Chapter 19

Experimental Approaches Used to Examine Single Glutamate-Receptor Ion Channels in Locust Muscle Fibers

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1. Introduction

Fluctuation analysis has allowed estimation of the mean parameters of synaptic channels at vertebrate (Katz and Miledi, 1972, 1973) and invertebrate nerve–muscle junctions (Crawford and McBurney, 1976; Anderson *et al.*, 1976; Dudel *et al.*, 1980). By means of patch-clamp recording, it has been possible to directly observe single-channel currents activated by acetylcholine or glutamate in various muscle membranes (Neher and Sakmann, 1976; Neher *et al.*, 1978; Patlak *et al.*, 1979).

We have been using the patch clamp recording technique on glutamate receptors in locust muscle to address several specific problems. First, we have attempted to examine general channel characteristics and, in particular, the concentration–response relationship of the individual receptor–channel complex (Cull-Candy *et al.*, 1981a). Second, since the conductance of the glutamate-activated channel is large, rapid kinetics of the channel have been examined using wide-band recording (Cull-Candy and Parker, 1982a) as described for acetylcholine receptor channels (Colquhoun and Sakmann, 1981). Third, the rapid kinetics of agonist-activated channels are presently being examined (Cull-Candy and Parker, 1982b).

2. Single Glutamate-Activated Channels

With conventional patch clamp recording, it is possible to obtain glutamate-activated single-channel currents from the extrajunctional membrane of normally innervated locust muscle fibers. Although the resistance of the seal formed between the patch electrode and the muscle fiber is low (< 20 MΩ), the signal/noise ratio is reasonably good since the conductance of the single channel is large. The ease with

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which channels can be detected was improved by first treating the fibers with concanavalin A to reduce desensitization (Mathers and Usherwood, 1976). Figure 19-1 illustrates typical records made with an electrode containing 100 μM glutamate, from fibers voltage clamped at -110 mV to increase the current amplitude. The density of the extrajunctional D-receptors appeared rather variable compared with measurements using ionophoretic glutamate application (Cull-Candy, 1976), and some fibers showed no apparent sensitivity. However, in fibers in which we found sensitivity, the receptors were randomly distributed rather than occurring in pairs or small groups. Because of their low density, it was possible to select membrane patches where only a single active channel was present. This was routinely done, since data from a single channel are easier to interpret.

3. Distribution of Lifetimes

Channel lifetimes usually fit well to an exponential distribution. Figure 19-2A,B shows histograms of a sample of single-channel lifetimes plotted on linear coordinates and on log–linear coordinates. The average lifetime of all events was 3.4 msec. Because of the limited bandwidth of the recording system, brief events are attenuated; the mean lifetime was therefore also estimated from an exponential distribution (dashed line) fitted to the data. The mean lifetime from the fitted exponential was 3.1 msec. It was also possible to estimate the mean lifetime by performing spectral analysis on the data. Figure 19-2C is a power spectrum of the same data. As expected, the power spectrum of single-channel currents can be reasonably well fitted by a single Lorentzian; the mean channel lifetime, calculated from the half-power frequency, f_c, according to $\tau = 1/(2\pi f_c)$, was $\tau = 3.54$ msec, in reasonable agreement with estimates obtained from the distribution histograms.

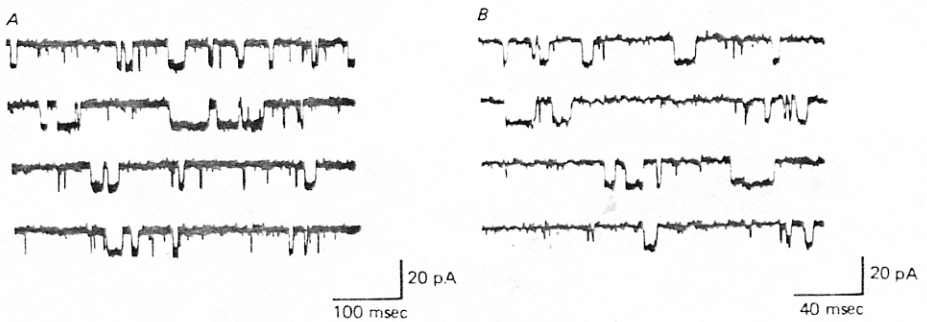

Figure 19-1. A,B: Records of glutamate-induced single-channel activity from two con-A-treated muscle fibers; note the difference in sweep speeds. Chloride-free solution was used for the bathing medium and in the patch pipette; in both experiments, the pipette contained 100 μM glutamate. Fibers were voltage clamped to a potential of -110 mV with a conventional two-microelectrode clamp, and the patch pipette was placed between the recording and current-passing electrodes. Recording bandwidth was DC to 1 kHz. Inward current is shown as a downward deflection. $T = 22°C$; calibration, 100 or 40 msec and 20 pA. (From Cull-Candy *et al.*, 1981.)

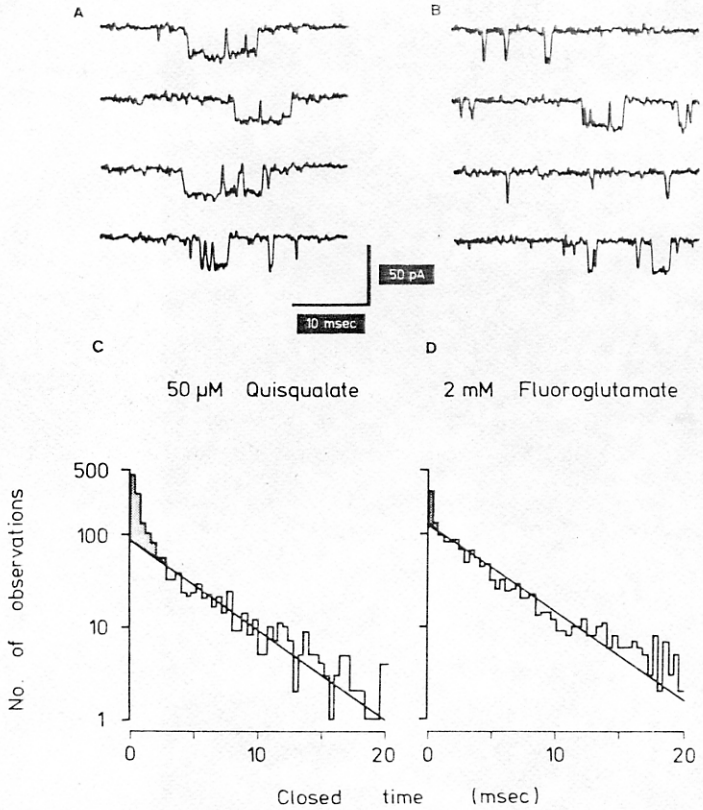

Figure 19-8. A,B: Recordings of single-channel currents activated by 50 μM quisqualate (A) and 2 mM fluoroglutamate (B) made with a bandwidth of DC–3 kHz. For both agonists, some channel openings can be seen to be interrupted by brief closings, but the mean number of brief closings per burst is higher in the case of quisqualate. C,D: Distribution of channel-closed times for channels activated by 50 μM quisqualate (C) and 2 mM fluoroglutamate (D), plotted on semilogarithmic scales: 3288 events were analyzed in C, and 3336 in D. Over much of the time scale, the distributions can be fitted well by single exponential functions (straight lines), except that for both agonists there is a clear excess of very brief closings (shaded areas). The relative number of excess brief closings is greater for quisqualate than for fluoroglutamate.

estimate that the mean duration of the individual openings that constitute the bursts is roughly doubled when the agonist changes from fluoroglutamate to quisqualate.

Although we have not observed any clear differences in mean duration of the brief closings with different agonists, this remains a possible source of error in these measurements, since very short closings would remain undetected and hence lead to an underestimate of the number of openings per burst. Further improvements in resolution of the recordings are still required.

ACKNOWLEDGMENTS. We are grateful to the MRC and the Wellcome Trust for support.

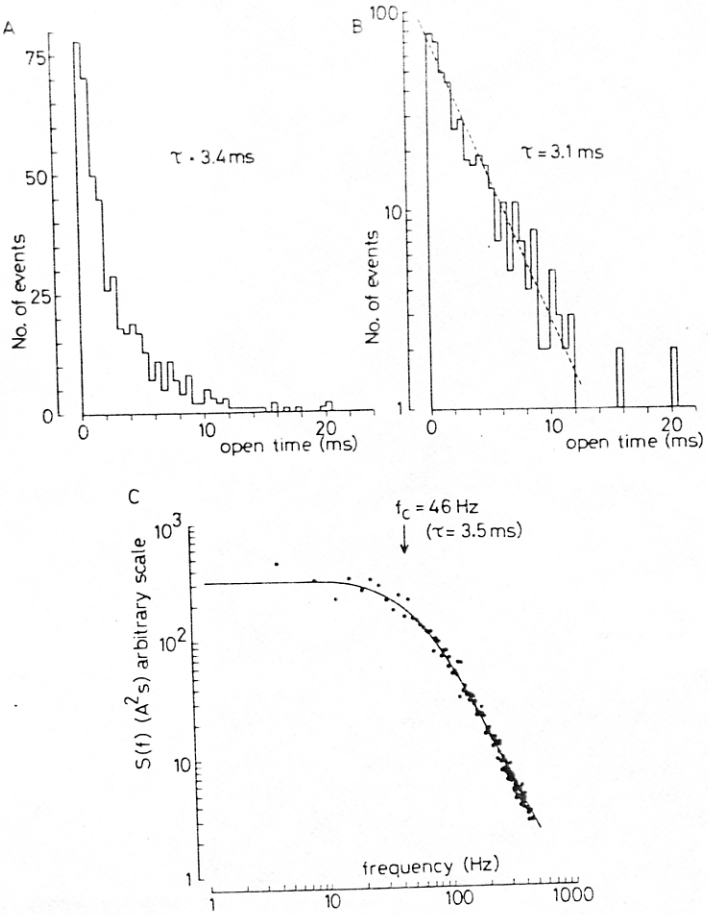

Figure 19-2. A,B: Distribution of channel-open times induced by glutamate. Patch pipette contained 100 μM glutamate, and 468 channel openings were collected. The data are shown on linear coordinates in A and on semilogarithmic coordinates in B. The line fitted to the data in B has a time constant of 3.1 msec. C: Averaged power spectrum derived from the same data record as in A and B. Blocks of 512 data points were sampled at a digitization interval of 1 msec, and power spectra were computed using a fast Fourier transform algorithm. Background noise in the recordings contributed relatively little power as compared to the channel openings and was not subtracted for the spectrum illustrated. The half-power frequency (f_c) of the spectrum is indicated by the arrow.

4. Recording of Miniature Currents with Patch Clamp

Single-channel currents have not so far been recorded at junctional glutamate receptors in locust fibers. However, if a patch pipette is positioned close to the neuromuscular junction, mejcs are readily detected. The signal/noise ratio is better than that obtained with a conventional two-electrode voltage clamp. Figure 19-3A–C shows examples of mejcs recorded either with a patch clamp electrode (A, B) or under voltage clamp (C). Although resolution of the mejc time course recorded with

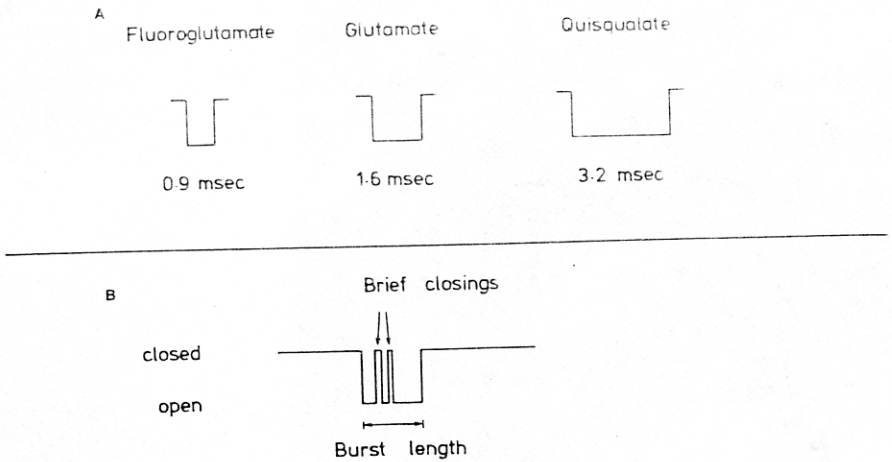

Figure 19-7. Diagrammatic illustration of channel openings. A: In recordings having a limited bandwidth (≤ 1 kHz), channel openings appears as simple rectangular current pulses. The mean open lifetimes are different for channels activated by different glutamate agonists. B: Recordings with a more extended bandwidth (~ 5 kHz) indicate that many channel openings are interrupted by intervening brief closings. The duration of the "burst" of openings therefore corresponds to the mean open lifetime measured with restricted-bandwidth recording.

increase in the number of openings per burst or in the mean duration of each individual opening.

Figure 19-8A,B illustrates examples of channels opened by 2 mM fluoroglutamate and 50 μM quisqualate. These two concentrations gave roughly similar opening rates, indicating that the receptor affinity for fluoroglutamate is probably low compared with the affinity for quisqualate. Brief closings occured when the channels were opened by either agonist, although from the recordings in Fig. 19-8, these are more evident for quisqualate-activated channels.

This was borne out when distributions of closed times were plotted for channels opened by fluoroglutamate and quisqualate (Fig. 19-8C,D). As with channels opened by glutamate, the closed times could be fitted to an exponential distribution except for a marked excess of brief closings. The closings falling outside the exponential distribution are shown as the stippled area in Fig. 19-8C,D, and this area is proportionally greater for channels opened by quisqualate. The mean burst lengths of the drugs examined have been estimated as described in the previous section and, in these experiments, were fluoroglutamate 0.9 ± 0.06 msec, glutamate 1.62 ± 0.16 msec, and quisqualate 3.15 ± 0.5 msec. Since the number of closings per burst went in the order quisqualate > glutamate > fluoroglutamate, there was a concomitant sequence in the number of openings per burst. The estimated openings per burst in these experiments were fluoroglutamate 1.42 ± 0.06, glutamate 1.58 ± 0.04, and quisqualate 2.1 ± 0.12. This significant increase in the number of openings per burst is not, by itself, sufficient to account for the difference in burst length. The influence of agonists on burst length therefore presumably results from a change in both the number of openings per burst and the mean duration of each opening. We

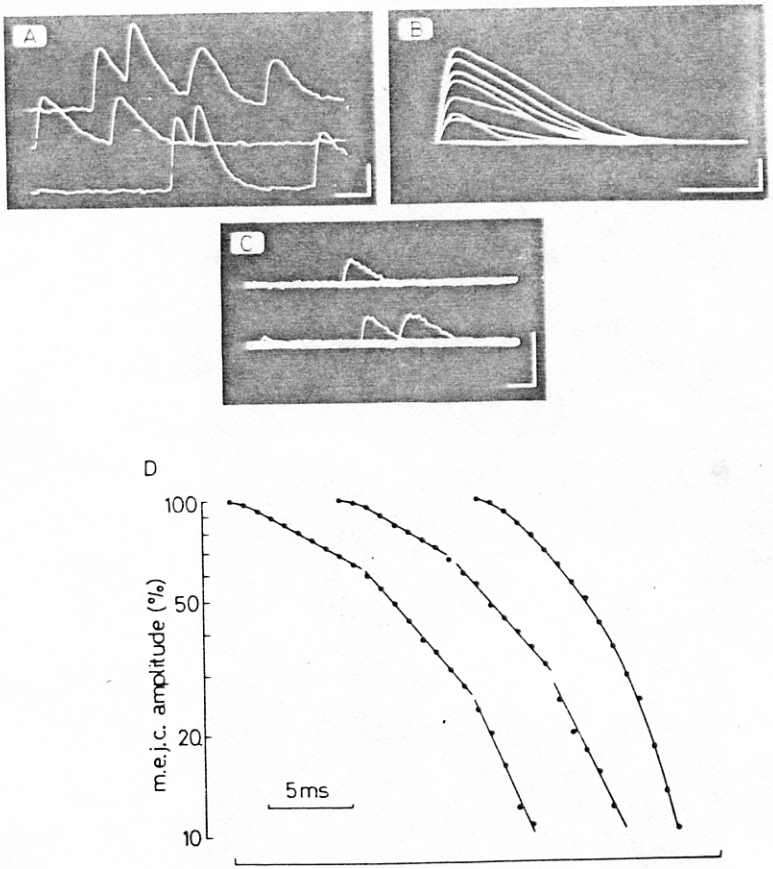

Figure 19-3. A,B: Examples of mejcs recorded with a patch-clamp electrode. The patch pipette was filled with normal locust medium. C: Miniature ejcs recorded using a conventional two-point voltage clamp. The muscle was bathed in Cl-free medium to improve the space clamp. Different fiber from that in A and B. D: Semilogarithmic plot of decay time courses of three mejcs recorded using a patch-clamp electrode.

patch clamp is good, the amplitude of the currents is less meaningful, since the seal resistances obtained were not high and because some events may occur at a distance from the pipette tip.

Some mejcs recorded with the patch clamp do decay exponentially and with a time course similar to intracellularly recorded mejcs. These therefore appear to give a reasonable estimate of the mean lifetime of the junctional channel for comparison with extrajunctional single-channel currents. However, other mejcs show more complex time courses (Fig. 19-3B,D). It was found that when pressure was exerted with the patch pipette, the proportion of mejcs that decay nonexponentially was increased as fibers were mechanically distorted, and in some experiments, all the events recorded had complex time courses that required several exponentials to give a good fit (Fig. 19-3D). This resembled the so-called "compression artifact" observed when miniature currents are recorded with a focally positioned micropipette at

To examine brief closings quantitatively, it was necessary to form histograms of the closed-time distribution as illustrated in Fig. 19-6D,E. The closed times can be fitted with a single exponential, in agreement with earlier studies, except that there were a large excess of closings with durations < 200 μsec. These increased sharply at shorter intervals but rolled off, probably because of events remaining undetected, in the 0–50 μsec range, as expected from the frequency resolution of the system.

The total number of brief closings in a record has been estimated by assuming that both "brief events" and "normal events" are exponentially distributed. Histograms were therefore fitted by the sum of two exponentials corresponding to the "brief" and "normal" distributions. The histograms in Fig. 19-6D,E show closed times on two different time scales. The time constants of the exponentials are $\tau_{fast} = 100$ μsec and $\tau_{slow} = 2$ msec. The predicted total excess of brief closings (including the undetected ones) estimated from the area under the fast exponential is 383, compared with 616 "normal" closings, fitted by the slow exponential. There are therefore about 0.6 closings/burst and hence 1.6 openings.

The mean open time of a burst can be estimated from the total open time during a record divided by the number of interburst intervals and is 1.64 msec. Thus, the burst length is ~1.7 msec, and the mean duration of each opening within a burst is 1.0 msec. The total burst length of 1.7 msec is therefore equivalent to the "apparent channel lifetime," previously measured with noise analysis and patch clamp (Anderson *et al.*, 1976; Patlak *et al.*, 1979; Cull-Candy *et al.*, 1981).

The possibility that brief closings may result from transient channel blocking by agonist molecules seems unlikely since the number of brief closings per millisecond open time did not increase with glutamate concentration. An attractive possibility is that, in a sequential reaction scheme consisting of binding steps followed by an isomerisation step, the intraburst closings result from reisomerization of the channel to the open state after closing without agonist dissociation (Colquhoun and Hawkes, 1977; Colquhoun and Sakmann, 1981; Cull-Candy and Parker, 1982).

7. Agonist-Activated Channels

At the vertebrate end plate, the lifetime of the synaptic channel is dependent on several factors including the agonist that opens the channel (Katz and Miledi, 1973). Similar observations have also been made for amino-acid-activated channels in locust and crustacean muscle fibers examined with noise analysis (Anderson *et al.*, 1976; Crawford and McBurney, 1976) and patch clamp recording (Gration *et al.*, 1981; Cull-Candy *et al.*, 1981). Mean parameters of the channels opened by fluoroglutamate, glutamate, and quisqualate, estimated with low-frequency-resolution patch clamp recording, are illustrated in Fig. 19-7A. Although conductances are virtually identical, the mean lifetime is 0.9 msec for fluoroglutamate, 1.6 msec for glutamate, and 3.2 msec for quisqualate. With improved time resolution recording, it became apparent that the previous estimates of lifetime may correspond to the mean burst length (Fig. 19-7B) of a burst of openings. The question therefore arose as to whether differences in burst length with different agonists result from an

esterase-blocked vertebrate end plates (Katz and Miledi, 1973) and suggests that at the locust nerve–muscle junction diffusion may be the major mechanism involved in the removal of transmitter from the synaptic cleft (Cull-Candy *et al.*, 1981).

5. Internal Perfusion of Patch Electrodes

In order to examine the concentration dependence of individual receptor–channel complexes, we have used a simple system for the internal perfusion of patch electrodes, which enables changes of drug solutions to be made while recording from a single channel (Cull-Candy *et al.*, 1981a). The method, illustrated in Fig. 19-4, allows rapid internal perfusion of the tip of the patch electrode by means of a multibarrel (usually six barrels) pipette, which is inserted down the inside of the patch electrode. The patch electrode and perfusion pipette are held on the two arms of a Zeiss double tool holder. The fine control of the arm on which the perfusion pipette is mounted is then used to position the tip of the perfusion system within 100

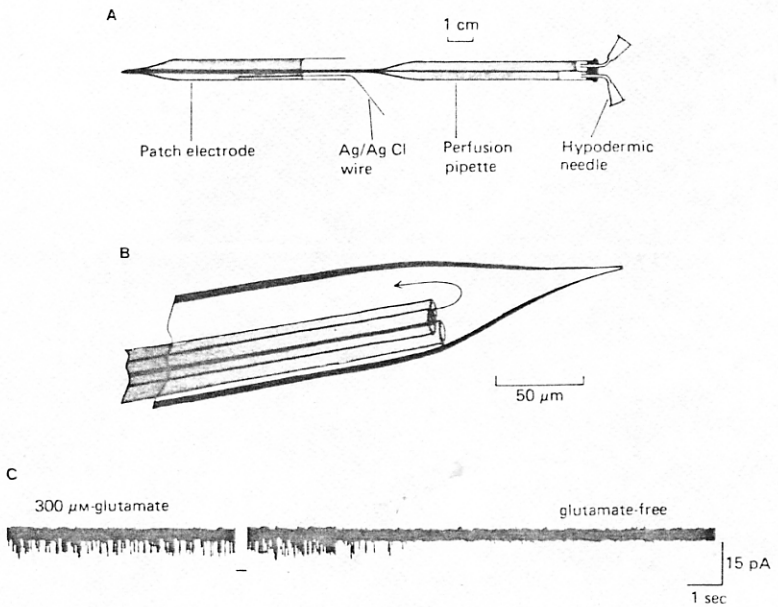

Figure 19-4. A: Diagram showing the arrangement of a perfusion pipette inserted in a patch electrode. For clarity, the diameter of the pipettes has been exaggerated, and only two barrels are shown on the perfusion pipette. B: Tracing from a photomicrograph of the tip of a patch-clamp pipette containing an internal triple-barrel perfusion pipette. The arrow indicates the direction of flow of solution from a perfusion barrel during the application of pressure. C: Continuous record of channel activity during internal perfusion of a pipette with 300 μM glutamate, followed by glutamate-free medium. Channel activity disappeared about 5 sec after perfusion of the electrode with glutamate-free solution. (From Cull-Candy *et al.*, 1981.)

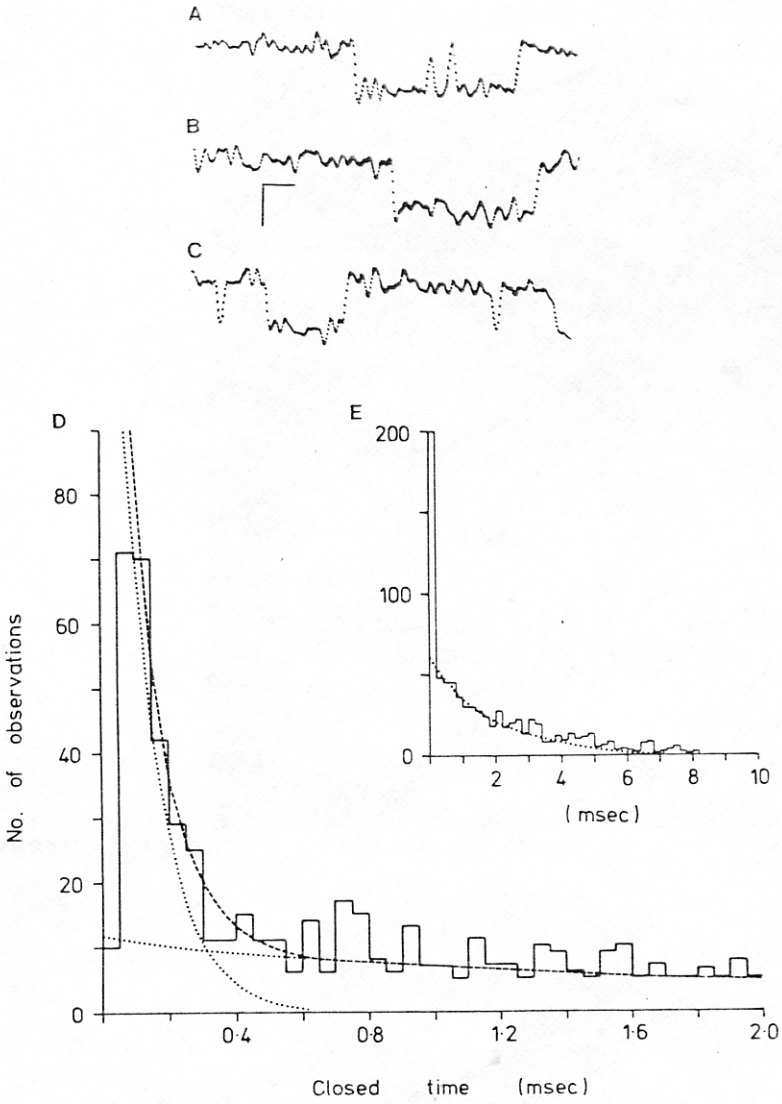

Figure 19-6. A,B,C: Examples of single-channel currents recorded with high time resolution (DC–3.2 kHz). Channel openings correspond to downward deflections. Calibration 20 pA, 1 msec. D,E: Histograms (on different time scales) of distributions of single-channel closed times, showing the excess of brief events. Data are measurements from 832 channel openings from a membrane patch in which only one channel was active. The distribution in D has been fitted by the sum of two exponentials; the component exponentials are indicated by dotted curves, and the sum by dashed curves; only the slow exponential is shown in E. Time constants of the exponentials are $\tau_{fast} = 100$ μsec; $\tau_{slow} = 2$ msec; area under the exponentials is fast 383, slow 616. (From Cull-Candy and Parker, 1982a.)

μm of the patch electrode tip (Fig. 19-4B). The whole operation is carried out under visual control using a compound microscope (6.3 × 16). Air pressure can then be applied to one of the hypodermic needles that are cemented into the back of the perfusion pipette. Drug solution is thereby released from the desired barrel to produce an excess in the patch-pipette tip (see Cull-Candy *et al.*, 1981, for further details).

It was possible to accomplish a change of fluid to a new one within about 10 sec. Figure 19-4C shows a record obtained during perfusion with 300 μM glutamate followed by glutamate-free solution (applied at the time indicated by the bar). Single-channel currents ceased within 5–10 sec after the start of perfusion with glutamate-free solution.

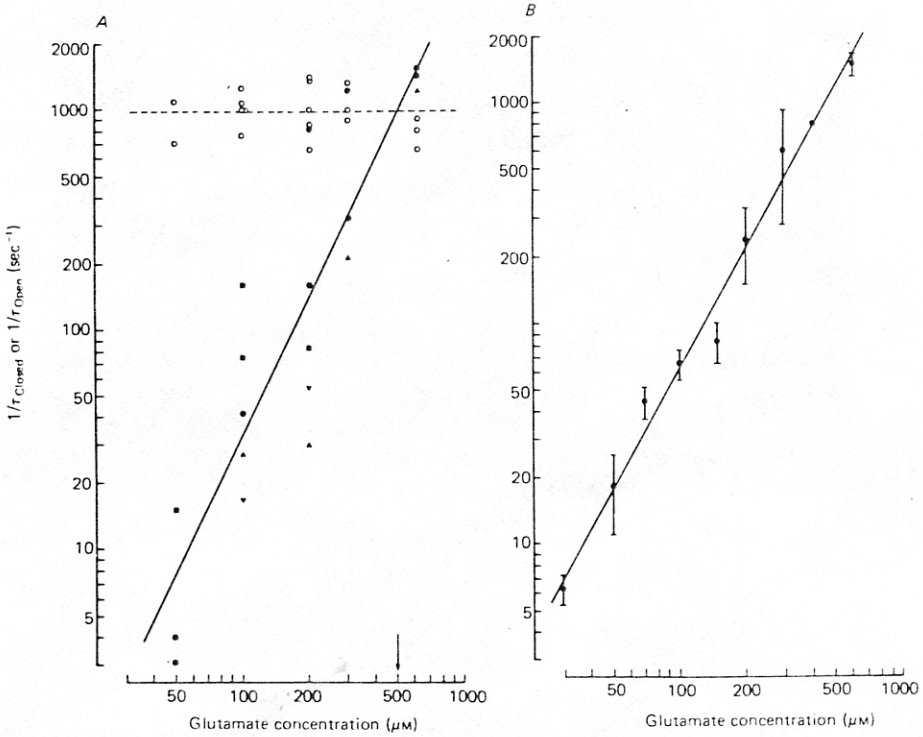

Figure 19-5. Relationship between the reciprocal of mean channel closed time, $1/\tau_{closed}$, and glutamate concentration. A: $1/\tau_{closed}$ (filled symbols) and the reciprocal of the mean channel-open time, $1/\tau_{open}$ (open circles) are plotted against the concentration of glutamate contained in the patch pipette (logarithmic coordinates). Measurements of $1/\tau_{closed}$ were obtained from four membrane patches in different muscle fibers (●, ■, ▲, ▼). Several glutamate concentrations were tested at each site, and $1/\tau_{closed}$ was estimated from the time constant of the distribution histogram. The continuous line through the points is fitted by the least-squares method and has a slope of 2.1. The mean value of $1/\tau_{open}$ (○) obtained from the same experimental records is indicated by the dashed line. Open and closed times are equal at the intercept of the lines fitted to $1/\tau_{closed}$ and $1/\tau_{open}$, which occurs at a glutamate concentration of 500 μM (indicated by arrow). B: Relationship constructed from estimates of $1/\tau_{closed}$ obtained at 17 membrane patches. The continuous line through the points has a slope of 1.85. All values are mean ± S.E. (except at 400 μM glutamate, which is only one value). (From Cull-Candy *et al.*, 1981.)

As expected, the frequency of openings increases with glutamate concentration. To obtain a quantitative measure of the relationship, we have determined the mean closed time (i.e., interval between events) for an individual channel in the presence of a range of glutamate concentrations.

For a first-order reaction, the rate constant for channel closing is concentration independent. The net forward rate constant, β', is an apparent rate constant, as its value depends on the binding steps preceding channel opening. It should therefore increase with agonist concentration, as expected of a second-order step. For this scheme, the net forward reaction rate is $\beta' = 1/\text{closed time}$. Figure 19-5 shows double-log plots of the dependence of 1/closed time on the glutamate concentration contained in the patch electrode tip. The data in Fig. 19-5A are from four different membrane patches in which varying glutamate concentrations were applied to individual receptors by internal perfusion of the electrode. The reciprocal of open time, α, for the same data was not apparently dependent on concentration in the range examined, although some apparent dependence has been described for higher concentrations (Gration *et al.*, 1981). The apparent dissociation constant is $K_{App} = \alpha/\beta'$. Hence, when 50% of channels are open (i.e., open time = closed time), K_{App} = agonist concentration. For the data in Fig. 19-5A, the mean open time is 1.0 msec, and K_{App} is 500 μM glutamate (intersect of the continuous and dashed lines). Figure 19-5B illustrates pooled results from 17 individual channels, and the slope of the relationship is close to 2. For these data, where the mean open time is 1–2.5 msec (not shown), K_{App} is around 300 μM. The dissociation constant for the glutamate receptor is therefore roughly an order of magnitude larger than K_{App} for the ACh receptor (Sakmann *et al.*, 1980).

6. Burst Kinetics of Glutamate-Activated Channels

By using a recording system with improved frequency resolution, we have found an excess of brief closings and brief openings over that expected from a single exponential distribution. The recording technique was similar to that used in earlier experiments except that fibers were clamped at more hyperpolarized levels (-110 to -150 mV) to increase the signal/noise ratio. More importantly, the time resolution of the recordings was improved by means of a frequency compensation circuit after the headstage amplifier. The final bandwidth was DC–5 kHz, although data were usually low-pass filtered at ~3 kHz before analysis. As previously described, the resolution of the recording system was such that events of 50 μsec or longer could be readily identified (see Cull-Candy and Parker, 1982a, for further details).

As can be seen in Fig. 19-6A, some single-channel currents were interspersed with brief closings. In our previous experiments with more restricted bandwidth, such closing would not have been detected, and the currents would have appeared as single events of long duration. Some events of long duration did not show brief closings. In addition, an excess of brief openings was present (Fig.19-6C). However, it should be mentioned that a deficiency rather than an excess of brief openings has also been described (Gration *et al.*, 1982). The reason for this contrasting observation will require further examination.