not used to fit the open-duration distribution.

Throughout this analysis of single-channel events it was assumed that the timing of all opening and closing transitions had been accurately measured. However, given the resolution limits that were imposed, both the measured open durations and closed intervals between events would necessarily have been corrupted on occasion by unresolved, very brief, open and closed transitions. Because it is the statistical treatment of event durations together with the intervals between events that have allowed us to estimate the kinetic rates of AChR activation, the question of how detrimental these unresolved transitions might be to our estimates must be examined. Below we will estimate the expected number of occurrences of the two most probable kinds of unresolved phenomena: brief open events, and brief closed episodes during what appeared as a single open event.

The unresolved number N_O, of brief open events which were of duration <300 μs may be estimated by integration of the open duration distribution between $t = 0$ and 300 μs. This gives the expression

$$N_O = N(e^{0.0003\alpha} - 1).$$

For the data set in this paper with $N = 2305$ and $\alpha = 593$ s^{-1}, $N_O = 449$.

Although this is a rather large number, it would have a noticeable effect only on our estimate of β, because that rate reflects the frequency of channel activity; an increase of $\leq$19% would be expected. There would be no effect on the estimate of α and only a very minor decrease in k_{-2} might be expected—much less than the uncertainty we attach to this value.

The unresolved number of brief closed episodes during what appeared as a single open event could be estimated in a similar manner as N_O, by extrapolation of the waiting time histogram (Fig. 5). However, this would not be a very satisfactory approach given these data. A more suitable estimate may be obtained by directly deriving the probability with which such closed episodes occur. These episodes are built of two sequential occurrences: the initial closing transition and the subsequent opening. Either one or two channels could be involved. Consider a hypothetical event of duration T during which only one channel appears to be open. The probability that a closing transition occurs during T is given by $p_c(T)$:

$$p_c(T) = \int_0^t \alpha e^{-\alpha t} dt = 1 - e^{-\alpha T}.$$

This must be multiplied by the probability $p_0(\tau_c)$ that an opening transition will occur within a minimum time τ_c following the closing transition. More precisely, we want the probability that only one opening will occur before τ_c transpires. Eq. 4 gives the correct probability density for this event; it needs to be sumed from $t = 0$ to τ_c to give

$$p_o(\tau_c) = \int_0^{\tau_c} \beta[\bar{n} + \exp[-(\beta + k_{-2})t]]Z(0, t)\, dt.$$

Then the probability that unresolved closed episodes interrupt an event of duration T is the product $p_o(\tau_c)p_c(T)$:

$$p_o(\tau_c)p_c(T) = p_o(\tau_c)[1 - e^{-\alpha T}].$$

For the exponential distribution of N resolved open intervals, it follows that

$$N_c = Np_o(\tau_c)[1 - (e^{-\alpha\tau_0})/2].$$

where τ_0 is the minimum open duration that we accepted (here 300 μs). We evaluated $p_o(\tau_c)$ conservatively with a two-segment piecewise linear approximation as $p_o(\tau_c) \doteq 0.08$. For the data in this paper we obtained $N_c = 118$ unresolved closed episodes during the set of 2,305 events. That is, ~5% of the events were really made by two or more successive openings. These events contaminated the open duration histogram with erroneous entries at longer times that should have contributed to the earlier bins. Correction for N_c would be expected to cause only minor changes in the three estimated rates α, β, and k_{-2}; the biggest change would probably occur in β with an increase of $\leq$5%.

In sum, the populations of the two major types of unresolved events that must be expected to contaminate any set of single channel data have been estimated. Under the conditions of these experiments unresolved events were relatively few in number and should not necessitate major corrections to the rate constants α, β, and k_{-2} that we have estimated.

It is useful to examine the internal consistency of the fitted kinetic model. Consider first the measured variable $\bar{n}$ which estimates the equilibrium number of AChR in the A_2R state at low agonist concentration. Typically, $\bar{n} << 1$ under our experimental conditions, and this must account for the low incidence of multiple events that occurred. This also suggests why the measured probability expressions had such prominent time-dependent components, inasmuch as they appear to have been derived from the 20–100-fold increase in the population of the doubly liganded closed channel state that occurred when one open channel closed. An estimate of the total number N of AChR in each membrane patch can be obtained from $\bar{n}$ if reasonable values for the equilibrium binding constants are used. Assuming 300 μM for both K_1 and K_2 with carbamylcholine and 30 μM with ACh we find an average $N \simeq 1{,}100$ channels per patch.

As a second point, we note that the macroscopic forward binding rate k_{+2} for the second agonist molecule may be estimated from the equilibrium binding constant and the

measured unbinding rate k_{-2} for the agonist. We obtain $k_{+2} \simeq 1.3 \times 10^7\ M^{-1}\ s^{-1}$ for carbamylcholine and $\sim 1.5 \times 10^8\ M^{-1}s^{-1}$ for ACh. These are one to two orders of magnitude less than a diffusion-limited rate and are similar to other published estimates. Sheridan and Lester (1977), in a study of evoked postsynaptic currents in *Electrophorus electricus* electroplaques, obtained a comparable forward rate of $10^7\ M^{-1}\ s^{-1}$ for carbamylcholine. In *Torpedo* AChR the association rate of ACh with the receptor has been estimated at $5.7 \times 10^7\ M^{-1}\ s^{-1}$ using a rapid-mixing ultrafiltration method (Boyd and Cohen, 1980).

The kinetic treatment of channel activation which we have produced tacitly omitted all second-order effects on the probability functions $P(t, \Delta t)$ and $P(t, \Delta t; t)$. For example, transient increases in the population of A_2R caused by the near simultaneous closing of two or more channels were neither considered theoretically nor excluded from the data set. Nor was the possibility considered that some of the larger events that appeared to be single-channel currents could have been produced by the simultaneous activation of two independent channels. If we estimate the frequency of occurrence of second-order effects from the observed rate at which single opening transitions occurred, <0.1% of the opening transitions should have been altered by an undetected closing transition, and the likelihood of mistaken identification of a double event as a single should have been even less. Because the average number of single events in our record sets was $\sim 10^3$, second-order effects should have been quite negligible. Although we have not evaluated the accuracy of our estimates of the transition rates, we have observed that changes of as little as 100 and 250 s^{-1} in β and k_{-2}, respectively, noticeably reduce the quality of the fit in Fig. 4 and 5. Nevertheless, our estimates of the transition rates must be regarded as preliminary given the resolution of our recordings.

A great variety of single-channel mechanisms are susceptible to the analytical method that we have described here. The essential assumption is that the process as a whole is quasistationary. Given that, if the channels operate independently, shuttling between two kinetic states (opened and closed), the probability of occurrence of opening transitions $P(t, \Delta t)$ will be time independent while the probability of opening transitions occurring after a waiting period $P(t, \Delta t; t)$ will have a single exponential distribution in time. This would be the simplest behavior possible for a channel mechanism, and fitting the probability measurements would allow both transition rates in a two-state kinetic scheme to be evaluated. The appearance of greater temporal complexities in the probability descriptors, such as we have observed for the AChR, would be evidence of at least three kinetically distinct states of the channel, only one of which represents the open conformation.

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