GLUTAMATE RECEPTOR CHANNEL KINETICS

The Effect of Glutamate Concentration

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ABSTRACT Single channel recordings from the locust muscle D-glutamate receptor channel were obtained using glutamate concentrations ranging from 10^{-6} to 10^{-2} M. Channel kinetics were analyzed to aid in the development of a model for the gating mechanism. Analysis of channel dwell time histograms demonstrated that the channel possessed multiple open and closed states at concentrations of glutamate between 10^{-5} and 10^{-2} M. Correlations between successive dwell times showed that the gating mechanism was nonlinear (i.e., branched or cyclic) over the same glutamate concentration range. The glutamate concentration dependence of the channel open probability, and of the event frequency, was used to explore two possible allosteric gating mechanisms in more detail.

INTRODUCTION

Single channel recording is now the primary tool for investigating the gating mechanisms of both receptor- and voltage-gated ion channels. The most intensively studied receptor-gated ion channel is the nicotinic acetylcholine receptor (nAChR), but it remains to be established whether this is a model for receptor-gated channels in general. If a general picture of the properties of receptor-gated channels is to emerge, then it is important to investigate other systems in comparable detail. Single channel studies undertaken in our laboratory over the past nine years have concentrated on the quisqualate-sensitive, glutamate receptor–gated channel (GluR) of locust skeletal muscle (Usherwood, 1978*a*, *b*, 1981; Gration et al., 1979), several properties of which have made it particularly amenable to single channel analysis of gating kinetics (Patlak et al., 1979; Gration et al., 1981*a*, *b*; Cull-Candy et al., 1981; Gration et al., 1982; Cull-Candy and Parker, 1982). One advantage of this system is that desensitization can be inhibited by exposure of the muscle membrane to concanavalin A (Mathers and Usherwood, 1976, 1978), thus permitting channel gating to be studied in isolation from desensitization. The high conductance of the GluR (115-150 pS) enables recordings to be obtained using megaohm seals, thus avoiding pretreatment of the muscle membrane with proteolytic enzymes.

Previous analysis of the GluR gating mechanism focused on the channel behavior in the presence of 10^{-4} M glutamate (Ashford et al., 1984*a*, *b*; Kerry et al., 1987). The gating mechanism was shown to contain at least three open states, at least four closed states, and at least three isomerization pathways connecting the closed and open states. The work described in this paper extends such analysis over a range of glutamate concentrations, and leads to the construction of a working hypothesis for the GluR gating mechanism. As such, it prepares the way for a fuller description of the molecular details of GluR gating.

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METHODS

Experimental

The experimental methods were the same as those described in Kerry et al. (1987), and earlier publications from this laboratory (Patlak et al., 1979; Gration et al., 1982). Female adult locusts (*Schistocerca gregaria*) were used at 7–10 d post-fledgling. These were fed exclusively on a diet of home-grown wheat and dried food supplement, in an attempt to eliminate possible diet-dependent changes in extrajunctional GluR density.

Data Reduction

Recorded data was filtered at f_c = 3 kHz on playback, and reduced to vectors of dwell (open and closed) times using the dual threshold crossing algorithm described by Gration et al. (1982), and by Kerry et al. (1987).

Computation

General Considerations. The channel dwell time vectors were input to a PDP 11/34 computer, which was used for the majority of the calculations. Some of the later stages of the analysis used an ICL 2900 series mainframe.

Dwell Time Distributions

Dwell time distributions are expressed as histograms, normalized to give estimates of dwell time probability density functions (pdfs) (Colquhoun and Sigworth, 1983). To facilitate the inclusion of dwell times (t) ranging over up to four orders of magnitude in the same pdf, the histogram intervals were varied as follows. The minimum (t_{min}) and maximum (t_{max}) times to be represented in the histogram were input, alongside the desired

number of intervals (m), and used to calculate

$$g = (t_{max}/t_{min})^{1/m}.$$

The ith interval is then defined by

$$t_{min}\, g^{i-1} < t < t_{min}\, g^{i}$$

and its width is

$$t_{min}(g^{i} - g^{i-1})$$

and it is centered about

$$t_i = t_{min}\, g^{(i-0.5)}.$$

As discussed by Landaw and DiStefano (1984), such exponential sampling is optimal for data to be interpreted in terms of multiexponential decays, as it allows components with differing time constants to be accurately estimated.

The dwell time pdfs were displayed on log-log plots, as used by Blatz and Magleby (1986), to reveal multiexponential components more readily. Sums of exponential functions were fitted using previously described procedures (Kerry et al., 1987). The minimum number of components required for a satisfactory fit was determined using the AIC method (Akaike, 1974).

Autocorrelation Function Analysis

The use of autocorrelation functions (acfs) to analyze single channel data has been discussed by Labarca et al. (1985), Kerry et al. (1987), and by Colquhoun and Hawkes (1987). Briefly, the acf for successive open times is given by

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

where $r_o(k)$ is the autocorrelation between pairs of openings separated by $k-1$ intervening openings, $t_o(i)$ is the ith open time, Cov denotes covariance and Var variance. A similar term applies for the closed times.

Adjacent Dwell Time Analysis

The theoretical background to this approach is given by McManus et al. (1985) and Fredkin et al. (1985). Briefly, a closed time (t_c) is assigned to interval i if

$$t_{min}\, g^{i-1} < t_c \le t_{min}\, g^{i},$$

where t_{min} and g are defined as above. For each interval i, the mean open times for the preceding and following openings are evaluated, and are then plotted against $t_c(i)$.

Fitting Procedures

In all but one case, the fits of equations to data were optimized using Gauss-Newton algorithms. The exception was the use of a simplex algorithm (NAG routine E04CCF) when fitting the event frequency data. In this latter case, it was also necessary to restrict the parameter space to non-negative values of the closed ⟶ open isomerization rates (h_i) by searching for optimal values of log h_i.

RESULTS

Summary of the Database

Single channel recordings from glutamate receptor–gated ion channels (GluR) have been obtained at 20 different glutamate concentrations, ranging from 10^{-6} to 10^{-2} M (see Table I). The database used in all subsequent analysis corresponds to recordings with a signal to noise ratio of ≥3:1. All events of duration <0.2 ms have been discarded so as to impose a consistent minimum dwell time (Colquhoun and Sigworth, 1983). Such event omission distorts the observed channel kinetics. However, corrections for event omission (Roux and Sauvé, 1985; Blatz and Magleby, 1986; Ball and Sansom, 1988*b*) are model dependent, and so have not been applied at this stage of the analysis. Earlier studies (Kerry et al., 1987) have shown that efficiency of event detection is 100% for event durations of >0.18 ms. So, 0.2 ms is a conservative estimate of the deadtime for the recording and detection system. Overall, the database contains 240,000 events recorded from 60 different membrane sites. In all except two cases, multiple recordings have been obtained for each glutamate concentration. In the presence of 10^{-4} M glutamate, the site-to-site variation in kinetic parameters was comparable to that observed within a recording from a single site (Kerry et al., 1987). In the current database, the maximum site-to-site variation is seen at glutamate concentrations ~5×10^{-4} M.

TABLE I
SUMMARY OF DATABASE

[Glu]	No. of sites	No. of events	Total recording time
M			*s*
1×10^{-6}	4	57	1,690
6×10^{-6}	1	21	538
1×10^{-5}	2	3,860	1,560
2×10^{-5}	3	1,570	864
5×10^{-5}	2	5,470	241
6×10^{-5}	4	11,000	833
8×10^{-5}	3	16,000	1,080
1×10^{-4}	6	50,600	1,160
2×10^{-4}	4	32,500	415
4×10^{-4}	4	21,900	156
5×10^{-4}	3	10,200	68
6×10^{-4}	3	23,400	110
8×10^{-4}	2	5,970	30
1×10^{-3}	3	30,200	263
2×10^{-3}	2	9,231	85
3×10^{-3}	1	10,800	210
4×10^{-3}	2	1,010	99
5×10^{-3}	5	1,640	278
8×10^{-3}	2	759	117
1×10^{-2}	4	3,410	383

Summary of the database used in studying the gating kinetics of the GluR. The number of events refers to the total number of channel openings of duration ≥0.2 ms. All openings and closings of lesser duration are discarded so as to impose a consistent minimum dwell time.

Overall Kinetic Properties of the GluR

The overall kinetic parameters (probability of being open, P_o; event frequency, f; mean open time, m_o; and mean closed time, m_c) as functions of glutamate concentration are displayed in Fig. 1. To calculate average parameter values the total time spent open ($T_{o,i}$) and the total time spent closed ($T_{c,i}$) are evaluated for each membrane site (i). The average probability of the channel being open

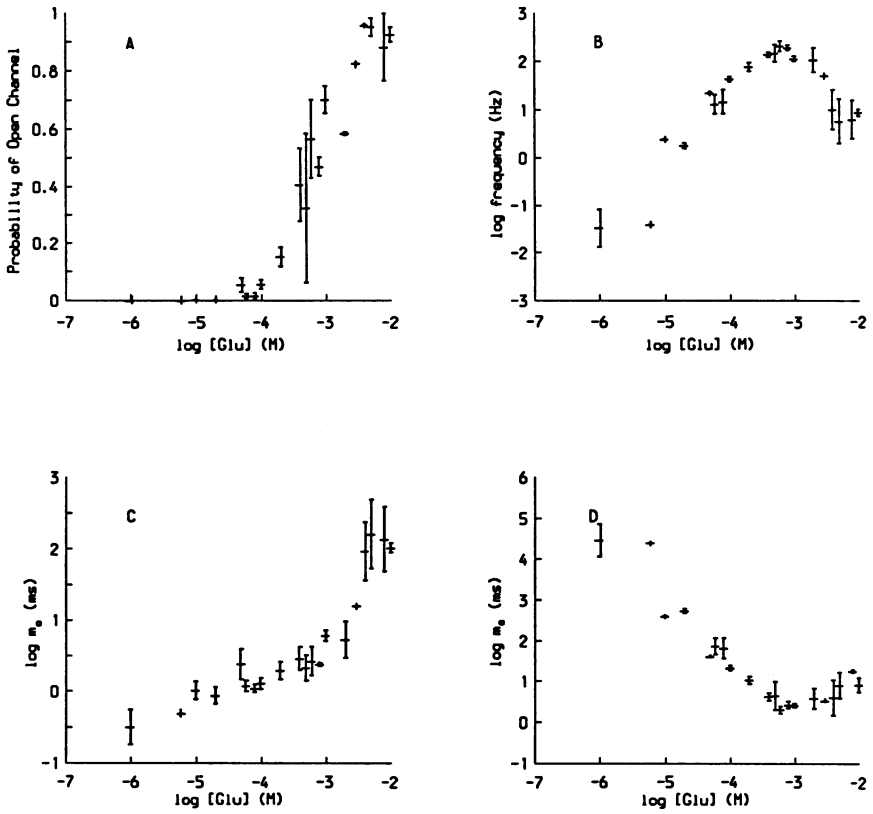

FIGURE 1 Overall kinetic parameters for the GluR single channel database. (*A*) The probability of the channel being open as a function of [Glu]. Each point is the average, calculated as described in the text, over all sites for a given concentration. The error bars indicate the standard deviation from the mean. (*B*) The channel event frequency (where an event is defined as an opening plus the following closing) as a function of [Glu]. Again, the points are averages for each concentration (see text). (*C* and *D*) The mean open times and mean closed times, respectively, as functions of [Glu]. The values are those calculated from *A* and *B* using Eqs. 3 and 4 of the text.

($\langle P_o \rangle$) is given by

$$\langle P_o \rangle = \sum_{i=1}^{M} T_{o,i} \Big/ \sum_{i=1}^{M} (T_{o,i} + T_{c,i}), \tag{1}$$

where summation is over the M sites for a given glutamate concentration. Similarly, the average event frequency ($\langle f \rangle$) is given by

$$\langle f \rangle = \sum_{i=1}^{M} N_i \Big/ \sum_{i=1}^{M} (T_{o,i} + T_{c,i}), \tag{2}$$

where N_i is the number of channel events recorded at site i. The average mean open time ($\langle m_o \rangle$) and mean closed ($\langle m_c \rangle$) times are then derived using

$$\langle m_o \rangle = \frac{\langle P_o \rangle}{\langle f \rangle} \tag{3}$$

and

$$\langle m_c \rangle = \frac{(1 - \langle P_o \rangle)}{\langle f \rangle}. \tag{4}$$

The graph of $\langle P_o \rangle$ vs. glutamate concentration is a dose-response curve for the GluR determined at the level of the single ion channel. Inspection of the curve reveals that the maximum value of $\langle P_o \rangle$ is close to unity (i.e., an efficacy of 1), and that half-maximal response is obtained at [Glu] = 8×10^{-4} M. These results are in close accord with those obtained in earlier studies (Gration et al., 1981*a*; Cull-Candy et al., 1981). Hill plot analysis of the dose-response curve (Fig. 2) yields a Hill coefficient of $n_H = 1.6$, comparable to the value of 1.5 for extrajunctional D-receptors of locust muscle obtained in macroscopic studies by Cull-Candy (1976), and 2.0 for junctional receptors obtained by Walther and Usherwood (1972). This implies positive cooperativity in the activation of the GluR and hence the existence of more than one glutamate binding site per receptor-channel molecule. As will be

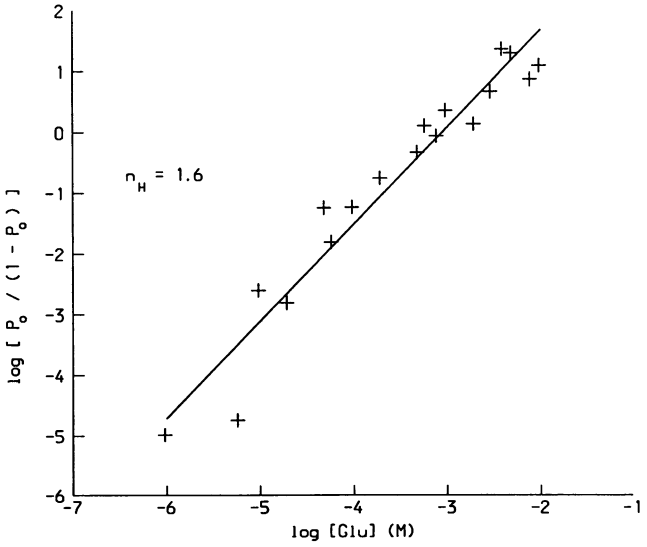

FIGURE 2 Hill plot for the GluR $\langle P_o \rangle$ vs. [Glu] data. The fitted line corresponds to $\log[(P_o)/(1 - P_o)] = n_H \log[\text{Glu}] - \log K$, with $n_H = 1.6$ and $K = 1.4 \times 10^{-5}$ M.

discussed in more detail below, the $\langle P_o \rangle$ vs. [Glu] curve can be used to determine equilibrium constants for proposed gating mechanisms.

Maximal event frequency occurs at [Glu] = 8×10^{-4} M, i.e., at the same agonist concentration as that giving $\langle P_o \rangle = 0.5$. A qualitatively similar concentration dependence of channel event frequency was obtained in previous studies using a lower time resolution (f_c = 1 kHz) (unpublished results). This lends support to the view that the rise and fall of event frequency is a genuine feature of GluR gating, and not an artifact resulting from event omission.

If, instead of expressing the concentration dependence of channel gating in terms of P_o and f, the mean open and closed times are used, a more complex picture is obtained. The mean open time ($\langle m_o \rangle$) increases with glutamate concentration from 0.32 ms at 10^{-6} M to ~100 ms at saturating agonist concentrations. This is compatible with the earlier results (using f_c = 1 kHz) of Gration et al. (1981*a*, *b*), although the increased time resolution of the current study reveals a lower mean open time for [Glu] $\leq$ 10^{-4} M. The mean closed time ($\langle m_c \rangle$) decreases from ~30 s at 10^{-6} M glutamate, to a minimum of ~2 ms at 6×10^{-4} M, and then rises to ~10 ms at saturating concentrations. Initially we were concerned that the rise in $\langle m_c \rangle$ at high glutamate concentrations might reflect residual desensitization of the GluR that had escaped the concanavalin A inhibition. However, inspection of the single channel dose-response curve fails to reveal a decline in $\langle P_o \rangle$ at high concentrations, although the maximum value of $\langle P_o \rangle$ is <1.0, and so we are reasonably confident that the rise in the mean closed times is an inherent property of the channel gating kinetics.

Data for Detailed Kinetic Analysis

For detailed kinetic analysis of channel properties, four glutamate concentrations were selected: 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M. These were chosen to represent GluR kinetics over a wide range of agonist concentrations. A low event frequency ($\langle f \rangle = 0.034 \pm 0.031$ s^{-1}) precluded detailed analysis of data obtained in the presence of 10^{-6} M glutamate. At this frequency, 16 h total recording time would be needed to obtain 2,000 events for, e.g., dwell time pdf analysis.

Representative single channel recordings at the four glutamate concentrations are shown in Fig. 3. The effect of agonist concentration on $\langle P_o \rangle$ is clearly seen in these recordings. Also evident is the modal behavior of the channel, there being distinct periods of high and low frequency activity, as originally noted by Patlak et al. (1979), and discussed by Gration et al. (1981*b*), Ball et al. (1985), and Ball and Sansom (1988*a*).

Open Time Distributions

The minimum number of open states entered by the GluR at different agonist concentrations can be obtained by determining the number of exponential components necessary to fit the observed open time pdfs. Such an analysis was carried out at all four glutamate concentrations, the results being presented in Fig. 4 and Table II. The distributions are presented using log-log plots (see Methods). The primary result of this analysis is to confirm that the complexity of open time distributions, first noted at 10^{-4} M glutamate (Kerry et al., 1987), is conserved across the concentration range. Thus, between [Glu] = 10^{-4} and 10^{-2} M, the channel has access to three or four kinetically distinct open states. At [Glu] = 10^{-5} M the pdf may be fitted with two components, although there is some suggestion that a third may be required for a complete fit. Verification of this is made difficult by the smaller number of openings measured at 10^{-5} M glutamate (Table I). Inspection of the pdf parameters (Table II) reveals that at all four concentrations of glutamate there is a component to the open time distribution, with time constant $\tau_1 \approx 0.4$ ms, corresponding to brief channel openings. At 10^{-5} M glutamate, this represents ~60% of the openings, which falls to ~20% at higher concentrations. At 10^{-6} M glutamate $\langle m_o \rangle$ is 0.3 ms. This implies that the brief-lived open state is the only one occupied at low glutamate concentrations.

With respect to the other components of the open time distributions the situation is less clear. This is in part due to the proximity of the time constants for the different components, which results in relatively high correlations

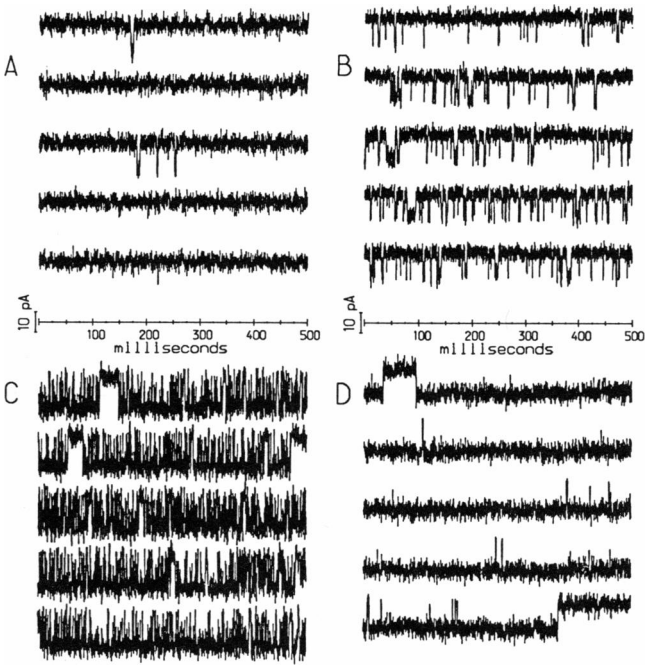

FIGURE 3 Representative single channel recordings for the four glutamate concentrations which are analyzed in detail. (*A*) 10^{-5} M; (*B*) 10^{-4} M; (*C*) 10^{-3} M; (*D*) 10^{-2} M. For each concentration, five consecutive traces are shown, each of 0.512-ms duration. The data were filtered at f_c = 3 kHz on playback, and digitized at 8 kHz before display. Channel openings are downwards.

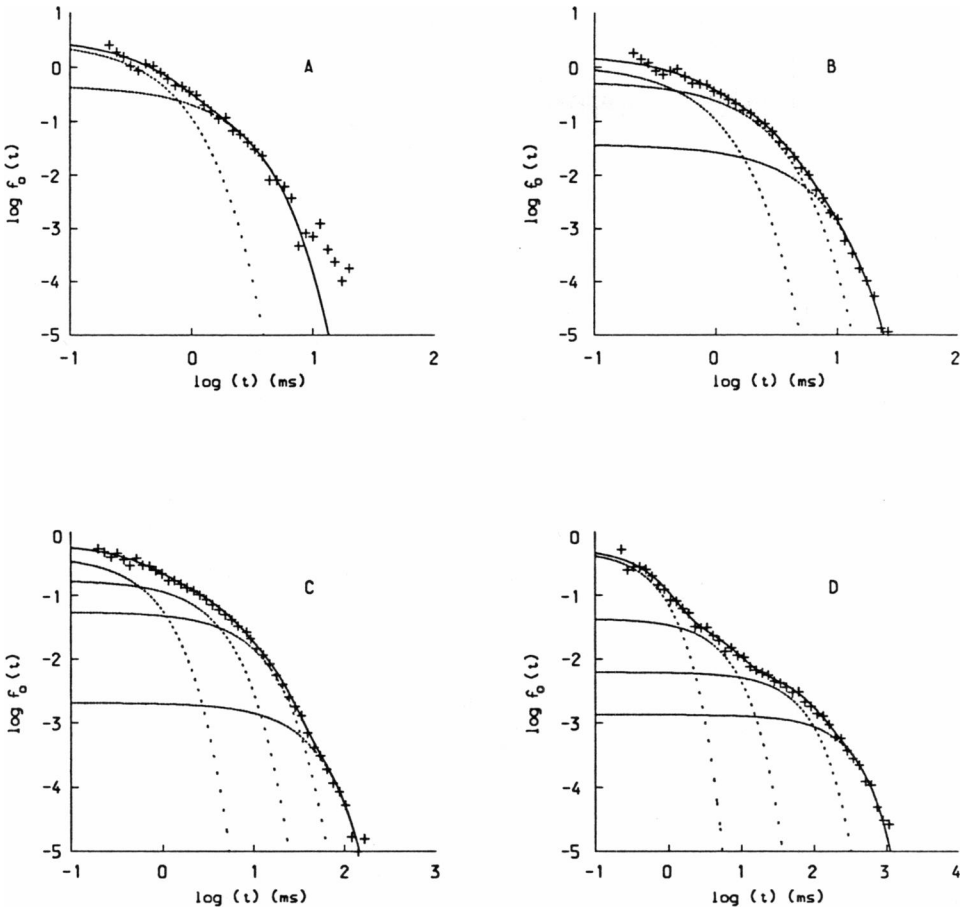

FIGURE 4 Open time pdfs for the four glutamate concentrations. (*A*) 10^{-5} M, 3,860 events; (*B*) 10^{-4} M, 50,600 events; (*C*) 10^{-3} M, 30,200 events; (*D*) 10^{-2} M, 3,410 events. For each concentration, events of duration ≥ 0.2 ms from all membrane sites are combined in the same distribution before fitting using

$$f_o(t) = \sum_{i=1}^{N_o} (\alpha_i/\tau_i) \exp(-t/\tau_i).$$

The parameter estimates are listed in Table II. The distributions are shown as log-log plots. The crosses represent the observed distribution and the solid lines the fitted pdfs. The broken lines indicate the individual exponential components which go together to make up the fitted pdf.

between parameter estimates. $\tau_2 \approx 1.2$ ms at both 10^{-5} and 10^{-4} M, but α_2 is greater at the higher concentration. At 10^{-4} M the third component also makes a significant contribution to the pdf.

Attempting to relate pdf components at 10^{-3} and 10^{-2} M proved quite difficult. Simulation studies suggest that such an approach can be problematic, even when the underlying mechanism is known (unpublished results). Consequently, at this stage of the analysis it suffices to conclude that at higher glutamate concentrations (*a*) at least four open states are accessible to the GluR, and (*b*) the occupancy of the longer lifetime open states increases with increasing glutamate concentration.

Closed Time Distributions

Analysis of closed time distributions yields information on the minimum number of kinetically significant closed states. Closed time distributions at four glutamate concentrations have been fitted with sums of exponential functions. The results are presented in Fig. 5 and Table III. The analysis of closed time distributions extends the earlier observation of four closed states in the presence of 10^{-4} M glutamate (Kerry et al., 1987). Four closed states are also observed at 10^{-3} and 10^{-2} M. At 10^{-5} M the closed time pdf is biphasic, i.e., $N_c = 2$. However, bearing in mind the relatively low number of events recorded with [Glu] = 10^{-5}

TABLE II
OPEN TIME PROBABILITY DENSITY FUNCTIONS

[Glu]	N_o	i	α_i	CV	τ_i	CV
M					*ms*	
10^{-5}	2	1	0.616	0.086	0.310	0.141
		2	0.384	0.138	1.25	0.092
10^{-4}	3	1	0.379	0.366	0.424	0.269
		2	0.532	0.178	1.22	0.229
		3	0.089	0.908	2.94	0.279
10^{-3}	4	1	0.192	0.172	0.512	0.160
		2	0.386	0.143	2.49	0.177
		3	0.371	0.166	7.49	0.126
		4	0.051	0.357	27.7	0.140
10^{-2}	4	1	0.241	0.077	0.516	0.109
		2	0.176	0.117	4.39	0.160
		3	0.291	0.103	50.6	0.135
		4	0.292	0.111	232.0	0.082

The open time pdfs are defined by

$$f_o(t) = \sum_{i=1}^{N_o} (\alpha_i/\tau_i) \exp(-t/\tau_i).$$

For each concentration the pdf is derived by combining open times from all membrane sites in a single normalized histogram, and fitting the equation above as described in Methods. N_o was determined by obtaining fits for successively greater numbers of components until a significantly better fit, as judged by use of the AIC, cannot be obtained. The coefficient of variation (CV) is given for each parameter estimate.

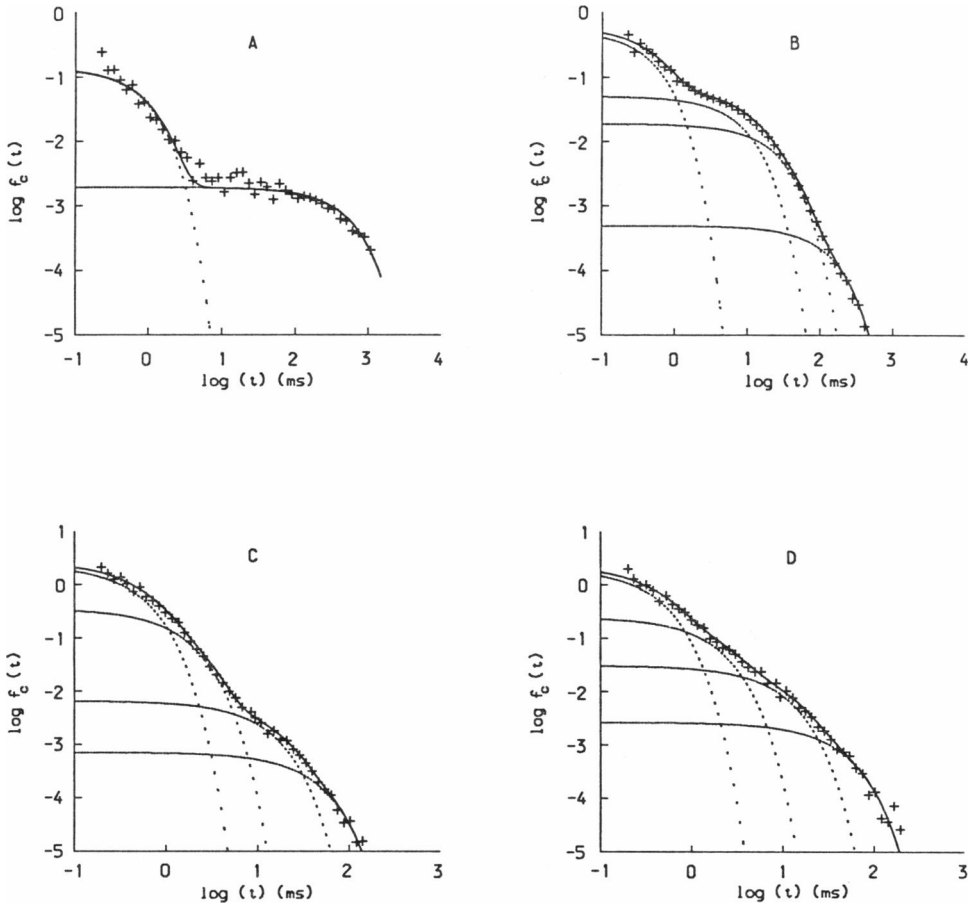

FIGURE 5 Closed time pdfs for the four glutamate concentrations. (*A*) 10^{-5} M, 3,860 events; (*B*) 10^{-4} M, 50,600 events; (*C*) 10^{-3} M, 30,200 events; (*D*) 10^{-2} M, 3,410 events. The pdfs are described by

$$f_c(t) = \sum_{i=1}^{N_c} (\alpha_i/\tau_i) \exp(-t/\tau_i)$$

and the parameter estimates are listed in Table III. Other details are as for Fig. 4.

TABLE III
CLOSED TIME PROBABILITY DENSITY FUNCTIONS

[Glu]	N_c	i	α_i	CV	τ_i	CV
M					*ms*	
10^{-5}	2	1	0.103	0.237	0.746	0.135
		2	0.897	0.027	468.0	0.047
10^{-4}	4	1	0.206	0.048	0.432	0.070
		2	0.350	0.153	7.52	0.114
		3	0.389	0.131	22.5	0.093
		4	0.055	0.259	122.0	0.105
10^{-3}	4	1	0.633	0.087	0.380	0.091
		2	0.303	0.168	1.22	0.129
		3	0.047	0.508	9.74	0.312
		4	0.017	1.483	32.4	0.357
10^{-2}	4	1	0.486	0.088	0.311	0.129
		2	0.264	0.133	1.41	0.216
		3	0.181	0.176	7.76	0.192
		4	0.069	0.492	34.1	0.160

The closed time pdfs are defined by

$$fc(t) = \sum_{i=1}^{N_c} (\alpha_i/\tau_i) \exp(-t/\tau_i).$$

The tabulated parameters are obtained in the same manner as for the open time pdfs.

M (Table I), $N_c = 2$ must be regarded as a minimum estimate of the number of closed states. A constant feature at all four glutamate concentrations is the component ($\tau_1 \approx$ 0.4 ms) corresponding to brief channel closings. The proportion of this component (α_1) increases with glutamate concentration until its peak value at 10^{-3} M, and then declines somewhat.

When attempting to analyze the α_i as functions of concentration, a similar difficulty is encountered to that already described for the open time distributions. Consequently, at this stage our conclusions are again limited to the following (*a*) there are at least four kinetically distinct closed states when [Glu] $\geq 10^{-4}$ M; and (*b*) as glutamate concentration increases, the relative contributions of the components shift in favor of briefer closings, until a maximum is reached at $\sim 10^{-3}$ M.

Autocorrelation Functions

Dwell time autocorrelation functions (acfs) provide information on the number of channel isomerization pathways (N_p) linking the closed states with the open states, and vice versa (Fredkin et al., 1985; Labarca et al., 1985; Ball and Sansom, 1988*b*; Colquhoun and Hawkes, 1987; Kerry et al., 1987). Earlier studies at 10^{-4} M have now been extended to the four glutamate concentrations. The results are presented in Figs. 6 and 7. Non-zero acfs indicate that

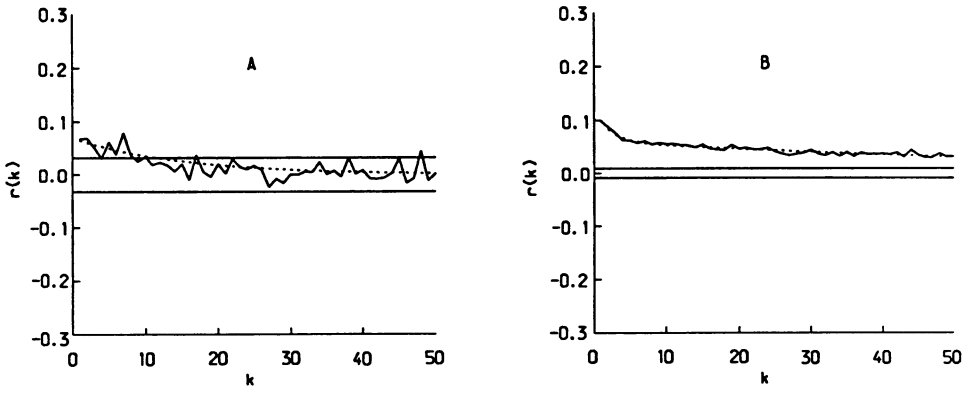

FIGURE 6 Autocorrelation functions for successive open times determined at (*A*) 10^{-5} M glutamate; (*B*) 10^{-4} M; (*C*) 10^{-3} M; and (*D*) 10^{-5} M. For each concentration, the acf is the average for all membrane sites. The jagged lines represent the observed acfs and the broken lines the fits (see Table IV) of

$$r(k) = \sum_{i=1}^{Np-1} A_i \pi_i^{|k|}.$$

The two horizontal lines are the 95% confidence limits for a null acf, given N_{evt} events at each concentration. These limits are defined by

$$-N_{evt}^{-1} \pm 2N_{evt}^{-1/2}.$$

Only those values of the observed acfs outside these limits were used in fitting.

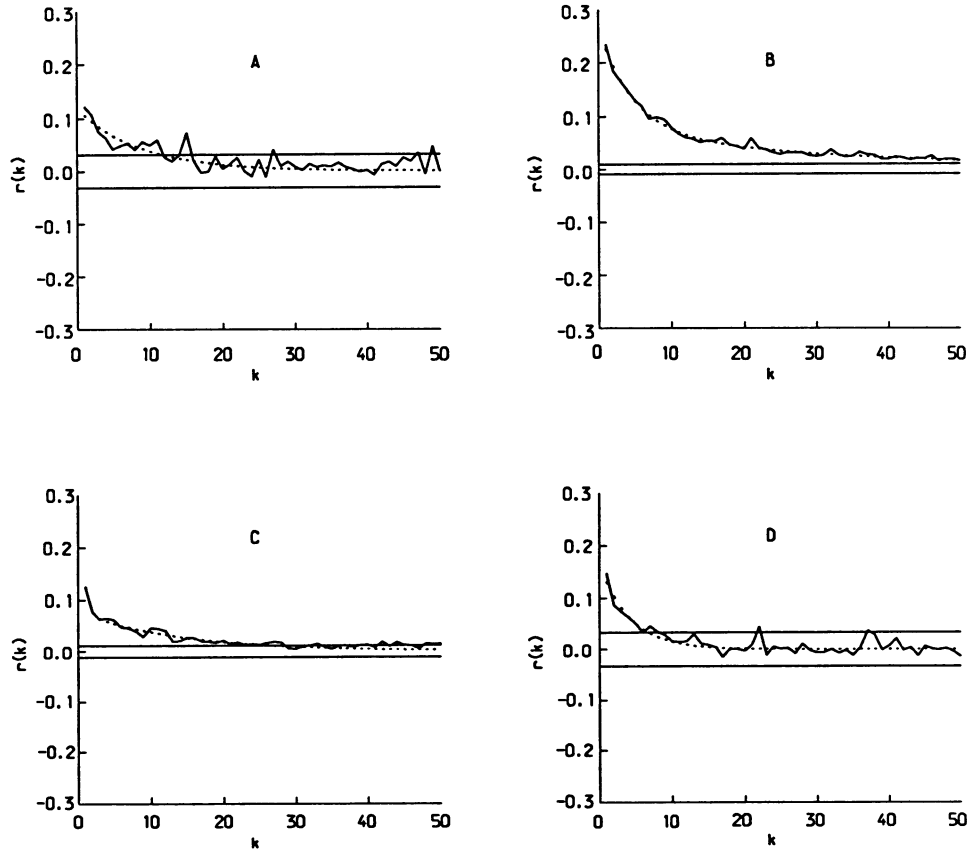

FIGURE 7 Autocorrelation functions for successive closed times determined at (*A*) 10^{-5} M glutamate; (*B*) 10^{-4} M; (*C*) 10^{-3} M; and (*D*) 10^{-5} M. Other details are as for Fig. 6.

TABLE IV
DWELL TIME AUTOCORRELATION FUNCTIONS

[Glu]	E	$N_p - 1$	i	A_i	CV	π_i	CV
M							
10^{-5}	O	1	1	0.069	0.203	0.936	0.038
	C	1	1	0.119	0.126	0.894	0.026
10^{-4}	O	2	1	0.064	0.141	0.623	0.095
			2	0.060	0.033	0.987	0.001
	C	2	1	0.194	0.046	0.810	0.020
			2	0.072	0.139	0.971	0.004
10^{-3}	O	2	1	0.058	0.086	0.701	0.001
			2	0.165	0.067	0.979	0.085
	C	2	1	0.278	0.723	0.189	0.741
			2	0.079	0.076	0.932	0.006
10^{-2}	O	2	1	0.203	0.187	0.723	0.142
			2	0.115	0.330	0.977	0.016
	C	1	1	0.167	0.102	0.791	0.035

The acfs are defined (see text) by the equation:

$$r_E(k) = \sum_{i=1}^{N_p-1} A_i \pi_i^{|k|},$$

where E = O or C. That is, the acf is described by the sum of $N_p - 1$ geometrically decaying components, where N_p is the number of isomerization pathways linking the closed states with the open. The values of N_p are obtained in a similar manner to that used in analysis of the pdfs. As in Tables II and III, the parameter error estimates are expressed as coefficients of variation (CV).

there is more than one isomerization pathway, i.e., the gating mechanism is nonlinear. Therefore, there must be either branches and/or cycles in the gating mechanism. The acfs have been analyzed as sums of geometrically decaying components (Table IV). As discussed by Fredkin et al. (1985) and by Kerry et al. (1987), if there are N_p pathways linking the open states with the closed, then there should be $N_p - 1$ geometrically decaying components to the acf. The results suggest that at 10^{-5} M, $N_p \geq 2$ and that $N_p \geq 3$ at the higher glutamate concentrations. These conclusions place quite strong constraints on possible models of the gating mechanism.

Adjacent Dwell Time Analysis

The results described suggest that the following is a reasonable description of the classes of models describing the GluR gating mechanism:

$$\begin{array}{ccccccc} C_1 & \rightleftharpoons & C_2 & \rightleftharpoons & C_3 & \rightleftharpoons & C_4 \cdots C_n \\ \updownarrow & & \updownarrow & & \updownarrow & & \updownarrow \\ O_1 & \rightleftharpoons & O_2 & \rightleftharpoons & O_3 & \rightleftharpoons & O_4 \cdots O_n. \end{array} \quad (5)$$

Increasing glutamate concentration shifts the equilibrium from the left- to the right-hand side of the equation. If such a mechanism applies, the durations of adjacent open and closed intervals should be correlated. The magnitude of the correlation will be dependent on, amongst other things, the relative rates of the horizontal and vertical steps in the above scheme, being increased if the horizontal steps are relatively slow. Therefore, analysis of adjacent open and closed intervals (McManus et al., 1985) can provide a confirmation of the results of autocorrelation function analysis. Adjacent dwell time analysis has been applied to the GluR data. In the presence of 10^{-4} M glutamate there is a particularly clear negative correlation, i.e., briefer openings are adjacent to longer closings and vice versa (Fig. 8). This observation also places constraints on possible gating mechanisms.

Possible Mechanisms

The analyses described above provide firm indications of the class of gating mechanisms applicable to the GluR. One attractive class of models is that originally proposed by Monod et al. (1965) with respect to allosteric proteins, and discussed in the context of channel gating by Karlin (1967) and by Colquhoun and Hawkes (1977, 1981, 1983). A cooperative model with four identical glutamate binding sites has been explored, in advance of more detailed analysis of the GluR gating mechanism:

$$\begin{array}{ccccccccc} C & \rightleftharpoons & CA & \rightleftharpoons & CA_2 & \rightleftharpoons & CA_3 & \rightleftharpoons & CA_4 \\ \updownarrow & & \updownarrow & & \updownarrow & & \updownarrow & & \updownarrow \\ O & \rightleftharpoons & OA & \rightleftharpoons & OA_2 & \rightleftharpoons & OA_3 & \rightleftharpoons & OA_4. \end{array} \quad (6)$$

("A" represents an agonist, i.e., glutamate, molecule in the above scheme.) Such a model, with $N_o = 5$ and $N_c = 5$, has been selected since it displays a large (i.e., ≥ 3) number of kinetically significant open and closed states over a relatively wide glutamate concentration range.

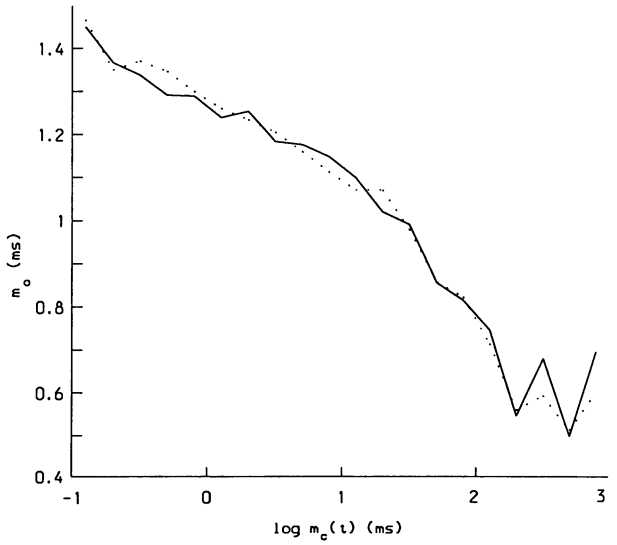

FIGURE 8 Adjacent dwell time analysis for the GluR in the presence of 10^{-4} M glutamate. Channel closings are sorted into 50 intervals on the basis of closed time duration, and, for each interval, the mean open time for the preceding and following openings determined. A marked negative correlation is observed, indicating that long closings are adjacent to brief openings and vice versa. The superimposition of the curves for the preceding (*solid line*) and following (*broken line*) openings is as predicted for a channel gating mechanism at thermodynamic equilibrium.

The equilibrium properties of mechanism 6 are determined by three parameters: α, K_B, and L, where L is the equilibrium constant for the reaction C $\leftrightarrow$ O, K_B is the microscopic binding constant for association of the glutamate with a closed channel site, and αK_B is the microscopic binding constant for an open channel site. So, the equilibrium constants for all 13 steps of mechanism 6 may be expressed in these terms. The concentration dependence of P_O is given by

$$P_O = (1 + a\alpha K_B)^4/[(1 + a\alpha K_B)^4 + L^{-1}(1 + aK_B)^4], \quad (7)$$

where a is the glutamate concentration. Thus the three equilibrium parameters may be estimated by fitting Eq. 7 to the $\langle P_O \rangle$ vs. [Glu] data (see Fig. 9 *a* and Table V).

A modification of mechanism 6 in which the unliganded open state has been omitted has also been considered

$$\begin{array}{ccccccc} C & \longleftrightarrow & CA & \longleftrightarrow & CA_2 & \longleftrightarrow & CA_3 \longleftrightarrow CA_4 \\ & & \updownarrow & & \updownarrow & & \updownarrow \quad \updownarrow \\ & & OA & \longleftrightarrow & OA_2 & \longleftrightarrow & OA_3 \longleftrightarrow OA_4, \end{array} \quad (8)$$

for which the [Glu] dependence of $\langle P_O \rangle$ is given by

$$P_O = [(1 + a\alpha K_B)^4 - 1]/\{[(1 + a\alpha K_B)^4 - 1] + L^{-1}(1 + aK_B)^4\}, \quad (9)$$

TABLE V
PARAMETER ESTIMATES FOR THE COOPERATIVE MECHANISMS

Parameter	Mechanism 6	Mechanism 8
α	14.2 (±5.7)	9.6 (±3.3)
K_B/M^{-1}	2.7×10^3 ($\pm 0.7 \times 10^3$)	2.3×10^3 ($\pm 0.7 \times 10^3$)
L	1.4×10^{-4} ($\pm 2.3 \times 10^{-4}$)	7.6×10^{-4} ($\pm 11.4 \times 10^{-4}$)
h_1/s^{-1}	1.6×10^{-9} (±53)	
h_2/s^{-1}	7.3×10^{-7} (±210)	1.3×10^{-6} (±150)
h_3/s^{-1}	150 (±370)	260 (±320)
h_4/s^{-1}	530 (±290)	460 (±290)
h_5/s^{-1}	1.5×10^{-12} (±92)	6.8×10^{-12} (±99)

The parameter estimates are for the basic cooperative mechanism 6, and for the modification 8 from which the unliganded open state (O) is excluded. Parameter error estimates are given as standard deviations.

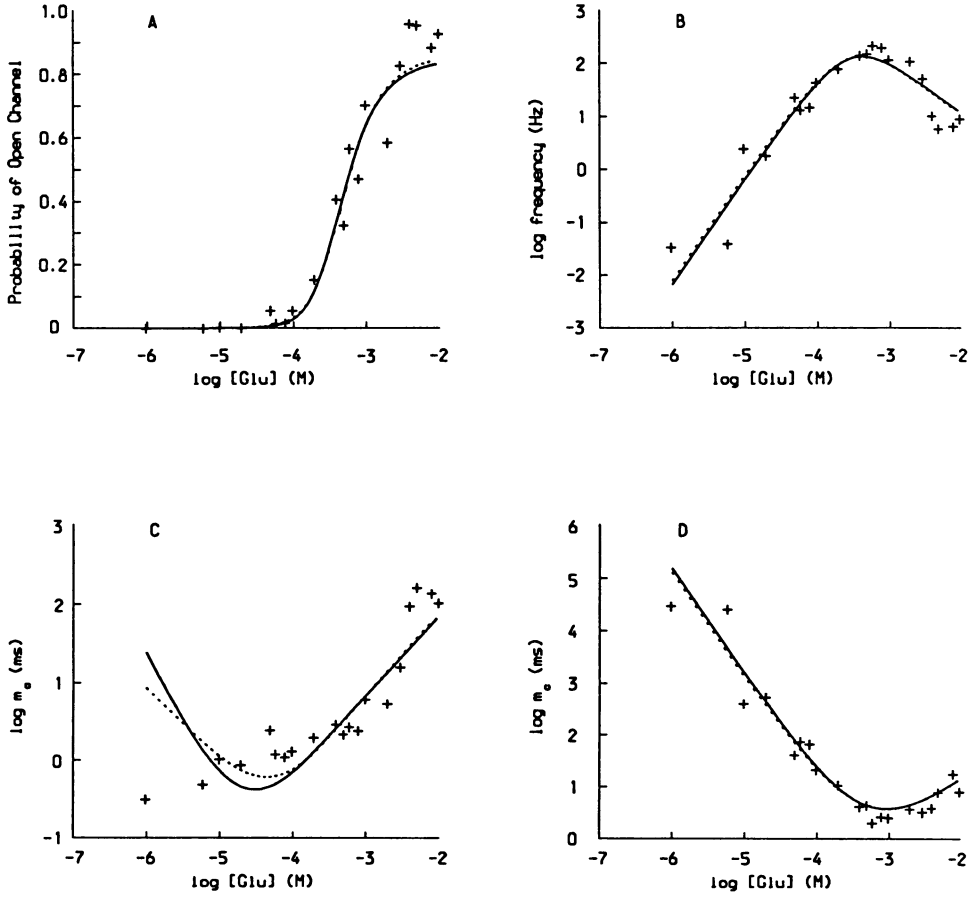

FIGURE 9 Overall kinetic parameters for mechanisms 6 (*solid lines*) and (*broken lines*) compared with the experimental (*A*) single channel dose-response; (*B*) event frequency; (*C*) mean open time; and (*D*) mean closed time data. The $\langle P_o \rangle$ vs. [Glu] curve arises from fitting Eqs. 7 or 9 to the data and the $\langle f \rangle$ vs. [Glu] curve corresponds to Eq. 10. The m_o and m_c data and curves are derived using Eqs. 3 and 4, respectively.

where α and K_B are defined as before, and where L is defined in terms of the equilibrium constant for the reaction CA $\rightarrow$ OA (αL). The equilibrium parameter estimates for this modified mechanism are also given in Table V, and the corresponding fit illustrated in Fig. 9 *a*.

One may extend the description of mechanism 6 to include consideration of the channel isomerization rates. Defining h_1 as the rate constant for C $\rightarrow$ O, h_2 as that for CA $\rightarrow$ OA, etc., it is relatively easy to show (Colquhoun and Hawkes, 1981) that the concentration dependence of the event frequency is given by

$$f = \sum_{i=1}^{5} h_i p_i, \tag{10}$$

where p_1 is the equilibrium probability of state C, p_2 that of CA, . . . and p_5 is the probability of CA_4. The equilibrium probabilities are derived from the three equilibrium parameters using

$$p_1 = (1 - P_0)/(1 + aK_B^4) \tag{11}$$

and

$$p_i = \binom{4}{i-1} (aK_B)^{i-1} p_1. \tag{12}$$

If the three equilibrium parameters are known, then by using Eqs. 7 and 12–14, the glutamate concentration dependence of $\langle f \rangle$ may be used to obtain estimates of the isomerization rates h_i. Application of such analysis to the $\langle f \rangle$ vs. [Glu] data, for both mechanism 6 and for the modified mechanism 8, yields the results shown in Fig. 9 *b* and Table V. Fitting either model suggests that there are at least two kinetically significant isomerization pathways, in broad agreement with the results of the autocorrelation function analysis. One should interpret the results cautiously. In particular, the estimates of h_1, h_2, and h_5 indicate that the corresponding reaction rates are indistinguishable from zero. More accurate parameter estimates must await more detailed modeling.

DISCUSSION

The results of single channel recording from the GluR over four orders of magnitude of glutamate concentration have been presented in terms of overall kinetic parameters. In particular, a single-channel dose-response curve has been determined. Single channel kinetics have been investigated in detail at four glutamate concentrations where large numbers of events are available, and have been used to derive a minimal model of the gating mechanism. The dose-response and event-frequency curves have been used to exemplify the initial stages of construction of a detailed gating mechanism for the GluR.

Dose-Response Curve Analysis

In the absence of detailed biochemical characterization, Hill plot analysis of the single channel dose-response curve ($n_H = 1.6$) is the main evidence for multiple binding sites on the GluR. Such analysis involves only limited assumptions concerning the nature of the underlying gating mechanism. However, it is important to stress that the $\langle P_o \rangle$ data can, in principle, be used to determine the equilibrium parameters for any gating mechanism, and hence provide an important starting point in trying to understand the gating mechanism of the GluR.

It is important to note that there is no indication of a decrease in $\langle P_o \rangle$ at high glutamate concentrations. This excludes the possibility of channel block by the agonist, a complication that occurs with the nAChR (Ogden and Colquhoun, 1985).

Event Frequency Curve Analysis

The display of $\langle f \rangle$ vs. [Glu] shows the rise and fall of the event frequency with increasing glutamate concentration. Colquhoun and Hawkes (1981) have shown that, for any gating mechanism, the event frequency is given by

$$f = \sum_{i=1}^{N_c} p_i h_i, \tag{13}$$

where p_i is the equilibrium probability of closed state i and h_i is the sum of the rate constants for all steps linking closed state i to an open state, the summation being over the N_c closed states. As the equilibrium probabilities at a given glutamate concentration may be calculated from the equilibrium parameters of the model, the values of h_i may be readily determined. Furthermore, if the proposed gating mechanism is such that each closed state is linked to a single open state, then the h_i values are equal to the corresponding closed $\rightarrow$ open isomerization rates. Consequently, the event frequency curve is also of importance in providing initial parameter estimates for models of the gating kinetics.

Analysis of Dwell Time PDFs

Before detailed modeling, it is advisable to maximize the constraints on possible gating mechanisms. One way of achieving this is to analyze the dwell time pdfs in terms of the numbers of exponential components. Previous analysis of GluR kinetics in the presence of 10^{-4} M glutamate (Kerry et al., 1987) revealed at least three open states and four closed states of the receptor channel. Such estimates are necessarily minimal estimates. Additional states may remain undetected for three reasons: (*a*) the time constant (τ) of a component may be briefer than the cutoff time of the data recording and analysis system; (*b*) the τ value for a component may be sufficiently close to that of another component that the two are not resolved; and (*c*) the entry of the receptor-channel into a particular kinetic state may be so infrequent that a very large number of channel events must be recorded before the corresponding component is detected in the dwell time pdf.

The first two problems are not easy to circumvent for a fixed experimental protocol. However, *c* can be resolved by

exploring channel kinetics over a wide range of glutamate concentrations, and by recording for extended periods of time. Kinetic states rarely entered at one glutamate concentration may be quite highly populated at another.

Analysis of GluR pdfs over three orders of magnitude of glutamate concentration confirms the kinetic complexity first observed for 10^{-4} M glutamate. At high [Glu] there are at least four open and at least four closed states; at low [Glu] fewer states are occupied. Attempts to relate pdf components at different concentrations were difficult. Simulation studies suggested this to be an inherent difficulty of analysis of pdfs for multiple state cyclic gating mechanisms. In part this difficulty is due to the redistribution of the channel between the different states with change in glutamate concentration. Furthermore, the time constant associated with a kinetic state that may be left via a glutamate-binding reaction will change with the concentration of glutamate, thus confusing assignment of time constants to states. That such a difficulty occurs may be due to the underlying gating mechanism containing >4 open and >4 closed states. So, when considering the constraints put upon gating mechanisms by the pdf analysis, one must remember that N_o and N_c are minimum estimates.

Analysis of Correlation Between Successive Dwell Times

The dependence of successive dwell times has been examined using two complementary procedures: autocorrelation function analysis and analysis of mean adjacent dwell times. As discussed by Fredkin et al. (1985), both methods of analysis provide information on the same aspect of receptor-channel gating, i.e., the number of pathways linking the closed states with the open states of the channel (N_p). Both analyses confirm that multiple channel isomerization pathways exist for the GluR. Fitting the acfs with sums of geometrical components suggests that $N_p \geq 3$. At this stage of the analysis it is pertinent to enquire into the effect of the 0.2 ms cutoff imposed on single channel events on the conclusions drawn from the kinetic analyses. With respect to estimating the number of components in pdfs, both Colquhoun and Sigworth (1983) and Roux and Sauvé (1985) have demonstrated that, while event omission does distort the pdfs, it is not expected to introduce spurious additional components. With respect to autocorrelation function analysis, a similar situation applies (Ball and Sansom, 1988*b*). In particular, event omission does not appear to generate positive acfs from gating mechanisms with $N_p = 1$. Therefore, even allowing for event omission, the constraints on possible gating mechanisms still apply. On this basis it is reasonable to conclude that a mechanism such as 5 applies to the GluR. As discussed above, theoretical studies suggest that the horizontal steps (i.e., those involving interconversions between closed states only, or between open states only) should be slow compared with (some of) the vertical steps (i.e., channel isomerizations). Adding the requirement for brief openings to be paired with long closings, and vice versa, one has quite firm constraints on possible gating mechanisms. It is important to note that any model satisfying these constraints will explain the modal behavior of GluR gating (dubbed the "gearshift" phenomenon by Moczydlowski [1986]), with the duration of the different kinetic modes being determined by the (low) rates of switching between different closed → open isomerization pathways.

A Cooperative Model for GluR Gating

The results discussed so far lead us to investigate cyclic gating mechanisms such as 5. As noted by Fredkin et al. (1985), a problem of identifiability can arise with some cyclic schemes. In brief, if a gating mechanism comprises N_o open states and N_c closed states, then if the total number of independent parameters is greater than $2N_oN_c$ it is not identifiable. This places restrictions on the classes of mechanisms that can realistically be considered in terms of the single channel data. In the early stages of analysis there is an advantage in restricting consideration to those models where there are internal constraints on the rate constants, leading to a reduced number of free parameters. One way of achieving such constraints is via symmetry in the model, such as in mechanism 6. In the absence of additional constraints it has 26 rate constants. However, if the constraints implied by the assumption of four identical binding sites are included, then the number of independent parameters is reduced to 10 (i.e., α, L, K_B, h_i where $i = 1$–5, and the microscopic association rates for closed channel sites and for open channel sites). Consequently, this class of models is an attractive starting point for further investigations. A model with four binding sites was selected, on the basis of simulation studies, as being likely to result in $N_o \geq 3$ and $N_c \geq 4$ over a wide range of glutamate concentrations.

It should be stressed that this mechanism is a current working hypothesis, and is not intended as a definitive statement concerning the GluR gating mechanism. In particular, we have not as yet excluded models where multiplicity of open states does not correspond to multiplicity of binding sites, i.e., models where $N_o - 1$ is greater than the number of ligand binding sites on the receptor. Such models have recently been implied, for the nAChR, by the results of several authors (e.g., Colquhoun and Sakmann, 1985; Auerbach and Lingle, 1986; and Chabala and Lester, 1986). Models with multiple open states for a constant number of bound ligands lack the internal constraints alluded to above, and consequently have a relatively large number of free parameters. We have therefore reserved their analysis until a later stage of this series of investigations.

Analysis of the single channel dose-response curve in terms of mechanism 6 resulted in a reasonable fit to the $\langle P_o \rangle$ vs. [Glu] data (see Fig. 9 *a*). The microscopic association constant (K_B) for closed channel sites was 2.7 ×

10^3 M^{-1}, and was α (=14.2)-fold higher for open channel sites. In the absence of glutamate:

$$P_{o,min} = L/(1 + L) = 1.4 \times 10^{-4}(\pm 2.3 \times 10^{-4}). \quad (14)$$

Channel openings for the GluR have not been detected in the absence of glutamate (unpublished results), although such events are well documented for the nicotinic acetylcholine receptor (Jackson, 1984). The above estimate of $P_{o,min}$ is compatible with the experimental result, but we also thought it worthwhile to investigate mechanism 8, which does not include the unliganded open state O. For this latter model, $P_{o,min}$ is zero by definition. For both mechanisms, the maximum value of P_o is

$$P_{o,max} = \alpha^4 L/(1 + \alpha^4 L). \quad (15)$$

For the basic mechanism 6, this gives $P_{o,max}$ = 0.85 (±0.03), whilst for the modified mechanism 8, $P_{o,max}$ = 0.87 (±0.03). Both values are reasonably close to that observed (~0.9). If the P_o curves predicted from the fitted parameters is subjected to Hill plot analysis, $n_H \approx 1.3$ is obtained, again comparable to that derived from the experimental data (n_H = 1.6). So, both mechanisms (6 and 8) provide a reasonable description of the dose-response curve of the GluR.

A second feature of this class of mechanisms is that each closed state is linked to a single open state, and hence the concentration dependence of event frequency may be used to estimate closed → open isomerization rates. Some difficulties were experienced when attempts were made to fit the event frequency data, requiring the use of the simplex algorithm for their solution. It is reasonable, therefore, to proceed cautiously, and interpret the results (Table V) as indicating that h_1, h_2, and h_5 (and hence h'_1, h'_2, and h'_5) are all indistinguishable from zero, and that two channel isomerizations are predominant:

$$CA_2 \rightarrow OA_2 \quad h_3 = 150\ s^{-1}(260\ s^{-1});$$

and

$$CA_3 \rightarrow OA_3 \quad h_4 = 530\ s^{-1}(460\ s^{-1}).$$

The corresponding open → closed rates are

$$OA_2 \rightarrow CA_2 \quad h'_3 = h_3/\alpha^2 L = 5{,}300\ s^{-1}(3{,}700\ s^{-1});$$

and

$$OA_3 \rightarrow CA_3 \quad h'_4 = h_4/\alpha^3 L = 1{,}300\ s^{-1}(680\ s^{-1}),$$

where the figure in brackets represent the parameter estimates for the modified mechanism 8. Thus, negative correlations between adjacent dwell times would be expected when the channel is primarily in the bi- and tri-liganded forms. Such negative correlations are seen experimentally, particularly in the presence of 10^{-4} M glutamate.

One may also evaluate the model in terms of the predicted [Glu] dependence of the mean open and closed times (Fig. 9, *c* and *d*) (noting in passing that this information is already implicit in the predicted dose-response and event frequency curves). Between [Glu] = 10^{-5} and 10^{-2} M the agreement between the model and the data are quite good. However, at lower concentrations, there is an overestimation of the mean open time, especially for mechanism 6. This may reflect inadequacies of the modeling procedures, although studies using simulated data suggested that this should not be the case.

So, in terms of such a model, only the two microscopic glutamate association rates (k^o_{on} and k^c_{on}) remain undetermined. Initial calculations and simulation studies suggest that both rates are of the order of 10^4 $M^{-1}s^{-1}$ which, should this prove to be correct, suggests that the association between the glutamate and its binding site is rather slow, compared with the diffusion limited rate (~10^8 $M^{-1}s^{-1}$). However, further consideration of this must await the application of more powerful methods of fitting gating models to the data, such as the maximum likelihood technique (Horn and Lange, 1983; also see Fredkin et al., 1985, and Blatz and Magleby, 1986). It must be stressed that the model proposed is not necessarily unique, and that considerable effort will have to be invested into future modeling studies to objectively assess the simplest mechanism capable of adequately accounting for all features of the experimental data. Such investigations are currently underway, and will form the basis of a future publication. However, one should remember that, even given such methods, the problem of identifiability inherent to single channel kinetic analysis still remains. For example, one might wish to relax the constraint of equivalent binding sites used in the cooperative model. This would lead to an increase in the number of parameters, and consequent difficulties in identification. We are attempting to formulate the exact nature of such identifiability problems when single channel data are available over a range of glutamate concentrations.

Comparison with Other Ion Channels

The emerging picture of a relatively complex gating mechanism for the GluR may be compared with the results of similar studies of other ion channels. Invertebrate glutamate receptors have also been looked at in the crayfish (Dudel and Franke, 1987; Franke and Dudel, 1987). In the latter work the open time distributions do not appear to consist of more than a single exponential component, and the closed time distributions are composed of two or three components. A possible reason for the difference between these results and those described in Kerry et al. (1987), and in the current paper lies in the greater number of single channel events that have been recorded from the locust GluR. Resolution of the differences awaits a more detailed comparison. Excitatory amino acid receptors in vertebrate systems appear to have a complex relationship between the

receptor(s) and the channel (Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987), precluding direct comparison with the invertebrate GluR.

The concentration of L-glutamate required to give an open probability of 0.5 is high, but this was anticipated from the results of macroscopic studies of junctional glutamate receptors on locust muscle (Clements and May, 1974). The sensitivity of glutamate receptors on embryonic locust muscle in vitro, which have recently been studied using the giga-ohm seal technique, is much higher, although the reason for this difference is unclear (Cook et al., 1985; Duce and Usherwood, 1986). Dudel and Franke (1987) have shown that the glutamate receptor channels of crayfish muscle also exhibit a low sensitivity to L-glutamate, and have concluded that concentrations of the transmitter in the range of 1×10^{-3} M would be required to generate postsynaptic currents at the glutamatergic excitatory synapses of this muscle. The low sensitivity of the transmitter glutamate receptors on adult arthropod muscle may be related to the high levels of L-glutamate, and other agonists, which are often present in the blood of the open circulatory systems of these animals.

The most intensively studied receptor-gated ion channel is the nAChR. Many authors (e.g., Labarca et al., 1985; Colquhoun and Sakmann, 1985) have provided evidence for the existence of two open states in the nAChR gating mechanism. Sine and Steinbach (1984) have emphasized the presence of multiple closed states. At present, there is some debate concerning the nature of the two open states (Sine and Steinbach, 1986). It is of particular interest to note that Labarca et al. (1985) have proposed a branched ($N_p = 2$) model for *Torpedo* nAChR gating.

The work of Blatz and Magleby (1986) on rat skeletal muscle Cl^- channel gating has also resulted in the proposal of a branched gating mechanism. Their working hypothesis for the major mode of Cl^- channel gating is characterized by $N_c = 5$, $N_o = 2$, and $N_p = 2$, of comparable complexity to the model for the GluR. In a somewhat different context, cooperative allosteric models have also been proposed to account for the effects of dihydropyridines on the kinetics of L-type Ca^{2+} channels (Kokubun et al., 1986).

In the light of these studies our working hypothesis for GluR gating does not seem to be unreasonably complex. Two major tasks remain to be undertaken. First, for mechanisms 6 and 8, the values of k^o_{on} and k^c_{on} remain to be determined. Second, alternative gating mechanisms need to be compared in an objective manner. Although initial results suggest that the ligand association rates may be estimated using the glutamate concentration dependence of the acfs, adoption of maximum likelihood procedures (Horn and Vandenberg, 1984) should enable us to fufill both tasks simultaneously. Work is underway on the implementation of such an approach, and should result in a more detailed understanding of the molecular mechanism of GluR gating.

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