Rapid kinetics of single glutamate-receptor channels

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Drug-induced ionic channels are known to operate by opening briefly when agonist molecules bind to receptor sites[1-7] to produce rectangular current pulses, the amplitude and lifetime of which can be derived from noise analysis[1-3], or directly recorded using the patch-clamp technique[4-7]. If desensitization is neglected, the distributions of channel open and closed times can generally be fitted by single exponential functions[5-7], suggesting that a simple two-stage reaction scheme is sufficient to account for the observed kinetics[1]. Using patch-clamp recording with an improved time resolution, we have now found that glutamate-activated channels in locust muscle membrane show a large excess of both brief openings and closings over those expected from single exponential distributions. Many events which, with poorer frequency resolution, would have appeared as single openings were found to consist of two or more openings, interspersed with brief (<300 μs) closings. These observations have interesting implications for receptor/channel functioning.

Patch-clamp recordings of single glutamate-activated channels were obtained from the extensor tibiae muscle of the locust (*Schistocerca gregaria*), using techniques similar to those described previously[4,6]. Patch-clamp pipettes contained 200 μM glutamate, which gave a high frequency of channel openings, and muscle fibres were usually voltage-clamped at a potential of −110 mV to increase the size of the single channel currents. Muscles were pretreated with 1 μM concanavalin A to reduce receptor desensitization[5], and recording patches were selected where only one active channel was present (that is, where no simultaneous openings were observed[6]). The time resolution of the recordings was improved significantly by using a circuit, after the headstage amplifier, which partially compensated for high-frequency roll-off caused by capacitance across the feedback resistor. This gave an overall frequency response of d.c. to ~5 kHz, but records were usually filtered at 3 kHz to reduce noise, before being digitized for computer analysis. Data were sampled in blocks of 512 points, at a digitization rate of 40 or 20 kHz.

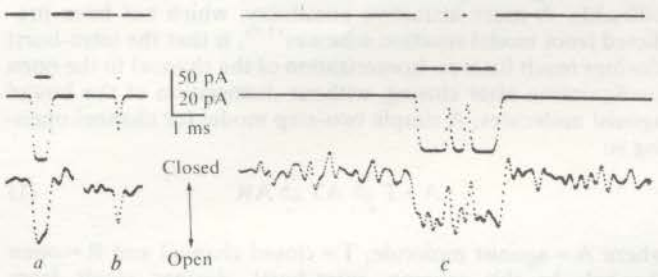

Fig. 1 Patch-clamp recordings of test pulses (*a*, *b*) and single channel currents (*c*) illustrating the time resolution of the recording system and the method of measurement. In each record the lower trace is a computer-digitized signal from the path-clamp amplifier, sampled at 25-μs intervals after sharp cut filtering ($1/f^4$) at 3.2 kHz. The upper traces show computer-generated traces used to help measure the data. A rectangular pulse (upper trace) with a height equivalent to the single channel current was generated and the position and width of this could be altered manually. A smoothing function applied to this pulse approximated the step response function of the recording system and the resultant smoothed pulse (middle trace) was displayed on a screen superimposed on the data. The position and width of the generated rectangular pulse was adjusted so that the smoothed pulse gave the best match to a channel opening and these parameters were then stored. Channel openings in each 512-point data block were fitted sequentially and a display of previously fitted openings was maintained to assist in matching brief channel closings (*c*). After analysis of several hundred data blocks, histograms of open and closed times of the generated rectangular pulses were automatically constructed. *a*, *b*, Lower traces show recordings of rectangular test pulses with durations of 500 and 50 μs respectively. Upper traces are computer-generated pulses fitted to the recordings. Calibrations, 50 pA and 1 ms. *c*, Lower trace shows single channel currents recorded from a membrane patch. Channel openings are shown as downward deflections. Upper trace shows the rectangular current pulses considered to underlie the records, whilst the middle trace shows the expected distortion of these pulses due to the frequency response characteristics of the recording system. Calibrations, 20 pA and 1 ms.

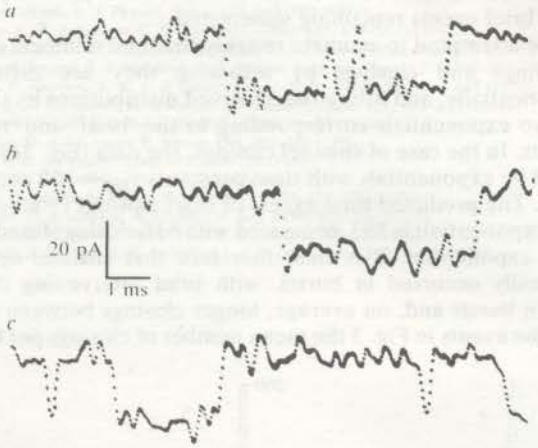

Fig. 2 Examples of single channel currents recorded with high time resolution (d.c. to 3.2 kHz). Channel openings correspond to downward deflections. Same membrane patch and recording parameters as in Fig. 1*c*. Temperature, 23 °C. Calibrations, 20 pA and 1 ms.

The frequency response of the patch clamp was such that test pulses lasting 500 μs could be recorded with little distortion (Fig. 1*a*), whilst 50 μs pulses were detectable but became broadened and attenuated (Fig. 1*b*). The rise and fall times of single channel currents were similar to those of the test pulses (Figs 1*c*, 2), suggesting that the true transition times of the channel were faster than the resolution of the recording system. To permit more accurate measurements of brief channel openings and closings, a computer program was developed which partially compensated for the restricted frequency response of the recordings (see Fig. 1 legend). The ultimate limiting factor in time resolution was then set by the signal-to-noise ratio. We were unable to obtain seal resistances against the muscle membrane of better than 20 MΩ, so noise from this source was high[4], but as large single channel currents (20 pA) can be recorded from locust muscle the problem was not too serious.

A striking feature in our records was that many channel openings were interrupted by one or more brief closings (Figs 1*c*, 2*a*), although some long openings showed no apparent intervening closings (Fig. 2*c*). Also, there seemed to be more brief openings than expected from an exponential distribution (Fig. 2*c*). A quantitative examination was made by forming histograms of open and closed time distributions; Fig. 3 illustrates data from 832 channel openings recorded from one membrane patch. The present results are consistent with previous findings that both the open and closed time distributions of single glutamate channels can be fitted reasonably by single exponentials[5,6] and that open times are exponentially distributed[3], except that at short intervals (<200 μs) there is a considerable excess of both channel openings and closings (Fig. 3*b*, *d*), which are probably too brief to have been detected in previous patch-clamp studies having a narrower frequency range.

Figure 3*a*, *c* shows the distributions of brief openings and closings on an expanded time scale. In both cases an excess of events over a single exponential distribution becomes apparent at intervals of <~300 μs, and increases steeply with shorter intervals, except for a falloff in the shortest histogram bin (0–50 μs). A duration of 50 μs is about the limit of resolution of

the recording system, so the falloff in this bin is probably due to very brief events remaining undetected.

We attempted to estimate roughly the total numbers of brief openings and closings by assuming they are distributed exponentially, and fitting the observed distributions by the sum of two exponentials corresponding to the 'brief' and 'normal' events. In the case of channel closings, the data (Fig. 3*c*) can be fitted by exponentials with time constants $\tau_{fast} = 100$ μs, $\tau_{slow} =$ 2 ms. The predicted total excess of brief closings (=area under fast exponential) is 383, compared with 616 closings fitted by the slow exponential. It is clear therefore that channel openings generally occurred in bursts, with brief intervening closings within bursts and, on average, longer closings between bursts. For the events in Fig. 3 the mean number of closings per burst is ~0.6 and hence the mean number of openings per burst is 1.6. The mean open time per burst can be estimated from the total area under the open time distribution (dashed line in Fig. 3*a*) divided by the number of inter-burst intervals and is 1.64 ms. Hence, the total burst length is ~1.7 ms and the mean duration of the openings within a burst ~1.0 ms. This estimate includes both the fast and slow components of the open time distribution. If the brief intra-burst closings were not detected, the apparent 'channel lifetime' would correspond to the mean burst length, and previous estimates of channel lifetime from noise analysis[3] and patch-clamp recording[5,6] are in reasonable agreement with our estimate of the mean burst length.

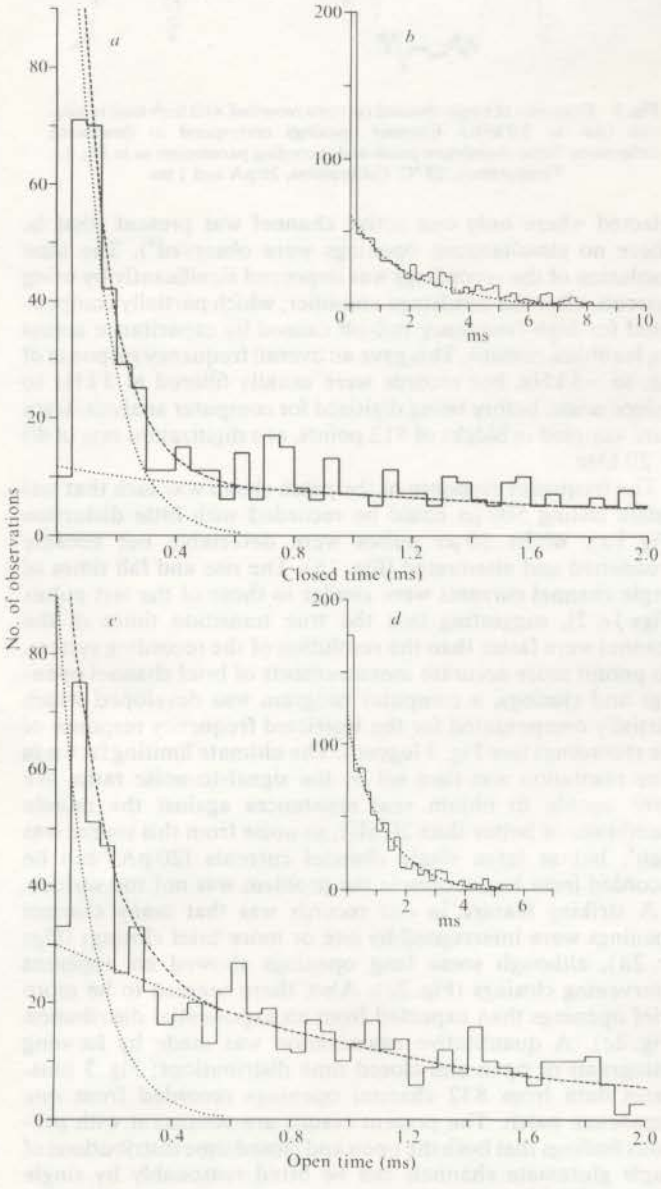

Fig. 3 Histograms of distributions of single channel closed (*a*, *b*) and open (*c*, *d*) times illustrating the excess of brief events. Data are measurements from 832 channel openings recorded from one membrane patch where only one channel appeared to be present. During the entire recording period the channel was in the 'normal' kinetic state[6]. Insets (*b*, *d*) show the same data as the main histograms, but on a different time scale. The distributions in *a* and *c* have been manually fitted by the sum of two exponentials; the component exponentials are indicated by dotted curves and the sum by dashed curves. Only the slow exponentials are shown in the insets. Time constants of the exponentials are: open times, $\tau_{fast} = 120$ μs, $\tau_{slow} = 1.5$ ms; closed times, $\tau_{fast} = 100$ μs, $\tau_{slow} = 2$ ms. Area under the exponentials are: open time, fast = 220, slow = 717; closed time, fast = 383, slow = 616. In preliminary experiments with different glutamate concentrations, estimated time constants for the closed time were: 100 μM, $\tau_{fast} = 110$ μs, $\tau_{slow} = 6.8$ ms; 600μM, $\tau_{fast} = 117$ μs, $\tau_{slow} = 0.86$ ms (mean values from 5–7 patches).

Considering next the excess of brief openings, the mean lifetime of this component estimated from the fitted fast exponential is 120 μs, and the predicted number in the recording period is 220, compared with 717 'normal' openings. Thus, about one-quarter of all channel openings were due to the brief component, but because of their short duration they would have carried only ~3% of the total current.

Records from seven other membrane patches (four muscles) using 200 μM glutamate were similar: a mean value of 1.6 ± 0.03 (s.e.) openings per burst, with a mean channel open lifetime of 0.97 ± 0.12 ms. However, in view of possible errors and assumptions in fitting the fast exponential functions to the tails of poorly resolved distributions, these values should be regarded as provisional.

Brief intervening closings during channel openings and an excess of brief openings have been observed from acetylcholine-receptor channels in frog twitch muscle[8] and the existence of intra-burst closings has been inferred from noise analysis in frog slow muscle fibres[9]. It seems likely therefore that these features may be general to most drug-activated channels. One possible mechanism for the intra-burst closings is that the channel is transiently blocked in a manner analogous to the action of local anaesthetics[10,11]. If this blocking resulted from glutamate molecules plugging the channel, then the number of brief closings would be expected to increase directly with glutamate concentration. In preliminary experiments we found that the number of brief closings per millisecond of open time showed little change over a range of glutamate concentrations (100, 200, 400, 600 μM). A more attractive possibility, which has been predicted from model reaction schemes[12,13], is that the intra-burst closings result from re-isomerization of the channel to the open configuration after closing, without dissociation of the bound agonist molecules. A simple two-step model for channel opening is:

$$A + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AT \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} AR \qquad (1)$$

where A = agonist molecule, T = closed channel and R = open channel. In this scheme, intra-burst closings result from the transitions AR → AT → AR, and their mean duration is $1/(\beta + k_{-1})$. For our data $\beta \approx 4 \times 10^3\ s^{-1}$. Similarly, $\alpha \approx 1/$ channel lifetime $\approx 10^3\ s^{-1}$. The mean number of openings per observed burst $= (1 + \beta/k_{-1}) = 1.6$; hence $k_{-1} \approx 6 \times 10^3\ s^{-1}$. In fact, this two-step model is probably an over-simplification; for example, opening of the glutamate channel seems to involve binding of two or more agonist molecules[6,14]. Such a cooperative model is expected to give rise to a three-component distribution of closed times[13]. The intermediate component (due to brief closings via the singly bound state) may be partially included in our estimated fast component, but calculations based on a cooperative model indicate that the maximum contribution would be smaller than our experimental error. The rough estimates of α, β and k_{-1} are therefore applicable to both cooperative and non-cooperative models.

The origin of the excess brief openings is more mysterious. One possibility, which may be readily discarded, is that two separate channels of different mean lifetimes, were present under the patch pipette. If this were the case, 47 simultaneous openings would have been expected in the record of Fig. 3, and the probability of observing zero simultaneous events is $\approx 10^{-20}$;

none was observed. Another possibility, which has been suggested to explain similar brief openings of acetylcholine-receptor channels[8], is that they arise from channel openings when only one agonist molecule is bound. Important clues as to the origins of both the brief openings and closings should be given by their dependence on agonist concentration and species of agonist. It is hoped to explore these possibilities further using internally perfused patch-clamp pipettes[6,14].

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