SINGLE CHANNEL KINETICS OF A GLUTAMATE RECEPTOR

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ABSTRACT The glutamate receptor-channel of locust muscle membrane was studied using the patch-clamp technique. Muscles were pretreated with concanavalin A to block receptor-channel desensitization, thus facilitating analysis of receptor-channel gating kinetics. Single channel kinetics were analyzed to aid in identification of the molecular basis of channel gating. Channel dwell-time distributions and dwell-time autocorrelation functions were calculated from single channel data recorded in the presence of 10^{-4} M glutamate. Analysis of the dwell time distributions in terms of mixtures of exponential functions revealed there to be at least three open states of the receptor-channel and at least four closed states. Autocorrelation function analysis showed there to be at least three pathways linking the open states with the closed. This results in a minimal scheme for gating of the glutamate receptor-channel, which is suggestive of allosteric models of receptor-channel gating.

INTRODUCTION

Most neurotransmitters act via receptor-mediated gating of ion channels in the membranes of excitable cells. Although the broad outlines of this process are understood, we remain uncertain concerning the molecular events of channel gating. The advent of single channel recording (Neher and Sakmann, 1976) has enabled kinetic analysis of channel gating mechanisms and it is anticipated that the results of such studies will complement structural and biochemical investigations and lead toward a detailed description of neurotransmitter action.

L-glutamic acid is probably an important excitatory neurotransmitter within vertebrate central nervous systems (Usherwood, 1978; Nistri and Constanti, 1979; McLennan, 1983; Fonnum, 1984) but for a variety of reasons transmitter receptor interactions at the putative glutamatergic synapses are not well understood. However, L-glutamate also mediates transmission at synapses on arthropod skeletal muscle and postjunctional receptors at these sites have somewhat similar properties to the quisqualate type of glutamate receptor that possibly occurs postjunctionally at vertebrate, central glutamatergic synapses. Here we present recent results of single channel recordings and kinetic analyses of the glutamate receptor-channel (GluR) found on locust muscle membrane. Access by patch pipette to postjunctional glutamate receptors on locust muscle would be difficult to achieve, but similar receptors for L-glutamate are found on immediately accessible extrajunctional muscle membrane in this system.

Most receptor-channels are subject to desensitization, i.e., inactivation during prolonged exposure to agonist concentrations optimal for channel opening (Gration et al., 1980; Sakmann et al., 1980) and this complicates analysis of single channel kinetics as it precludes recordings from a single-receptor channel for extended periods of time. Although the receptors for L-glutamate which are found both junctionally and extrajunctionally on locust muscle readily desensitize (Usherwood and Machili, 1968; Cull-Candy and Usherwood, 1973), this may be blocked by prior treatment of the muscle with either concanavalin A (Mathers and Usherwood, 1976, 1978) or with other lectins with similar sugar specificities (Evans and Usherwood, 1985). This allows long-duration recordings to be made from single receptor-channels over a wide range of glutamate concentrations. Furthermore, the low population density of the extrajunctional glutamate receptor-channels enables recordings to be made routinely from single channels, again facilitating subsequent kinetic analysis. A further advantage is that the GluR has a relatively high single channel conductance (115–150 pS; e.g., Patlak et al., 1979), which enables single channel recordings to be made with megaohm seals from intact adult muscle, without pretreatment with "cocktails" of proteolytic enzymes (Neher and Sakmann, 1976). In this way possible perturbation of single channel properties by limited proteolysis is avoided.

MATERIALS AND METHODS

Experimental procedures were similar to those described in previous publications (Patlak et al., 1979; Gration and Usherwood, 1980; Gration et al., 1982). L-glutamic acid was purchased from the Sigma Chemical Co. Ltd., Poole, Dorset, England, and were of the highest quality available. Single channel currents were recorded from extrajunctional D-receptors of innervated metathoracic extensor tibiae muscle fibers of female adult locusts (*Schistocerca gregaria*) 7–10 d post-fledging. Muscles were pretreated with a solution of 1–2 μM concanavalin A in saline for 30 min. During recordings this solution was replaced by standard

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locust saline (180 mM NaCl; 10 mM KCl; 2 mM $CaCl_2$; 3 mM HEPES; pH adjusted to 6.8 with NaOH). All studies were undertaken at room temperature (21–23°C). Patch-clamp recordings were restricted to fibers in the midventral region of the muscle that are exclusively innervated by a single glutamatergic neuron (Cochrane et al., 1972). Muscle fibers were clamped at −100 mV using a conventional 2-electrode technique. The signal-to-noise ratio was typically >13.

The patch amplifier was similar to that described by Hamill et al. (1981). Patch electrodes with ~2 μm fire-polished tips were pressed onto the surface of a muscle fiber and the resultant single-channel currents stored on an FM tape recorder. All data were analyzed off-line. On play-back, current records were filtered at 3 kHz. Channel openings were detected using the automated procedure described by Gration et al. (1982). The reduced data comprised a series of dwell times (open times and closed times) that were stored and further analyzed on a PDP 11/34 minicomputer (Digital Equipment Corp., Maynard, MA). Long channel records were reduced to a series of data sets, each comprising 2,000 channel events. A typical data record, with details of the data reduction scheme, is shown in Fig. 1.

Kinetic Analysis

The principles of analysis of single channel kinetics have been described by Colquhoun and Hawkes (1977, 1981, 1983) and by Labarca et al. (1985) (also see Fredkin et al., 1985). We routinely examined channel dwell-time distributions and correlations between successive dwell times.

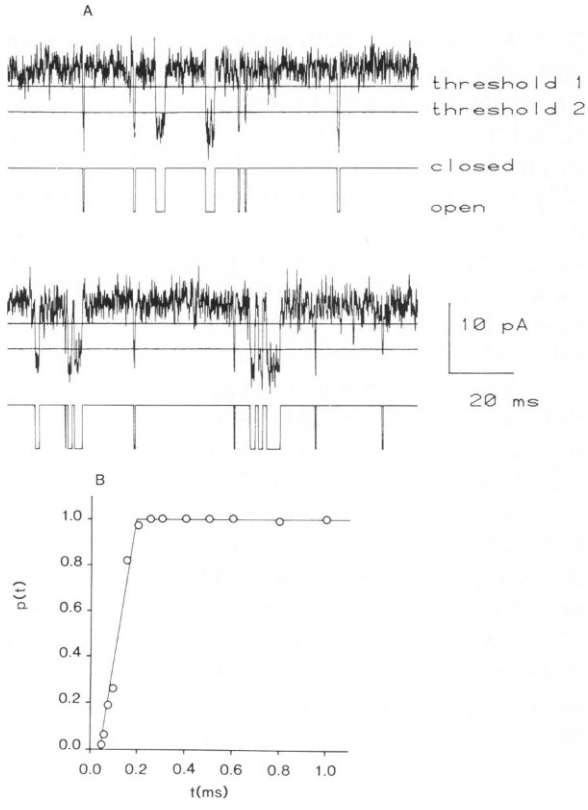

FIGURE 1 (*A*) A segment of a typical data record, obtained in the presence of 10^{-4} M L-glutamate in the patch electrode, with the channel detection thresholds depicted. The beginning of a channel opening (end of a closing) corresponds to the time when the current crosses threshold 2, having previously crossed threshold 1. Similarly, the end of an opening (beginning of a closing) corresponds to the crossing of threshold 1, threshold 2 having previously been crossed. (See also Gration et al., 1982). (*B*) The probability of detection ($p(t)$) of brief events is shown as a function of event duration (t; see text). The points represent the result of the calibration; the solid line is the fitted probability of detection function.

From analysis of channel dwell-time distributions in terms of the underlying dwell-time probability density functions (pdfs) one may estimate the numbers of open states (N_o) and closed states (N_c) of the receptor channel. The open and closed time pdfs ($f_o(t)$ and $f_c(t)$, respectively) are finite mixtures of exponential distributions with N_o and N_c components

$$f_o(t) = \sum_{j=1}^{N_o} (\alpha_j/\tau_j) \exp(-t/\tau_j);$$

and

$$f_c(t) = \sum_{j=1}^{N_c} (\alpha_j/\tau_j) \exp(-t/\tau_j),$$

where τ_j are the time constants ($1/\tau_j$ being the corresponding apparent closing or opening rates), and α_j the respective mixture proportions.

Dwell-time pdfs were estimated by analysis of dwell-time histograms. Since the histograms were required to encompass events of duration <0.3 ms to >300 ms, they were constructed using variable bin widths. Mixtures of exponential functions were fitted to dwell-time histograms using a Gauss-Newton-Marquardt algorithm (Marquardt, 1963), initial parameter estimates having been obtained using an interactive curve-peeling program.

Failure to detect brief channel events results in a deficiency of events in the early bins of the histograms (see below), which were omitted, therefore, from consideration when estimating the dwell time pdfs (see Roux and Sauvé [1985] for a discussion of this problem). To detect components of the pdfs with high τ values, it was necessary to obtain recordings of ~10^4 channel openings from the same membrane site.

The problems inherent in objective determination of the number of components in a mixture of exponential distributions have been discussed by Everitt and Hand (1981) and an approach suitable for the analysis of channel dwell-time histograms has been described by Colquhoun and Sigworth (1983). We have followed the approach described by the latter authors, aided by application of the Akaike information criterion (AIC) (Akaike, 1974) and the Schwarz criterion (SC) (Schwarz, 1978), as discussed by Landaw and DiStefano (1984). In brief, pdfs were fitted using successively higher values of N_o and N_c until no further improvement in fit, as estimated by both visual inspection and AIC and SC analysis, was achieved. This process was repeated for data collected from several sites under the same conditions and an estimate of N_o and N_c obtained in each case.

In investigating correlations between successive dwell times, the complexities of the observed dwell time pdfs rendered a simple binominal approach such as that of Jackson et al. (1983) inapplicable. Instead we approached the problem via estimation of channel open time and closed time autocorrelation functions (acfs) (Fredkin et al., 1985; Labarca et al., 1985). The acfs were used to probe the dependency of successive channel dwell times. For example, if one considers pairs of open times where the second opening is k openings after the first, then the acf is arrived at by evaluating the product of the deviations of the two open times from the overall mean open time. Averaging this product over all such pairs gives the covariance of the ith and $(i + k)$th openings. This covariance is calculated for values of k from $k = 1$ (neighboring pairs of openings) to $k = M/4$ (where M is the total number of openings), and is normalized (by division by the open time variance) to give the acf. In the absence of any correlation, $r(k)$ is normally distributed (Kendall and Stuart, 1966) with mean $-1/M$ and variance $1/M$. So, one can set approximate 95% confidence limits at $-1/M \pm z\sqrt{M}$ and use these in assessing the significance of any apparent correlation. More formally, by representing the open time series and closed time series of a reduced data set by $t_o(i)$ and $t_c(i)$, respectively, the dwell time acfs are given by

$$r_o(k) = \mathrm{Cov}[t_o(i), t_o(i + k)]/\mathrm{Var}[t_o(i)]$$

and

$$r_c(k) = \text{Cov}\,[t_c(i), t_c(i + k)]/\text{Var}[t_c(i)],$$

where $r_o(k)$ and $r_c(k)$ are the open and closed time acfs at lag k, where Cov denotes covariance and Var denotes variance.

In addition to analytical relationships between channel gating models and single channel kinetics, it is useful to be able to simulate the single channel behavior of a particular model. In these studies a simulation algorithm similar to that described by DeFelice and Clay (1983) was used but it was modified to simulate failure to detect all brief channel events in the following manner.

Let $p_d(t)$ be the probability of detecting an event of length t. If an event of length t is generated then, using $p_d(t)$ and a random number drawn from the unit rectangular variate, one can simulate whether or not it is detected. If one considers an open event that is not detected, then the preceding and following closed events will sum to give an artefactually long closed time. Calibration of Probability of Detection of Brief Events The recording and detection system causes reduced efficiency of detection of brief events. It is clearly of importance to calibrate such an effect, both in terms of analysis of the resultant reduced data and to estimate the function $p_d(t)$ used in the channel simulation program.

Square wave pulses of known duration were filtered at 3 kHz and a signal-to-noise ratio comparable to that in the experimental data imposed. The resultant records were reduced by the channel detection program in the usual manner. The efficiency of detection of the short pulses as a function of pulse duration was estimated (Fig. 1 *b*). Examination of the resultant calibration curve showed the following to be a reasonably accurate model of the probability of event detection $p_d(t)$ as a function of event duration (t):

$$t \leq 0.05 \text{ ms}, p_d(t) = 0$$

$$0.05 \text{ ms} < t \leq 0.19 \text{ ms}, p_d(t) = (t - 0.05)/0.14$$

$$0.19 \text{ ms} < t, p_d(t) = 1.$$

This function has been employed in simulation studies to judge the effect of failure to detect brief events on dwell time pdfs. The resultant pdfs (not shown) peaked at ~200 μs, comparable to the experimentally observed dwell time distributions. Similar studies were carried out to test whether or not spurious additional exponential components could be generated by failure to detect brief events. In none of the simulations was the number of exponential terms required to fit the resultant distribution greater than that predicted on the basis of the underlying gating model, a result that is in agreement with the recent work of Roux and Sauvé (1985).

RESULTS

Data Analyzed

Dozens of recordings were obtained in the presence of 10^{-4} M L-glutamate in the patch pipette. However, after application of the stringent criteria of W 3:1 signal-to-noise ratio, single channel activity, and long, continuous recordings, data from four recording sites on four muscle preparations remained (Table I). Subsequent analysis concentrated on these data. Similar data have been recorded over a range of glutamate concentrations — their analysis will form the subject of a later paper.

The single channel kinetics may be initially characterized in terms of the mean channel open time (m_o) and mean channel closed time (m_c), or in terms of the frequency of channel openings (f) and probability of the channel being in the open state (P_o). Comparison of these values for the four data sets revealed the variation between different membrane sites to be relatively small. It is important to check that there is no trend with increasing time in either m_o or m_c during a lengthy recording from a single membrane site. Data sets were divided into blocks of 2,000 channel events (see Methods). To test for such a trend, m_o and m_c were plotted against data block number (not shown). There was no obvious systematic trend in either parameter. Gration et al. (1981) pointed out that the number of events in successive intervals Δt did not follow Poisson statistics, and in particular that the ratios of the variance (v) to mean (m) number of events in Δt were greater than unity. We have subsequently shown that high limiting values (~100) of v/m may be generated from nonlinear receptor-channel gating schemes (Ball et al., 1985; Ball, personal communication), and so are not in themselves indicative of nonstationary channel kinetics.

TABLE I
DATASETS EMPLOYED IN THIS STUDY

Data set name	Total recording time(s)	Total number of events	p_o
G14CK1	261	16 000	0.0787
G14CK2	202	10 000	0.0348
G14CK3	223	10 000	0.0502
G14CK4	147	8 000	0.0626

Mean (±SD) 0.0566 (±0.0161).
Each dataset was derived from recordings at a single membrane site with 10^{-4} M L-glutamate inside the patch pipette. p_o is the probability of the channel being in the open state.

Open Time Distributions

All four data sets required a mixture of three exponential functions to obtain satisfactory fits to the observed open time distributions (Table II). Note that the open-time distribution (Fig. 2) is displayed using a log-linear scale — this is necessary if one is to be able to estimate whether a satisfactory fit had been obtained from a single graph.

The time constants for the three exponential components were of the order of 0.40 , 1.2, and 3.5 ms. These are not widely separated, a factor that makes their independent estimation difficult. However, attempts to fit the observed distributions with a smaller number of exponential components were unsuccessful.

In early work on the GluR, the apparent peak in the open time distribution was discussed in terms of possible mechanistic implications (see Methods). More recent evidence suggests that it is simply the result of the failure to detect brief (<200 μs) channel openings at full efficiency. First, as pointed out above, simulation studies incorporating a model of the probability of event detection generate a similar peak in the resultant open time histograms. Second, to test this hypothesis further, the number of observations in each bin of the open time distribution was corrected for

TABLE II
DWELL TIME PDFS AND ERRORS IN THEIR ESTIMATION

	i	α_i	CV_W	CV_B	$1/\tau_i$	CV_W	CV_B
			%	%	ms^{-1}	%	%
Open time $N_o = 3$	1	0.440	24	25	2.53	13	14
	2	0.527	15	18	0.869	43	19
	3	0.0330	130	54	0.294	350	17
Closed time $N_c = 4$	1	0.214	4.1	32	2.48	8.2	8.9
	2	0.373	19	20	0.135	12	18
	3	0.353	19	21	0.0499	16	21
	4	0.0604	23	42	0.0106	17	21

The dwell time pdfs are described by: $f_e(t) = \Sigma_{i=1}^{N_e}(\alpha_i/\tau_i)\exp(-t/\tau_i)$, where e = o (open) or c (closed).
CV_W and CV_B are the coefficients of variation for the parameter in the column to the left, within and between data sets, respectively. They are calculated as follows: $CV_W = \sigma_W/\mu$; $\sigma_W^2 = 1/4\ \Sigma_{i=1}^{4}\sigma_i^2$, $CV_B = \sigma_B/\mu$; $\sigma_B^2 = 1/4\ \Sigma_{i=1}^{4}(\mu_i - \mu)^2$, where μ is the overall mean value of a parameter, μ_i and σ_i are the estimated mean and standard deviation of that parameter for a single dataset, and summation is over the four data sets in question.
The parameter values given are the overall mean values for the four data sets.

the probability of detection. The corrected distribution (not shown) no longer shows a peak and the corrected observations correspond closely with those values predicted on the basis of the pdf fitted to data points for $t > 0.2$ ms. A similar analysis has been applied to the closed time data and similar results obtained.

Closed Time Distributions

In all data sets, four components were required for a satisfactory fit to the close time distributions (Table II and Fig. 3). The time constants for the four components are of the order of 0.41, 7.6, 21, and 99 ms. The first component, corresponding to very brief closings, is clearly separated from the other three. Again, attempts to fit three or fewer exponential components to the data were unsatisfactory.

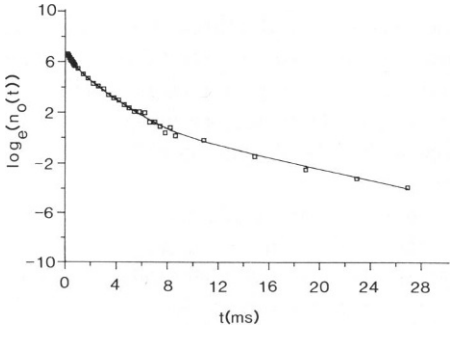

FIGURE 2 Open time distribution derived from data set G14CK1. The histogram was constructed using three binwidths — 0.04 ms for the first 20 bins; 0.4 ms for the next 20 bins, and 4.0 ms for the remaining bins. A log-linear plot of the distribution is shown ($\log_e(n_o(t))$ vs. t). Points ( ) represent the observed distribution and the solid line represents the function fitted using three exponential components (see text for details).

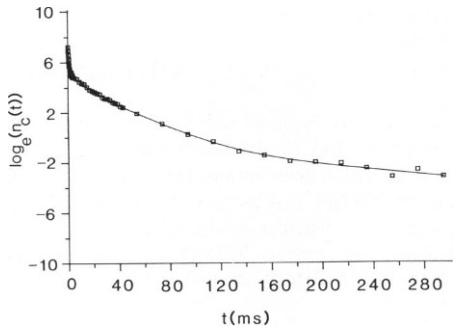

FIGURE 3 Closed time distribution derived from data set G14CK1. The histogram was constructed using three binwidths — 0.2 ms for the first 20 bins; 2.0 ms for the next 20 bins, and 20.0 ms for the remaining bins. As in Fig. 2, a log-linear plot of the distribution is shown ($\log_e(n_c(t))$ vs. t), which points ( ) representing the observed distribution and a solid line representing the function fitted (see text).

Errors in Estimation of Dwell Time Pdfs

Inversion of the normal matrix obtained during nonlinear least-squares estimation of dwell time pdf parameters allows one to calculate the standard deviations of the parameter estimates. Using these estimated standard deviations, it has been possible to calculate the coefficient of variation of each parameter within the data sets (CV_W). Comparison of these values with the coefficients of variation between the data sets (CV_B; see Table III) reveals that for most of the dwell time pdf parameters CV_W is of the same order as CV_B. This suggests that there is little variation between different membrane sites that cannot be accounted for by variation at a single membrane site. This strengthens the belief that our single channel recordings were obtained from members of a homogeneous population of receptor-channels.

Correlations Between Successive Dwell Times

Autocorrelation functions were calculated from both the open time and closed time series of all four data sets. In each case, weak positive acfs were observed (Table III and

TABLE III
DWELL TIME ACFS AND ERRORS IN THEIR ESTIMATION

	i	A_i	CV_W	CV_B	π_i	CV_W	CV_B
			%	%		%	%
Open time $N_p - 1 = 2$	1	0.061	18	25	0.97	1.6	2.4
	2	0.035	50	49	0.46	240	16
Closed time $N_p - 1 = 2$	1	0.063	34	38	0.97	1.6	1.1
	2	0.17	57	39	0.80	5.8	6.8

In both cases the acfs were fitted by $r(k) = A_1\pi_1^k + A_2\pi_2^k$
The coefficients of variation (CV_W and CV_B) were calculated as described in Table II. The parameter values given are the averages over the four datasets studies.

Fig. 4), with the correlations persisting up to (and beyond) lag $k = 40$. The strength and degree of persistence of the acfs are determined by, among other things, the relative rates of channel open-closed isomerizations compared with channel closed-closed and channel open-open transitions (see below).

As pointed out by Fredkin et al. (1985), if there are N_p isomerization pathways linking the channel open states with the channel closed states, then the dwell time acf will be a sum of geometrically decaying functions with up to $N_p - 1$ components

$$r(k) = \sum_{j=1}^{N_p \leq 1} A_j \pi_j^k.$$

Geometric decay of a function of a discrete variable (k) is comparable to exponential decay of a function of a continuous variable (t). So, plotting a geometrically decaying function on log-linear scales results in a series of points lying on a straight line. Plotting a sum of geometrically decaying functions results in a curve that can be dissected into a number of straight lines. This allows $N_p - 1$ to be estimated in the same way as N_o and N_c were estimated.

Replotting the acfs on log-linear scales (Fig. 4 *b*) reveals two geometrical components. Therefore, we may reasonably conclude that N_p R 3, i.e., that there are at least three pathways linking the open states of the GluR to the closed states.

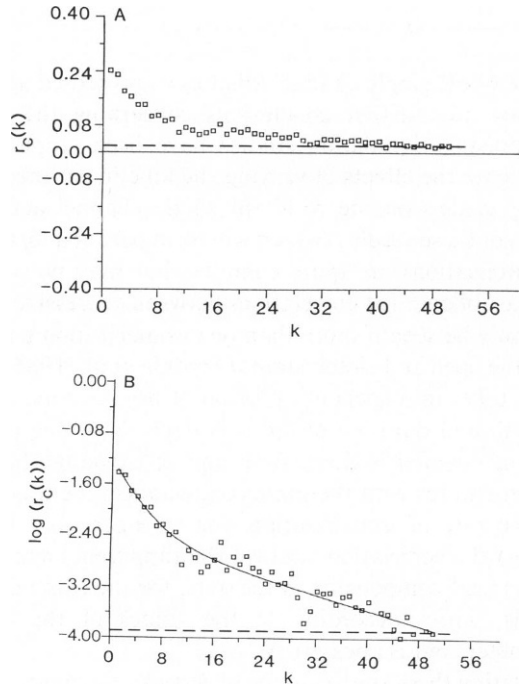

FIGURE 4 The autocorrelation function derived from the closed times of data set G14CK3. Similar results were obtained from analysis of the open times. *A* shows the autocorrelation function ($r_c(k)$) on a linear scale, with positive autocorrelation persisting to values of k of ~40. In *B* the same data are shown on a log-linear scale. The solid line shows the result of fitting the sum of two geometrical decays to the observed acf (see text for details). In both diagrams the broken line indicates the 95% confidence limit for zero correlation, set at $-1/M + 2/\sqrt{M}$, where $M = 10{,}000$ (see text).

The fitted acfs are given in Table III. Both the open and closed time acfs are biphasic, with an initial stronger correlation that decays rapidly with increasing k, and a weaker correlation that persists for longer. (Remember that k refers to the number of intervening channel events rather than time.) Analysis of coefficients of variation, comparable to that carried out for the dwell time pdfs, suggests that the variation between data sets can be accounted for by the variation within individual data sets.

DISCUSSION

Complex Kinetics of the Glutamate Receptor Channel

From analysis of the dwell time distributions certain conclusions may be drawn concerning the complexity of the underlying gating mechanism of the GluR. However, it should be remembered that the estimates of the number of channel states, N_o W 3 and N_c W 4, are minimum estimates.

Why is this so? Clearly the finite time resolution of the channel detection system (i.e., patch clamp amplifier plus window discriminator) sets an overall dead time. For the signal-to-noise ratio of the current studies this has been shown to be ~200 μs; any channel states with briefer mean lifetimes are unlikely to be detected. Therefore, the present description of single channel kinetics deals with only relatively long duration states. Second, the analysis of the dwell time distributions would be unlikely to detect two exponential components if their time constants differed by a factor of <2. In this way, channel states with similar lifetimes would be unresolved. Finally, and this is possibly the most important reservation, at any one agonist concentration only those receptor-channel states significantly populated at that concentration are likely to be detected. A range of concentrations may have to be investigated to provide kinetic evidence for each state, particularly if multiple agonist binding sites exist. To overcome this limitation we are at present pursuing a detailed investigation of GluR kinetics as a function of glutamate concentration.

The results of the acf analysis show that channel dwell times are correlated. Such correlations explain the "state-switching" of the GluR noted by Patlak et al. (1979), and the apparent nonstationarity of the GluR analyzed by Gration et al. (1981), the latter having been discussed further by Ball et al. (1985).

In light of the recent work of Roux and Sauvé (1985) on the effects of omission of brief channel events on the analysis of single channel kinetics, it is important to ask how such effects could distort the observed acf. We have approached this problem both theoretically and via simulation studies. Using techniques from stochastic process

theory, the situation in which brief events have been omitted has been analyzed (Ball, manuscript in preparation) to obtain a matrix/vector expression for the acf of successive detected open or closed intervals. For gating models producing acfs similar to those observed, numerical calculations have shown that the acf will still be a finite sum of geometrically decaying terms. The number of such terms is the same as when all events are detected but their values will be slightly modified. Similarly, the results of the simulation studies suggest that, for gating models producing acfs similar to those observed, omission of brief events changes the measured acf parameters somewhat, but does not increase the apparent number of geometrically decaying terms. Furthermore, simulations based on models that do not exhibit correlations between successive channel dwell times do not show artefactual correlations when omission of brief events is included.

Elimination of Simple Gating Models

The kinetic properties of the GluR may be summarized as follows: (*a*) number of open states, N_o R 3; and (*b*) number of closed states, N_c R 4; and (*c*) number of isomerization pathways, N_p R 3. How do these impinge upon views concerning models for the gating process? Such results could be explained by receptor channel heterogeneity, e.g.,

$$\begin{array}{ccccccc} C_1 & \longrightarrow & C_1A & \longrightarrow & O_1A & \longrightarrow & \\ C_2 & \longrightarrow & C_2A & \longrightarrow & O_2A & \longrightarrow & O_2A^* \end{array}$$

(where C denotes the closed channel, O the open channel, and A the agonist).

There are several lines of evidence against such a proposal. Principally, only a single conductance (Patlak et al., 1979) has been observed for the GluR — no subconductance levels have been detected; therefore, any receptor heterogeneity would have to reside in channel gating properties rather than channel conductance. The analysis of errors in estimation of the dwell time pdf parameters suggests that there is little, if any, site to site variation and so heterogeneity of receptor-channel gating kinetics seems unlikely. Furthermore, operation of two or more independent channels with different kinetics might be expected to result in higher correlations between successive channel dwell times that those observed.

To what extent may we be certain that our channel records do correspond to the operation of a single receptor channel? All of the records were extensively checked for multiple channel openings before being accepted for further analysis. It is possible to calculate the probability of multiple openings when more than one channel is present (Patlak and Horn, 1982; Colquhoun and Hawkes, 1983). From analysis of data collected in the presence of 10^{-4} M glutamate we estimate that the presence of two channels would be signaled by simultaneous openings for 0.4% of the total recording time. We are confident that had this been the case it would have been detected by our procedure. Furthermore, had different membrane patches contained different numbers of receptor-channels, we would have expected to see considerable site to site variation in channel kinetic parameters, which clearly was not the case.

The correlation between successive channel dwell times excludes linear gating schemes, e.g.

$$C \longrightarrow CA \longrightarrow OA$$

or

$$C \longrightarrow CA \longrightarrow OA \longrightarrow OA_2$$

because these have only a single pathway linking to the close channel states with the open states. Less obviously, gating mechanisms such as

$$C \longrightarrow CA \begin{array}{l} \nearrow O_2A \\ \searrow O_1A, \end{array}$$

which possess a single "gate-way" (Fredkin et al., 1985) closed state cannot result in correlation of successive dwell times and so are eliminated.

The types of model remaining are as follows:

$$\begin{array}{ccccccccc} C_1 & \longrightarrow & C_2 & \longrightarrow & C_3 & \longrightarrow & C_4 & \dashrightarrow & ? \\ \uparrow\downarrow & & & & & & \uparrow\downarrow & & \\ O_1 & \longrightarrow & O_2 & \longrightarrow & O_3 & \dashrightarrow & ? & & \end{array} \qquad \begin{array}{l} N_c \geq 4 \\ N_p \geq 3 \\ N_o \geq 3 \end{array}$$

Do the GluR single channel kinetics as presented allow us to draw any further conclusions concerning the gating mechanism? Simulation studies have been employed to investigate the effects of varying the kinetic parameters of gating models on the resultant single channel acfs. The results of these studies (which will form part of a forthcoming publication) are quite complex, but may be summarized as follows: (*a*) correlations between successive events may only be seen if more than one isomerization pathway links the open and closed states (Fredkin et al., 1985), even if one takes into account omission of brief events; (*b*) the strength and duration of the acfs depends on the relative rates of channel isomerization and of exchange between different states with the same conductance: the higher the relative rate of isomerization, the stronger (and longer-lasting) the correlation; and (*c*) the (apparent) number of geometrical components in the acfs, for the same class of models, varies according to the values of the kinetic parameters but is always r $N_p - 1$.

Relating these studies to the observed acfs suggests that, at 10^{-4} M L-glutamate, the channel isomerizations (open-closed) are of comparable rate to the open-open and closed-closed transitions. Studies currently underway in our laboratory will test this observation in a more quantitative manner.

It is helpful to note, at this stage, that one might account for the presence of multiple, heterogeneous channels under patch electrode in terms of a model with rapid channel

isomerizations and slow transitions between different closed states (representing switching between different channel types). In this situation the acf would be expected to be long-lasting, with a duration related to the rate of switching between the different channel types.

Relationship to Single Channel Kinetics of Other Systems

It is not entirely surprising that the single channel technique has revealed unexpected complexities in the gating mechanism of the GluR. Single channel kinetic studies on the nicotinic acetylcholine receptor (Sakmann et al., 1980; Colquhoun and Sakmann, 1981; Jackson et al., 1983; Auerbach and Sachs, 1984; Labarca et al., 1984; Leibowitz and Dionne, 1984; Montal et al., 1984; Sine and Steinbach, 1984; Labarca et al., 1985), the sodium channel (Aldrich et al., 1983), on Ca^{2+} channels (Hess et al., 1984) and on Ca^{2+}-activated K^+ channels (Magleby and McManus, 1984) have revealed comparable complexities.

Multiple open and closed states for the acetylcholine receptor have now been detected using several different preparations. Multiple states for the locust GluR were first alluded to in the publication of Patlak et al. (1979) and in subsequent publications from our laboratory (Gration et al., 1981, 1982). Cull-Candy and Parker (1982) described rapid kinetics for this system suggesting two open states for the GluR. However, the results presented here suggest that the GluR is exceptional in having at least three open states. Most observers have only detected two for the AChR, although Labarca et al. (1985) have suggested the existence of three open states for the *Torpedo* AChR. On the other hand, more closed states for the AChR have been reported than for the GluR. Perhaps this is related to our inhibition of desensitization in the latter system with concanavalin A.

It is interesting to compare the time constants for the GluR dwell time pdfs with those obtained from analysis of single channel kinetics of nicotinic AChRs of BC_3HI cells by Sine and Steinbach (1984). In the presence of 10^{-4} glutamate, the time constants for the GluR open time pdF were $\tau_1 = 0.4$ ms; $\tau_2 = 1.2$ ms, and $\tau_3 = 3.5$ ms. For the AChR in presence of 3×10^{-4} M acetylcholine, the open time pdf time constants were $\tau_1 = 0.20$ ms and $\tau_2 = 24.2$ ms. While the briefer components of the two systems are comparable, the two longer components of the GluR open time pdf are both shorter than τ_2 of the AChR open time distribution. This is a clear difference between the behavior of the two types of receptor.

Turning to the closed time distributions, the AChR kinetics for the BC_3HI cells reveal five exponential components to the closed time distribution in the presence of 3×10^{-4} M acetylcholine, with time constants $\tau_1 = 0.055$ ms, $\tau_2 = 0.37$ ms, $\tau_3 = 3.0$ ms, $\tau_4 = 35$ ms, and $\tau_5 = 1{,}220$ ms. Of these, the briefest (corresponding to $(1/\tau) = 18.2$ ms^{-1}) would not be detected with the megaohm recording system. The second component for the AChR closed time distribution ($\tau_2 = 0.37$ ms) is closely comparable to the first component ($\tau_1 = 0.41$ ms) for GluR. The nicotinic system also differs in having a much longer time constant ($\tau_5 = 1{,}220$ ms) component to the closed time distribution. The absence of such a component from the GluR kinetics may be due to the blockade of GluR desensitization by concanavalin A.

Correlations between successive dwell times have been observed for the AChR of cultured rat skeletal muscle (Jackson et al., 1983) and of *Torpedo* (Labarca et al., 1985). It seems unlikely, therefore, that either the nicotinic AChR or the GluR follow the simple linear gating scheme originally proposed by Katz and Miledi (1972). Furthermore, recent studies concerning the voltage dependent gating of Ca^{2+} channels (Hess et al., 1984) and the gating of Ca^{2+} dependent K^+ channels (Magleby and McManus, 1984) may also be explained in this manner.

The next step in analysis of the gating of the GluR will be to develop a kinetic scheme capable of explaining the single channel data. A reasonable starting point in the search for such a scheme is the allosteric model (Karlin, 1967), based on that for regulation of enzyme activity (Monod et al., 1965) and described in terms of single channel kinetics by Colquhoun and Hawkes (1977, 1983). Such a model would propose that the multiple open and closed states of the GluR correspond to different numbers of glutamate molecules bound per receptor-channel complex. This would correspond with the known oligomeric nature of membrane proteins in general (Klingenberg, 1981) and the AChR in particular (Changeux, 1981). Unfortunately, such biochemical information is not yet available for the GluR. Another feature of such a model for the gating mechanism is that it would predict a low level of channel openings in the absence of agonist — as has indeed recently been observed for the nicotinic AChR by Jackson (1984).

The analysis of the single channel kinetics of the GluR in terms of the gating mechanism is now possible, and with recent progress in techniques for receptor isolation and purification it seems likely that it will eventually prove possible to interpret the gating mechanism in molecular terms.

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