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ON THE MULTIPLE-CONDUCTANCE SINGLE CHANNELS ACTIVATED BY EXCITATORY AMINO ACIDS IN LARGE CEREBELLAR NEURONES OF THE RAT

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SUMMARY

1. Single-channel currents evoked by excitatory amino acids have been examined in outside-out patches from large cerebellar neurones (including Purkinje cells) in tissue culture. L-Glutamate (3-10 μM), L-aspartate (3–10 μM), NMDA (*N*-methyl-D-aspartate, 10–50 μM), ibotenate (50 μM), quisqualate (3–50 μM), and kainate (3–50 μM) all produced single-channel currents with multiple amplitudes.

2. Single-channel currents recorded over a range of patch potentials had a mean interpolated reversal potential of $-3{\cdot}8 \pm 0{\cdot}5$ mV. The directly resolvable multiple conductance levels could be classified into five main groups, with mean values (averaged for all agonists) of: 47.9 ± 0.7, $38{\cdot}5 \pm 0{\cdot}8$, $27{\cdot}8 \pm 1.4$, $18{\cdot}2 \pm 0{\cdot}5$ and $8{\cdot}3 \pm 0{\cdot}6$ pS.

3. From the relative areas under current amplitude histograms it was estimated that the percentage of openings with conductances greater than 30 pS was about 83% with NMDA, 79% with glutamate and 78% with aspartate. In some patches, the majority of > 30 pS events evoked by these agonists were to the maximum conductance of 48 pS, whereas in other patches there were more 38 pS openings than 48 pS openings. Only 27% of quisqualate openings, and about 10% of kainate openings, were > 30 pS.

4. Of the small amplitude (< 20 pS) events, 93% of quisqualate openings were to the 8 pS level whereas ~ 87% of < 20 pS currents produced by NMDA, glutamate and aspartate were to the 18 pS level (the remainder being 8 pS). Direct transitions could occur between certain levels (including events above and below 30 pS) suggesting that these are sublevels of multiple-conductance channels. The most frequently occurring transitions were between the 48 and 38 pS levels, and the 38 and 18 pS levels.

5. Channel openings occurred in bursts, within which individual openings were separated either by brief closures (gaps), or by direct transitions between the multiple conductance levels. The briefest of these gaps (< 200–400 μs) could represent a mixture of transitions to lower conductance levels as well as partially resolved complete shuttings. The mean duration of the longer gaps within bursts,

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thought to represent complete but partially resolved shuttings was $1{\cdot}05 \pm 0{\cdot}25$ ms (pooled for all agonists).

6. Burst-length distributions could be fitted with the sum of three exponentials. The briefest component may have arisen from brief single openings. The two slower components probably reflect the existence of two kinetically distinct open states. The mean time constants of the longer two components were $1{\cdot}7 \pm 0{\cdot}2$ and $13{\cdot}5 \pm 1{\cdot}8$ ms and were similar for all agonists. Spectra of single-channel currents in outside-out patches could also be fitted with two Lorentzian components, with mean time constants of $1{\cdot}6 \pm 0{\cdot}13$ and $17{\cdot}2 \pm 1{\cdot}4$ ms (pooled for all agonists). These were clearly similar to the directly measured bursts lengths.

7. When applied to isolated patches, kainate (and to a lesser extent quisqualate) gave a noise increase, accompanied in some patches by discrete single-channel openings (similar in conductance to those produced by other agonists). Analysis of kainate currents in outside-out patches gave two component spectra with time constants of $0{\cdot}9 \pm 0{\cdot}1$ and $16{\cdot}4 \pm 2{\cdot}4$ ms ($V_m = -70$ mV). The mean single-channel conductance estimated from the spectra was γ(kainate) $= 1{\cdot}03 \pm 0{\cdot}2$ pS. The fast component contributed most power to spectra of kainate and quisqualate single-channel currents.

8. Our results may be explained by assuming that NMDA, quisqualate and kainate receptors are coupled to separate channels, and that some, or all, of these receptor channels possess multiple conductance levels. All the channels appear to show two kinetically distinct open states, and two (or three) shut states. Differences in the properties of glutamate channels present in large cerebellar neurones and in certain other neurones are discussed.

INTRODUCTION

Single-channel recording originally indicated that NMDA and glutamate open channels with a maximum conductance of about 50 pS in embryonic mouse midbrain neurones (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984) and rat cerebellar granule neurones (Cull-Candy & Ogden, 1985). From later studies on membrane patches isolated from large cerebellar neurones and hippocampal neurones, it has become clear that glutamate receptor channels exhibit multiple conductance levels in these cells (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987). Indeed, it seems that the multiple conductances may well be a common feature of channels activated by excitatory amino acids, since they have recently also been observed in cerebellar granule cells (Cull-Candy, Howe & Ogden, 1988), mouse spinal and midbrain neurones (Ascher, Bregestovski & Nowak, 1988; Ascher & Nowak, 1988*a*), and in type-2 cerebellar astrocytes (Usowicz, Gallo & Cull-Candy, 1989).

We have used the methods of single-channel recording (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) and noise analysis in outside-out patches to examine further the multiple conductance levels and kinetic properties of channels activated by the three selective glutamate receptor agonists, *N*-methyl-D-aspartate (NMDA), quisqualate and kainate (see Watkins & Olverman, 1987) in large cerebellar neurones. A preliminary report of part of this work has appeared (Cull-Candy & Usowicz, 1987).

METHODS

The preparation and identification of cells, and the drugs and solutions used were as previously described (Cull-Candy & Usowicz, 1989).

Recordings

Single-channel currents were recorded from outside-out patches (excised from neuronal cell bodies) as described previously (Hamill *et al.* 1981), at room temperature (20–24 °C). All single-channel recordings were made with pipettes pulled from thick-walled capillaries, with external and internal diameters of, respectively, 1·5 and 0·86 mm ('hard' Pyrex glass). Their resistances were 5–12 MΩ when filled with 140 mM-CsCl internal solution (see Cull-Candy & Usowicz, 1989). The capacitance of each pipette was reduced by coating its shank with Sylgard resin (184, Dow-Corning), and pipette tips were fire-polished. The currents were recorded on FM tape (Racal Store 4; bandwidth DC to 2·5 or 5 kHz, −1 dB).

Analysis

Single-channel currents from magnetic tape were transferred to a PDP 11/40 computer via a low-pass filter set at 1, 1·5 or 2 kHz (−3 dB, eight-pole Bessel, Barr & Stroud). The filter cut-off frequency chosen was the setting at which single-channel currents that were approximately 10–17% of their full amplitude appeared distinct from the background noise. Clearly, there may have been smaller openings that were below the noise level. The larger currents could have been characterized with a wider bandwidth, if information about the small levels had not been required. Records were digitized continuously at 10–20 kHz (CED 502 interface, Cambridge Electronic Design) with a sampling rate 5–10 times the filter cut-off frequency (−3 dB). The digitized currents were displayed on a screen and openings were detected as crossings of a manually set threshold level. Amplitudes were then measured with two horizontal cursors, also displayed on the screen. The duration of the events was measured by the 'time-course' fitting method, i.e. the digitized currents were fitted with the measured step-response function of the recording system (patch-clamp, tape-recorder and filters; see Colquhoun & Sigworth, 1983; Colquhoun & Sakmann, 1985). The resultant lists of open times, shut times and amplitudes were revised by imposing minimum resolvable durations, which ranged from 60 to 200 μs, for openings and shuttings.

The presence of multiple current amplitudes makes the analysis of single-channel records more complex than when there is only one main current level. As a consequence, the amplitude and duration of incompletely resolved (brief) openings and gaps within openings is uncertain (see below). Gaps which could have been *either* transitions to lower conductance levels *or* complete closures were excluded from the analysis of shut-time times. This was done by calculating the gap duration at which a complete closure from the maximum 48 pS level would attain an amplitude of 4·8 pS (i.e. well below 8 pS, the smallest mean level that could be resolved). This was calculated for each experiment as w, according to $y_{max} = \text{erf}(2{\cdot}668fw)$; where y_{max} is the maximum response (in this case 90%, since the difference between 4·8 and 48 pS is 90%, taking the baseline as 100% gap amplitude) produced by a unit pulse of width w (Colquhoun & Sigworth, 1983); erf is the error function, and f is the −3 dB frequency of the Bessel filter. A sum of exponentials was fitted to distributions of shut times that were equal to, or longer than, w. This method of excluding undefined gaps was not ideal as it probably excluded many full closures, particularly those arising from conductance levels of less than 48 pS: the amplitude that a complete closure attains, at 90% of its full amplitude, clearly depends on the size of the current that it interrupts. For example, within an 18 pS opening such a gap will reach the 1·8 pS level. Hence, the gap duration necessary for a complete closure arising from the 38, 18 or 8 pS levels to attain an amplitude of 4·8 pS is briefer than the value calculated for w, as outlined above. Indeed, it may have been simpler to include all the shut times in the fit (with four exponentials), with the understanding that the first component probably represented a mixture of shuttings or rapid transitions to lower conductance levels. The revised record was used for displaying and fitting the current-amplitude distributions, and shut-time and burst-length distributions (Colquhoun & Sigworth, 1983).

In order to determine the value of the multiple current levels, amplitude measurements were made of only those openings with 'flat tops' (i.e. openings which seemed to be fully resolved). These measurements, however, will inevitably contain some erroneous values since events which are almost, but not quite, fully resolved can appear to have reached full amplitude in the presence of

noise. These values were displayed by forming amplitude histograms. Since the amplitudes of brief openings are unknown they cannot be included in the amplitude distributions which, as a result, underestimate the total number of openings in a patch. The histograms show amplitudes of *individual openings*, where an individual opening is defined as an opening in the normal sense, and as a subconductance level reached by a direct transition from another level within the same single-channel current. For example, a single-channel event consisting of a 38 pS opening, which is followed by a direct transition to the 18 pS level, which is then followed by a closing, comprises two individual openings (38 and 18 pS).

Although the duration, as well as the amplitude, of the brief openings is uncertain in the presence of multiple current levels, we attempted to measure the durations of all openings, to avoid biasing the distributions of shut times. Partially resolved openings were fitted as openings to the most frequently observed level in a patch, the amplitude of which was used to scale the step-response function. For example, for glutamate-evoked currents this was the maximum 48 pS level in some patches, whereas in others it was the 38 pS level. The fit of the function to some of the currents was clearly poor, indicating that the assumed amplitude was quite different from the true amplitude of the openings. Furthermore, in the presence of multiple current levels it is not immediately obvious whether gaps are partially resolved complete shuttings or brief sojourns in lower current levels. Indeed, two partially resolved complete shuttings of a unitary-conductance channel, in rapid succession, can easily be misinterpreted as a sublevel (see Colquhoun & Sakmann, 1985). Therefore, gaps were initially fitted as a single complete shutting, or two rapidly occurring complete shuttings. Only if these step-response functions gave a poor fit, were the currents then fitted as a transition to a subconductance level.

Amplitude histograms were fitted with the sum of several Gaussian distributions, by the method of maximum likelihood (Colquhoun & Sigworth, 1983). Mean current levels were determined from these Gaussian components, or they were measured directly from currents displayed on a digital oscilloscope. They were converted to slope conductances or to chord conductances, employing a mean reversal potential of −4 mV (Cull-Candy & Usowicz, 1987; and see Results section).

A burst was defined as a group of openings separated from another burst by a shut interval equal to, or longer than, a critical length, t_c (see Colquhoun & Sakmann, 1985). Briefly, t_c was calculated to make the proportion of intervals between bursts, that were misclassified as intervals within bursts, equal to the proportion of intervals within bursts misclassified as between bursts. The mean value for t_c was 2·2 ms. The average proportion of misclassified events was approximately 10%, and was greatest for channels activated by quisqualate, presumably because quisqualate activated many small conductance openings which were more difficult to characterize (see Results). Bursts were defined only according to t_c; they were not classified according to the conductance levels of their constituent individual openings. Distributions of shut times and burst lengths were fitted with the sum of several exponentials by the method of maximum likelihood (Colquhoun & Sigworth, 1983).

Single-channel currents were also analysed by the method of spectral analysis (Katz & Miledi, 1972; Anderson & Stevens, 1973). The currents from magnetic tape were transferred to a PDP 11/73 computer via a filter (bandwidth: DC or 0·2 to 500 Hz, −3 dB, eight-pole Butterworth, Barr & Stroud) and analysed as described for whole-cell noise in the previous paper (Cull-Candy & Usowicz, 1989).

RESULTS

Single-channel currents in outside-out membrane patches

Single-channel currents have been examined in outside-out patches excised from the cell body of large cerebellar neurones. Glutamate (3–10 μM), aspartate (3–10 μM), NMDA (10–50 μM), ibotenate (50 μM), quisqualate (3–50 μM) and kainate (10–50 μM) all gave single-channel currents with multiple current amplitudes. Figure 1 shows examples of single-channel currents in three different patches exposed to 50 μM-

NMDA (Fig. 1*A*, patch potential −70 mV), 50 μM-ibotenate (Fig. 1*B*, V_m = −100 mV), and 10 μM-quisqualate (Fig. 1*C*, V_m = −70 mV). The predominant current levels present in each of these records have been converted to slope or chord conductances (for a mean reversal potential of −4 mV, see below), and are indicated

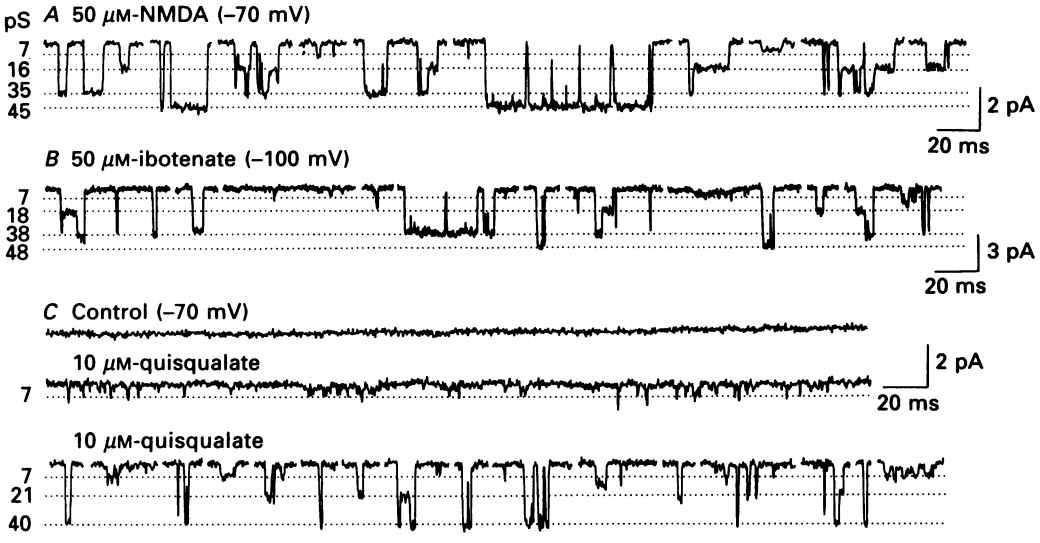

Fig. 1. Single-channel currents evoked by glutamate receptor agonists in three outside-out membrane patches. *A*, currents produced by 50 μM-NMDA at −70 mV to four levels (indicated by dotted lines). These correspond to slope conductances of 45, 35, 16 and 7 pS (interpolated reversal potential was −3 mV). Direct transitions between the 35 and 16 pS, and the 45 and 35 pS levels can be identified. Calibration, 2 pA and 20 ms. *B*, currents evoked by 50 μM-ibotenate at −100 mV. In this patch ibotenate produced openings to four conductance levels (dotted lines) of 48, 38, 18 and 7 pS (for a mean reversal potential of −4 mV). Direct transitions between the 38 and 18 pS levels are evident. Calibration, 3 pA and 20 ms. *C*, currents produced by 10 μM-quisqualate at −70 mV. Control noise level (upper trace). Where there were no openings to larger conductance levels (middle trace) smaller conductance changes were seen with quisqualate but could not be individually resolved; the dotted line indicates a conductance of 7 pS. Quisqualate also gave well-resolved single-channel openings (lower trace) to at least three conductance levels (dotted lines) of 40, 21 and 7 pS (for an extrapolated reversal potential of 0 mV). There are clear transitions between the 40 and 21 pS levels. Calibration, 2 pA and 20 ms.

by the dotted lines. The NMDA openings in Fig. 1 are to conductance levels of 45, 35, 16 and 7 pS; ibotenate gave openings of 48, 38, 18 and 7 pS; and quisqualate produced 40, 21 and 7 pS conductance openings (lower trace in Fig. 1*C*). Quisqualate also induced an increase in noise level in some patches. The quisqualate noise increase in Fig. 1*C* (middle trace) was recorded from the same patch as the discrete quisqualate openings (lower trace), during a section of the response that showed few of the larger amplitude events. Transitions that are apparently direct can be identified between the various multiple conductance levels; for example, transitions

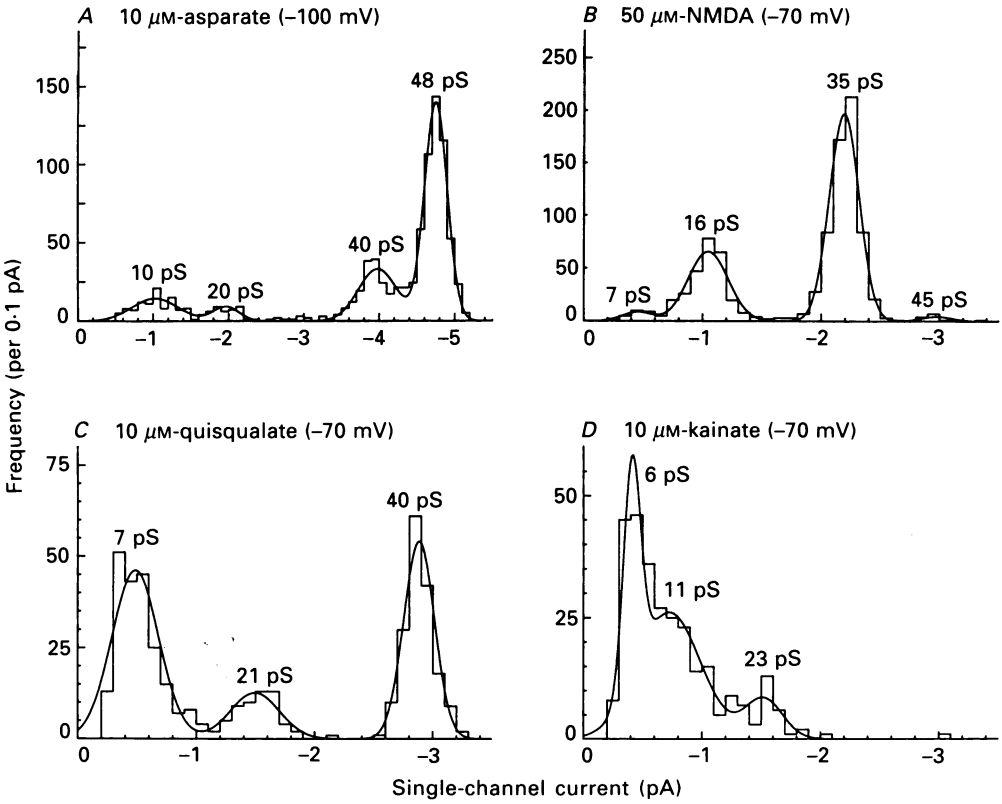

Fig. 2. Amplitude histograms of single-channel currents in four different patches. *A*, response to 10 μM-aspartate (−100 mV). *B*, response to 50 μM-NMDA (−70 mV, same patch as Fig. 1*A*). *C*, response to 10 μM-quisqualate (−70 mV, same patch as Fig. 1*C*). *D*, response to 10 μM-kainate (−70 mV). The peaks in the distributions of current amplitudes evoked by aspartate (*A*) and NMDA (*B*) are fitted by the sum of four Gaussian components (continuous line), while the distributions of quisqualate amplitudes (*C*) and kainate amplitudes (*D*) are described by the sum of three Gaussian components. Mean current levels were determined from the Gaussians and converted to conductances, which are given above each peak. The relative areas of the peaks (*A*, *B*) indicate that both aspartate and NMDA produced predominantly openings with conductances above 30 pS. The maximum conductance was also the most frequently visited (or main) level of aspartate channels, whereas the main conductance level of NMDA channels in this patch was the 35 pS, rather than the maximum (45 pS level). In contrast, quisqualate (*C*) and kainate (*D*) activated mainly conductances below 30 pS.

between 16 and 35 pS and between 35 and 45 pS are present within the NMDA currents in Fig. 1*A*; transitions between 18 and 38 pS can be seen in the presence of ibotenate in Fig. 1*B*, and between 21 and 40 pS in the presence of quisqualate in Fig. 1*C*.

The relative frequency of occurrence of the different conductance levels was examined more quantitatively by measuring openings with the time-course fitting method (see Methods, and Colquhoun & Sigworth, 1983), and forming amplitude histograms of the currents measured. Such amplitude distributions displayed clearly resolvable peaks and were usually fitted with the sum of several Gaussians. Figure 2

illustrates distributions of amplitudes of single-channel currents evoked by 10 μM-aspartate (Fig. 2*A*, −100 mV), 50 μM-NMDA (Fig. 2*B*, −70 mV, data from same patch as Fig. 1*A*), 10 μM-quisqualate (Fig. 2*C*, −70 mV; same patch as Fig. 1*C*) and 10 μM-kainate (Fig. 2*D*, −70 mV), in four different patches. It is apparent from the histogram in Fig. 2*D* (fitted with the sum of three Gaussian components), that kainate can activate multiple conductances, similar to those produced by the other agonists. However, kainate (10–50 μM) also produced a noticeable increase in the noise level of the patch, which presumably resulted from the activation of low-conductance events that were not individually resolved; this resembled the increase in noise level detected in some patches in response to quisqualate. The kainate currents produced in outside-out patches will be considered in further detail below.

To obtain an accurate estimate of the multiple conductance levels, single-channel currents produced by the various agonists were measured over a range of patch potentials. Current amplitudes were either calculated from the Gaussian components detected in each distribution, or were obtained by direct measurement from a digital oscilloscope. Figure 3 shows examples of the current–voltage relationships of the multiple conductance levels produced by the various glutamate agonists. The current–voltage plot in Fig. 3*A* is for the maximum-amplitude single-channel currents activated by 10 μM-glutamate, 10 μM-aspartate and 10 μM-NMDA, in four outside-out patches. The least-squares line through the points indicates a mean slope conductance of 48 pS and a mean reversal potential of −3 mV; the relationship appears to be reasonably linear over the range of potentials examined (−100 to +70 mV).

In Fig. 3*B* the amplitudes of all current levels, observed in a single patch exposed to 10 and 50 μM-NMDA and 10 μM-glutamate, are plotted against patch potential. Least-squares lines have been drawn through the points (for all but the smallest current level) and have slope conductances of 46, 35 and 18 pS and a reversal potential of −4 mV. In this patch the smallest current level could only be accurately measured at extremes of potential; the line was drawn through −4 mV, with an assumed slope of 8 pS (i.e. the mean conductance estimated for the smallest level from all patches). Figure 3*C* and *D* illustrates the relationship between single-channel current and patch potential for channels activated by glutamate, aspartate, NMDA, quisqualate and ibotenate in a total of eleven outside-out patches. The lines through the data have been drawn through a mean reversal potential of −4 mV, and with slopes corresponding to four of the mean conductance levels (48, 38, 18 and 8 pS, see below).

Reversing the direction of the currents in nine outside-out patches, gave a mean interpolated reversal potential of $-3{\cdot}8 \pm 0{\cdot}5$ mV (mean ± S.E.M.). When averaged for all agonists the conductance estimates (pooling both slope and chord conductances) could be classified into five main groups, with mean values (± S.E.M.) of $47{\cdot}9 \pm 0{\cdot}7$ pS (twenty-three patches), $38{\cdot}5 \pm 0{\cdot}8$ pS (twelve patches), $27{\cdot}8 \pm 1{\cdot}4$ pS (seven patches), $18{\cdot}2 \pm 0{\cdot}5$ pS (thirteen patches) and $8{\cdot}3 \pm 0{\cdot}6$ pS (seventeen patches). The 28 pS openings occurred infrequently and are therefore not present in Fig. 3.

The various agonists did not appear to activate all five conductance levels in all patches. Inspection of the records indicated that three or four levels were activated by glutamate (3–10 μM) in eighteen out of twenty-five patches, by aspartate (3–10 μM) in fourteen out of sixteen patches, by NMDA (10–50 μM) in eighteen out of

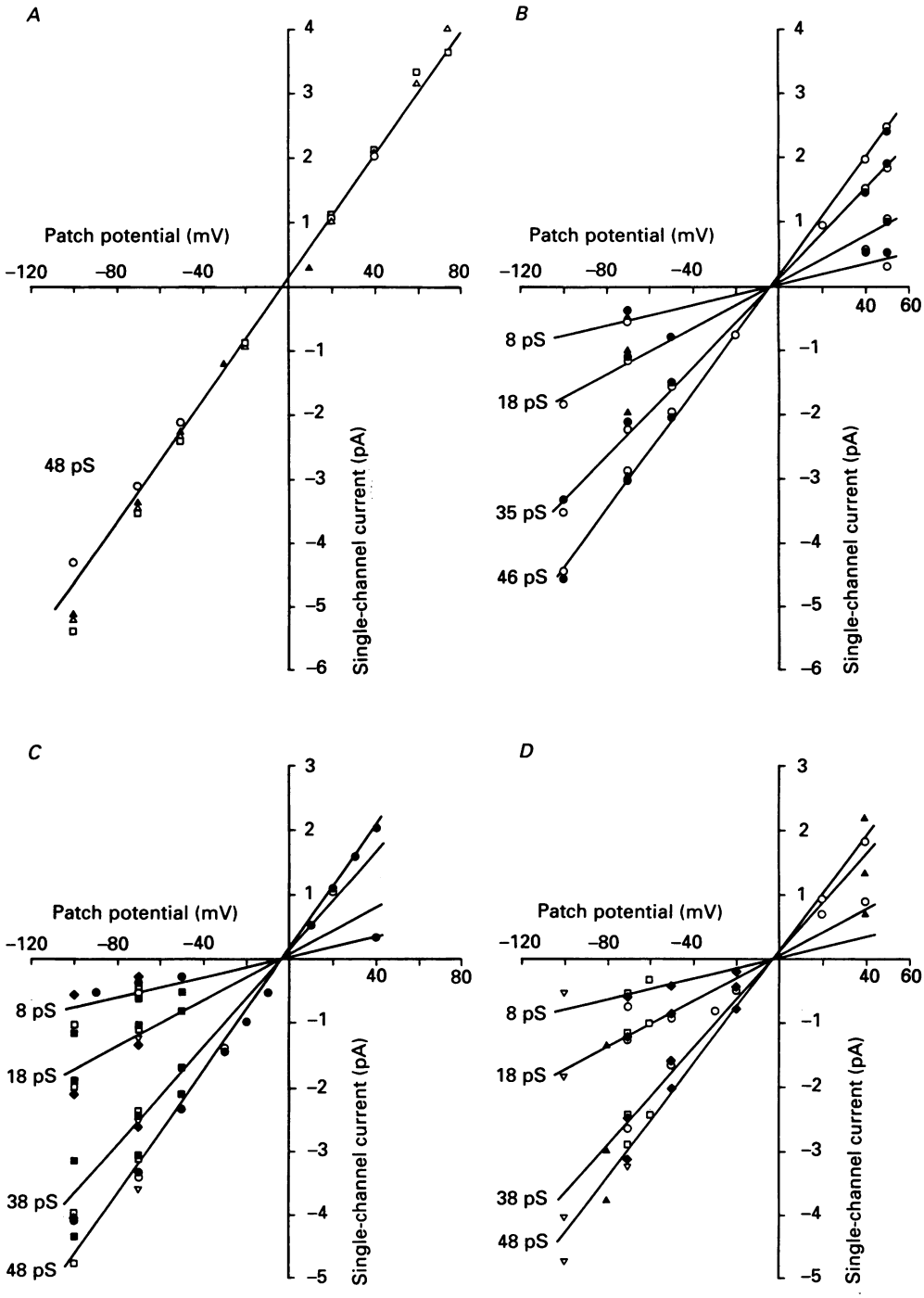

Fig. 3. For legend see opposite.

twenty patches, and by quisqualate (10–50 μM) in five out of twenty-six patches. The remaining patches usually displayed two levels. The fact that the number of conductance levels activated by any one of the agonists differed between patches could result from variation between cells (or patches). In addition, it may depend on the concentration of the agonists used (see below) or it could reflect differences between experiments in the level of extracellular glycine present (see Johnson & Ascher, 1987). These possibilities are being further examined.

Different agonists preferentially activate different conductance levels

It can be seen from the relative areas of the peaks in the current amplitude histograms (Fig. 2), that the various agonists activate the different conductance levels with differing relative probabilities. For each agonist the percentage of openings with conductances above 30 pS was calculated from the relative areas under the fitted Gaussian curves. Classification of openings according to whether their conductance exceeded 30 pS, or was below 20 pS, was convenient since 20–30 pS conductances occurred relatively infrequently, and agonists often preferentially activated *either* the 8 and 18 pS levels *or* the 38 and 48 pS levels.

Of the currents produced by NMDA (10–50 μM) 83$\pm$3% (mean$\pm$S.E.M., $n = 6$ patches) had conductances above 30 pS. Similarly, the putative transmitters L-glutamate (3–10 μM) and L-aspartate (10 μM) mainly evoked openings with conductances above 30 pS, the relative percentages being 79$\pm$2% (seven patches) for glutamate, and 78$\pm$6% (three patches) for aspartate. Ibotenate also appeared to preferentially activate conductances greater than 30 pS, although ibotenate currents were not analysed by the time-course fitting method.

The percentage of openings with conductances above 30 pS were clearly similar for glutamate, aspartate and NMDA, and pooling data for these three agonists gave a mean value of 79$\pm$3% (mean$\pm$S.E.M., sixteen patches). This is significantly different from the percentage of >30 pS openings obtained with 10–50 μM-quisqualate: 27$\pm$8% (mean$\pm$S.E.M., seven patches, $P < 0{\cdot}01$, two-tailed t test). Amplitude histograms of kainate-evoked openings were considered to be an

Fig. 3. Relationship between single-channel currents and patch potential for channels activated by glutamate receptor agonists in outside-out patches. *A*, maximum amplitude currents evoked by 10 μM-glutamate (▲, △) 10 μM-aspartate (□), 10 μM-NMDA (○), in four different patches. The least-squares line through the points indicates a mean reversal potential of -3 mV and a mean slope single-channel conductance of 48 pS. *B*, multiple current amplitudes produced by 10 μM-NMDA (○), 50 μM-NMDA (●), and 10 μM-glutamate (▲) in a single patch. Least-squares lines are drawn through points for all but the smallest current levels. These give a mean reversal potential of -4 mV and slope conductances of 46, 35 and 18 pS. The line through the points representing the smallest current level is drawn through -4 mV with a slope of 8 pS (the mean conductance estimated from all patches examined). *C*, multiple current amplitudes evoked by 3 μM-glutamate (▽), 10 μM-aspartate (□), 10 μM-NMDA (○), 50 μM-NMDA (●), 50 μM-ibotenate (■), and 10 μM-quisqualate (◆). *D*, multiple current amplitudes evoked by 3 μM-glutamate (▽), 10 μM-glutamate (▲), 10 μM-NMDA (○), 10 μM-aspartate (□), and 10 μM-quisqualate (◆) in a total of eleven patches. The lines in *C* and *D* are drawn through -4 mV with slopes corresponding to single-channel conductances of 48, 38, 18 and 8 pS. These conductances and the estimate of -4 mV for the reversal potential were the mean values obtained from all patches examined.

approximate representation of the various conductance levels present, since many of the small-conductance openings could not be measured individually, and the small noise increase that accompanied discrete kainate openings hampered the measurement of the discrete levels. Hence, our estimate of about 10% for openings above 30 pS, represents an upper limit. In summary, it is apparent from these experiments that the various glutamate receptor agonists preferentially activated different conductance levels, and at the concentrations tested (3–50 μM) none was specific for any one level. The percentage of openings with conductances above 30 pS followed the sequence: NMDA $\geqslant$ aspartate $\geqslant$ glutamate > quisqualate > kainate.

A further feature of glutamate receptor channels in large cerebellar neurones is that the relative proportions of the multiple levels activated by any one agonist may show concentration dependence. There was some evidence to suggest that as the concentrations of glutamate, aspartate and NMDA were increased these agonists became less 'selective' for the > 30 pS conductances. For example, the mean percentage of > 30 pS openings was about 87% for 3 μM-glutamate, but 77% for 10 μM-glutamate when the two concentrations were tested on the same patch; it was 88% for 10 μM-NMDA, but 77% for 50 μM-NMDA in a single patch. Moreover, inspection of currents produced by 3 μM-glutamate or 3 μM-aspartate (of which fewer were analysed by the time-course fitting method) indicated that not only may the relative proportion of > 30 pS openings be greater at lower concentrations, but also many more of these openings may be to the maximum 48 pS conductance level, rather than the 38 pS level. Indeed, when currents produced by both 3 and 10 μM-glutamate were analysed in one patch, the resultant amplitude histograms indicated that 48 and 38 pS openings accounted for, respectively, about 98 and 2% of > 30 pS openings produced by 3 μM-glutamate, whereas in the presence of 10 μM-glutamate, the corresponding values were 73% and 10%. At a concentration of 10 μM or more, depending on the patch, it was not uncommon for the proportion of 38 pS openings to exceed the proportion of 48 pS openings in response to glutamate, aspartate and NMDA. This occurred in two out of five patches with 10 μM-glutamate, one out of three patches with 10 μM-aspartate, and four out of six patches with 10–50 μM-NMDA. The histogram in Fig. 2*B* was from a patch where NMDA gave more 35 than 45 pS openings (equivalent to the mean conductances of 38 and 48 pS); whereas, in a different patch, aspartate evoked more 48 than 40 pS (equivalent to 38 pS) openings (see Fig. 2*A*).

The relative proportions of the conductance levels activated by quisqualate or kainate may also vary with concentration. At 3 μM, quisqualate activated predominantly the 8 pS level, while the larger conductances appeared relatively more frequently as the concentration was increased (up to 50 μM). An increase in the concentration of kainate, from 10 to 50 μM, was accompanied by an increase in the number of kainate-induced 48 pS openings (see Cull-Candy & Usowicz, 1987, Fig. 2).

For channels activated by 10–50 μM-quisqualate, the relative percentage of 8 pS openings exceeded the relative percentage of 18 pS openings in six out of seven patches; 8 pS openings comprised 93 $\pm$ 4% (mean $\pm$ S.E.M., six patches) of quisqualate currents with conductances smaller than 20 pS. In contrast, the proportion of 18 pS openings exceeded the proportion of 8 pS openings when channels were activated by 10 μM-glutamate, 10 μM-aspartate and 10–50 μM-NMDA in eight out of nine patches (data pooled for the three agonists); 18 pS openings comprised 87 $\pm$ 5% (mean $\pm$ S.E.M., eight patches) of currents with conductances less than 20 pS.

Variability in current amplitudes between patches

In the present experiments we have grouped the directly resolvable currents produced by the various agonists into five levels, rather than report each conductance value from every patch. For example, the estimate of the maximum conductance, 48 pS, represents a mean of values from different experiments, ranging from 44 to 50 pS. Of the four most commonly observed mean conductances (i.e. 48, 38, 18 and 8 pS) the variability between patches, in the conductance estimates, was smallest for the 48 pS level and greatest for the 8 pS level, the standard deviations being,

respectively, 6·6% and 27% of the mean. It is likely that the different standard deviations reflect, at least in part, the different signal-to-noise ratios of the various multiple conductances, being least good for the smallest level. However, the standard deviation of 6·6% for the 48 pS level, and indeed the standard deviation of all the levels, is greater than that observed with unitary-conductance channels such as the glutamate and acetylcholine receptor channels in muscle membranes (e.g. Cull-Candy, Miledi & Parker, 1981; Gardner, Ogden & Colquhoun, 1984), and seems to reflect the fact that the noise level of the 48 pS currents usually exceeded that of the baseline; the possibility of an increased noise level during the smaller discrete conductance openings has not been examined. By comparison, the variability in the conductances of acetylcholine-evoked currents at the frog end-plate is only about 2% between patches (Gardner *et al.* 1984). Recently, however, it has been reported that acetylcholine-induced single-channel openings in myotubes (Sigworth, 1985, 1986) and sympathetic neurones (Mathie, Cull-Candy & Colquhoun, 1987) show an increased noise level and wide variability between patches, although this variability does not seem to be as great as that described here for glutamate channels.

Therefore, it seems possible that some of our conductance values may represent the mean of several closely spaced levels. In particular this may apply to the smallest mean conductance (8 pS), as suggested by its large standard deviation. Indeed, it is worth noting that in the lower trace of Fig. 1*C* several quisqualate openings seem to be less, or more, than 7 pS, but these currents have not been resolved into separate peaks in the corresponding amplitude histogram (Fig. 2*C*). In some other patches quisqualate (10 μM) appeared to activate conductances of about 7 and 11 pS, which were resolved as separate peaks in the amplitude histograms. Interestingly, the histogram of kainate currents in Fig. 2*D* also indicates the activation of 6 and 11 pS conductances. It is not known whether the 8 pS peak in amplitude histograms of glutamate, aspartate, and NMDA currents also represents openings of two different levels.

Direct transitions between current levels

The resolution for detection of brief shuttings of the single-channel currents ranged from 60 to 200 μs. Therefore, openings to different levels, that were interrupted by gaps or sojourns at other levels lasting for less than 60–200 μs, would appear to represent direct transitions between levels. Given this limitation, the percentage of all openings that were followed by an apparently direct transition to another open level, rather than a closing, was calculated from some of the patches. The values were: 10 ± 4% with glutamate (3–10 μM, four patches); 14 ± 2% with aspartate (10 μM, three patches); 19 ± 5% with NMDA (10–50 μM, four patches); 9 ± 2%, with quisqualate (10–50 μM, seven patches).

Direct transitions were observed between most levels, but single-channel events composed only of transitions between two conductance levels were more common than events containing steps between three or more levels. The transitions that were most commonly detected were between the 48 and 38 pS levels, and the 38 and 18 pS levels; both of these commonly occurring transitions are evident in the records shown in Figs 1 and 6. The relatively large steps between the 38 and 18 pS levels were the most readily identifiable of the transitions. A particularly high frequency of

transitions between 38 and 18 pS was evoked by 50 μM-NMDA in the patch depicted in Fig. 1*A*; for example, one event recorded in this patch consisted of twenty-seven such steps.

It was possible that events of this sort, composed of steps between the 18 and 38 pS levels, could have arisen from two superimposed 18 pS openings (or perhaps several superimposed 8 pS openings). This is unlikely for several reasons. Firstly, in those patches displaying both 38 and 18 pS levels, glutamate, aspartate, quisqualate and NMDA gave a much higher frequency of 38 pS openings than of 18 pS events. The likelihood of observing superimposed 18 pS openings was therefore low. Secondly, opening or closing transitions of the 38 pS level usually lacked any detectable inflexion; the probability is extremely low that two independent 18 pS channels would open or shut within 60–200 μs on a sufficiently large number of occasions to account for this lack of inflexion. A similar argument applies to the possibility that transitions between the 38 and 48 pS levels could have arisen from superimposed openings. Although transitions between the 48 and 18 pS levels were observed, and these levels could be linked via a 38 pS opening within single-channel events, both these sorts of events were relatively rare.

The high frequency of occurrence of 38 and 18 pS transitions, and their time asymmetry (Cull-Candy & Usowicz, 1987) suggests that the 38 and 18 pS openings are substates of the same channel molecule. Similarly, the relatively high frequency of transitions between the 48 and 38 pS levels suggests that these levels are substates of a single channel molecule. The fact that the 38 pS level is common to both transition pairs raises the question of whether the 38 and 18 pS steps, and the 48 and 38 pS steps, represent the activity of a single channel, or two separate channels that are similar (i.e. two channels that both possess a 38 pS level). The occurrence (albeit at a low frequency) of events containing openings to all three levels (48, 38 and 18 pS) favours the idea that these three levels originate from a single channel. On the other hand, the fact that the transitions between the 48 and 18 pS levels were relatively infrequent could suggest that 48 and 18 pS openings actually represent separate channels. At present it is not possible to discriminate between these possibilities in the large cerebellar neurones, but it is possible to summarize the observations by regarding the 48, 38 and 18 pS levels as substates of a common *type* of channel (i.e. the levels may originate from several channels that have some or all levels in common, or they may arise as substates of a channel that can open to all levels).

Events containing what appeared to be transitions between 18 and 8 pS were also observed, and interestingly the 18 pS level is common to two types of transitions (between 18 and 8 pS, or between 38 and 18 pS). However, the identification of direct transitions between the 8 pS open level and other levels is less certain as the signal-to-noise ratio is less good for these small openings. For example, superimposed 8 and 38 pS levels may be difficult to distinguish from a direct transition between the 8 and 48 pS levels. However, there should have been no difficulty in identifying superimposed 8 and 48 pS openings, but these were rarely seen. While this supports the idea that transitions between the 8 pS level and the other open levels were not simply misclassified superimposed openings, the possibility cannot be excluded that such superimposed openings were not observed because of a low frequency of activation in a patch of an 8 pS channel or channels with subconductance states (48, 38 and 18 pS).

The relative number of openings of any one particular level, that showed direct transitions to another conductance level, depended on the agonist used. For

example, it is apparent from Figs. 1 and 6 that the 38 and 18 pS openings can occur independently, as well as in direct association. Of the total number of 18 pS openings, the percentage which occurred in isolation was $41 \pm 4\%$ with 10 μM-glutamate (mean $\pm$ S.E.M., three patches), $52 \pm 5\%$ with 10 μM-asparate (three patches), $22 \pm 5\%$ with 10–50 μM-NMDA (four patches) and $37 \pm 7\%$ with 10 μM-quisqualate (three patches), suggesting that NMDA was the least effective in giving *single* 18 pS openings.

Shut-time distributions

Many of the single-channel currents contained brief interruptions or 'gaps', suggesting that an individual glutamate receptor activation can give rise to a burst of openings that occur in rapid succession (see Figs 1 and 6). The correct interpretation of some gaps in uncertain due to the presence of multiple conductance levels. Some represent partially resolved complete closures (as interpreted for gaps occurring in single-channel currents of unitary-conductance receptor channels; see Cull-Candy & Parker, 1982; Sine & Steinbach, 1984; Colquhoun & Sakmann, 1985), but others may result from brief sojourns in lower conductance levels. The gaps which attain an amplitude corresponding to a conductance that is less than the smallest directly resolvable conductance level (8 pS) can be interpreted, with reasonable certainty, as complete closures (although the possibility remains that these are rapid transitions to the smallest conductance of 1 pS, see below).

Gaps which could have been interpreted *either* as transitions to a smaller conductance level *or* as complete closures were excluded from the analysis of shut times (see Methods). Figure 4*A* shows the distribution of shut times for channels activated by 10 μM-aspartate (at -70 mV), fitted with three exponential components (continuous line) with time constants (τ) and relative areas (a), of $\tau_1 = 0{\cdot}61$ ms and $a_1 = 30\%$; $\tau_2 = 19$ ms and $a_2 = 30\%$; $\tau_3 = 227$ ms and $a_3 = 40\%$. The same fit is shown on different time scales (Fig. 4 *B*, *C* and *D*) to display each of the three components separately. The leftmost bin in Fig. 4*B* is off-scale, and has not been included in the fit (as discussed above). If *all* gap times are included in the fit, the distribution can be described by the sum of four exponential components, as reported for shut-time distributions obtained with glutamate, aspartate and NMDA in cerebellar granule cells (Howe, Colquhoun & Cull-Candy, 1988). For the histogram in Fig. 4*A* the parameters of the *four-exponential* fit were $\tau_1 = 40$ μs and $a_1 = 48\%$; $\tau_2 = 0{\cdot}69$ ms and $a_2 = 15\%$; $\tau_3 = 20$ ms and $a_3 = 16\%$; $\tau_4 = 229$ ms and $a_4 = 21\%$. The parameters of these three slower components are clearly similar to those obtained with the three-exponential fit.

Distributions of gap-times (equal to, or longer than, w; see Methods) produced by glutamate (3–10 μM), aspartate (10 μM), NMDA (10–50 μM), and quisqualate (10–50 μM), could all be fitted with three exponentials. The first component had a mean time constant, $\tau_1 = 1{\cdot}05 \pm 0{\cdot}25$ ms (mean $\pm$ S.E.M., eighteen patches pooled for all four agonists, $V_m = -100$ to $+50$ mV), which was considered to be sufficiently brief to represent gaps between sequential openings of the same channel (i.e. gaps within bursts). On average, it occupied $23{\cdot}1 \pm 2{\cdot}5\%$ of the total area under the fitted curve. For comparison, a mean value of 0·43 ms has been reported for gaps within bursts of NMDA openings in mouse central neurones (Ascher *et al.* 1988). In

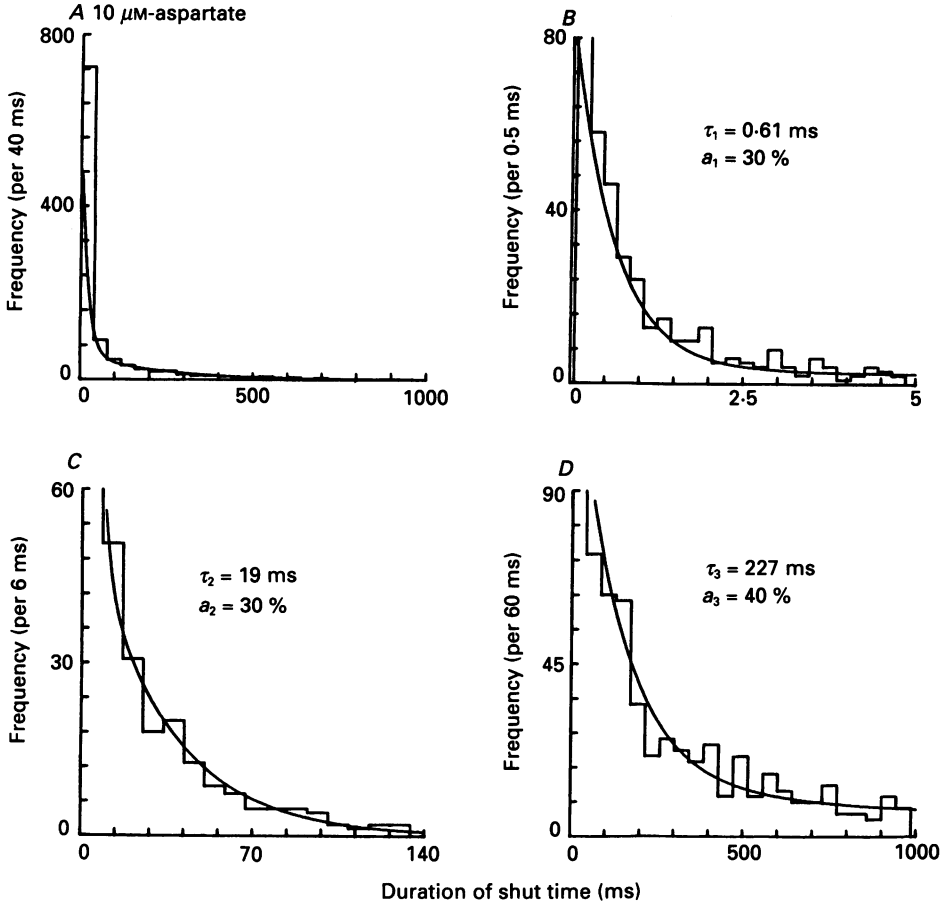

Fig. 4. Distribution of shut-times of channels activated by 10 μM-aspartate in an outside-out patch (patch potential, −70 mV). The sum of three exponentials is fitted to the distribution of durations equal to or longer than 210 μs (see text for details), with time constants and relative areas of 0·61 ms and 30%, 19 ms and 30%, and 227 ms and 40%. The resolution was set at 60 μs for shuttings and 80 μs for openings. The currents were filtered at 2 kHz (−3dB). *A*, complete fitted distribution. *B*, distribution up to 5 ms, to display the first component. The leftmost bin is off-scale and contains durations less than 210 μs that were not included in the fit. In this way only gaps thought to represent full closures (and not gaps which could be a mixture of full closures or rapid transitions to lower conductance levels) were included. *C* and *D*, distributions up to 140 ms (*C*), and up to 1000 ms (*D*), to display the second and third components.

cerebellar granule cells, the distribution of gaps within bursts produced by glutamate, aspartate and NMDA showed two components, with mean values of about 48 μs and 0·7 ms (Howe *et al.* 1988). In the present experiments, the second and third components had mean time constants of respectively, $\tau_2 = 21{\cdot}00 \pm 2{\cdot}47$ ms and $\tau_3 = 411{\cdot}0 \pm 94{\cdot}2$ ms; these were interpreted as the mean durations of shut times between bursts of openings. We have not determined whether the single-channel openings occur in long clusters, as they do in cerebellar granule cells (Howe *et al.* 1988), and whether the gaps between bursts are inter-cluster gaps.

Burst-length distributions

Bursts of openings were defined as a group of openings separated from another group by a shut duration equal to, or longer than, a 'critical gap duration', t_c, (mean value 2·2 ms) which was calculated for each experiment from the shut-time

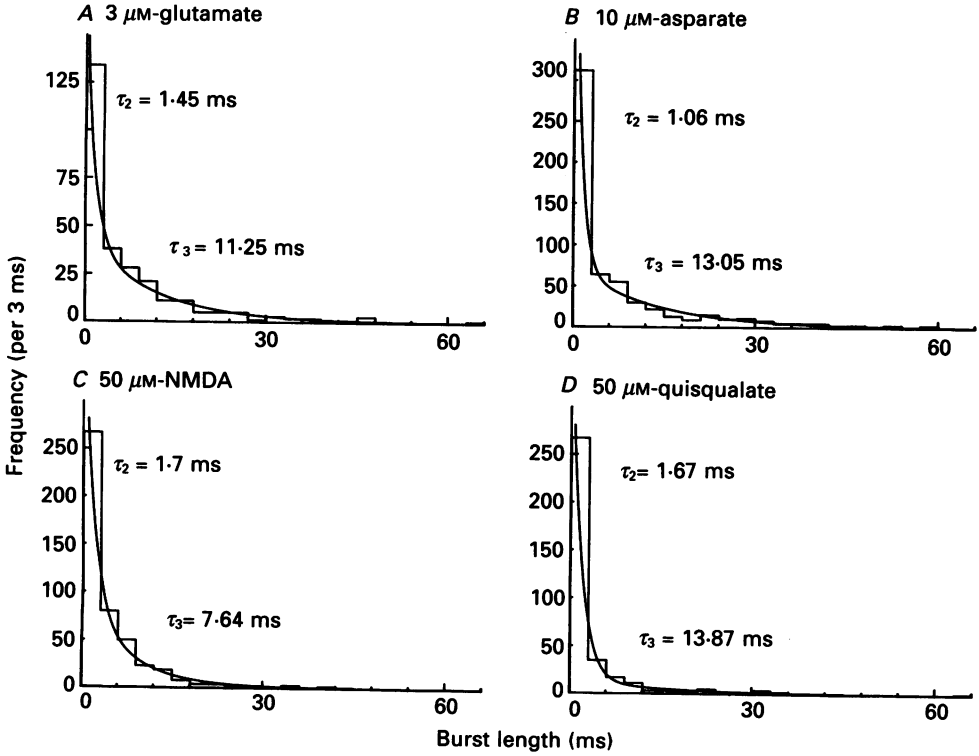

Fig. 5. Distributions of the burst length for channels activated in four outside-out patches by glutamate receptor agonists (patch potential, −70 mV). The distributions are fitted with three exponentials (continuous line) and are all plotted up to 66 ms to illustrate the two slower components and to facilitate comparison between the various agonists. *A*, 3 μM-glutamate, the time constants of the two slower components are $\tau_2 = 1{\cdot}45$ ms and $\tau_3 = 11{\cdot}25$ ms, and the relative area of the two slower components (a_2/a_3) is 0·36. Critical gap length for the definition of bursts was $t_c = 1{\cdot}25$ ms. *B*, 10 μM-aspartate, the time constants are $\tau_2 = 1{\cdot}06$ ms and $\tau_3 = 13{\cdot}05$ ms, and $a_2/a_3 = 0{\cdot}66$. Critical gap length, $t_c = 1{\cdot}55$ ms. *C*, 50 μM-NMDA, $\tau_2 = 1{\cdot}7$ and $\tau_3 = 7{\cdot}64$ ms, and relative areas (a_2/a_3) = 0·74. Critical gap length, $t_c = 1{\cdot}56$ ms. *D*, 50 μM-quisqualate, $\tau_2 = 1{\cdot}67$ ms and $\tau_3 = 13{\cdot}87$ ms, $a_2/a_3 = 3{\cdot}14$, $t_c = 1{\cdot}43$ ms.

distributions (see Methods). In the presence of multiple conductance levels the term 'burst' has been used to describe both a group of openings separated by brief gaps shorter than t_c *and* a single-channel event comprising directly associated openings to several levels, but not necessarily containing any visible gaps (see Methods).

Burst-length distributions were frequently fitted with the sum of three exponentials, although in some distributions, particularly those obtained with quisqualate, not all the components were well defined. Examples of burst-length distributions for channels activated by glutamate, aspartate, NMDA, and quisqualate are plotted in Fig. 5 to illustrate the slower two components. Most burst-

length histograms contained a fast component with a mean time constant of approximately 120 μs (pooled for all agonists). These extremely short 'bursts' may have resulted from brief (and therefore poorly resolved) single openings, the amplitudes of which were uncertain. Indeed, given that the smallest directly resolved conductance level was only about 8 pS the possibility of including random baseline fluctuations and other artifacts is greater than for the larger unitary-conductance channel openings in some previous studies. The durations of these brief events were well separated from the longer bursts in the burst-length distributions. Therefore, they could be easily excluded from the fit; in which case the burst-length distributions were well fitted with two exponentials, with time constants similar to τ_2 and τ_3 of the three-exponential fits given below.

It seems likely that the two slower components reflect the existence of two kinetically distinct open states, whose time constants are equivalent to the mean burst length of short and long bursts. Mean values of τ_2 and τ_3 were, $1{\cdot}8 \pm 0{\cdot}3$ and $13{\cdot}7 \pm 4{\cdot}9$ ms for glutamate (3–10 μM, five patches, $V_m = -70$ to -80 mV); $1{\cdot}5 \pm 0{\cdot}2$ and $11{\cdot}8 \pm 1{\cdot}2$ ms for aspartate (10 μM, three patches, $V_m = -70$ to -100 mV); $1{\cdot}3 \pm 0{\cdot}2$ and $12{\cdot}7 \pm 2{\cdot}6$ ms for NMDA (10–50 μM, five patches, $V_m = +50$ to -70 mV); $2{\cdot}3 \pm 0{\cdot}8$ and $15{\cdot}6 \pm 3{\cdot}3$ ms for quisqualate (10–50 μM, five patches, $V_m = -50$ to -70 mV). There was no marked difference in the time constants between agonists, and the mean burst lengths (averaged for all agonists) was $1{\cdot}7 \pm 0{\cdot}2$ ms (eighteen patches) for the short bursts, and $13{\cdot}5 \pm 1{\cdot}8$ ms (eighteen patches) for the long bursts. The ratio of the area of the second component (a_2) relative to the area of the third (a_3), a_2/a_3, was $0{\cdot}93 \pm 0{\cdot}22$ with glutamate, $2{\cdot}28 \pm 1{\cdot}23$ with aspartate, $1{\cdot}01 \pm 0{\cdot}63$ with NMDA, and $4{\cdot}33 \pm 0{\cdot}57$ with quisqualate. Therefore, the relative proportion of long bursts followed the sequence: glutamate > NMDA > aspartate > quisqualate. However, quisqualate gave many more small-conductance openings than glutamate, asparate, or NMDA, and the possibility cannot be excluded that many of these small-conductance openings occurred in long bursts but were overlooked or misclassified during the analysis to give an apparently greater proportion of short bursts for quisqualate channels. This has been further examined by means of fluctuation analysis of the single-channel currents.

Spectral analysis of single-channel currents

We have previously examined glutamate receptor channels in large cerebellar neurones by the analysis of agonist-induced noise under whole-cell clamp (Cull-Candy & Usowicz, 1987, 1989). The resolution of whole-cell noise will be limited at high frequencies by the series resistance of the pipette and the space clamp of the cell. Furthermore, the clamp currents needed to hold the cell away from its zero current potential could give voltage errors, resulting from the currents flowing across the series resistance of the pipette. It is therefore possible that due to low-pass filtering, some of our two-component whole-cell spectra may have been shifted to lower frequencies, while a second component may have remained undetected in those spectra fitted with one Lorentzian. Furthermore, the presence of some undetected synaptic currents, masked by the agonist-evoked noise increase, could have distorted the spectral parameters. Better frequency resolution is obtained in outside-out patches, although these only give properties of channels in the cell soma, and it is

possible that the channel kinetic properties may be altered in the excised patches. Nevertheless, for direct comparison with the whole-cell noise we have examined the kinetics of channels activated by glutamate (3–10 μM), aspartate (3–10 μM), NMDA (10–50 μM), and quisqualate (3–50 μM) in outside-out patches by applying spectral analysis to the single-channel currents.

Figure 6 shows examples of single-channel currents produced by 3 μM-glutamate (Fig. 6*A*), 10 μM-NMDA (Fig. 6*C*) and 10 μM-quisqualate (Fig. 6*E*) in three isolated patches at −70 mV. Both glutamate and NMDA are opening predominantly the maximum conductance level, while quisqualate is opening mainly lower conductance levels. The spectral densities of these currents (Fig. 6*B*,*D* and *F*) are well fitted with the sum of two Lorentzians. Indeed, spectral densities of single-channel currents invariably displayed two components. The mean time constants (at −70 mV) were: for glutamate, $\tau_1 = 22{\cdot}8 \pm 3{\cdot}6$ ms and $\tau_2 = 2{\cdot}4 \pm 0{\cdot}3$ ms (mean ± S.E.M.; nine patches); for aspartate, $\tau_1 = 12{\cdot}1 \pm 1{\cdot}1$ ms and $\tau_2 = 1{\cdot}6 \pm 0{\cdot}1$s (five patches); for NMDA, $\tau_1 = 15{\cdot}4 \pm 2{\cdot}3$ ms and $\tau_2 = 1{\cdot}6 \pm 0{\cdot}2$ ms (nine patches); and for quisqualate, $\tau_1 = 17{\cdot}6 \pm 3{\cdot}7$ ms and $\tau_2 = 1{\cdot}8 \pm 0{\cdot}4$ ms (eight patches). There was no apparent dependence of τ_1 or τ_2 on the nature of that agonist ($P > 0{\cdot}1$, one-way ANOVA). The ratios of the variances of the individual spectral components, $G_2(0)f_2/G_1(0)f_1$, (at −70 mV) differed widely between patches; for each agonist the mean values (± S.E.M.) were $0{\cdot}66 \pm 0{\cdot}25$ with glutamate, $0{\cdot}56 \pm 0{\cdot}14$ with aspartate, $0{\cdot}37 \pm 0{\cdot}13$ with NMDA, and $1{\cdot}18 \pm 0{\cdot}42$ with quisqualate.

Kainate currents in outside-out patches

Figure 7*A* shows a typical example of the response produced by 50 μM-kainate in an outside-out patch at −70 mV, compared with the control noise level in the absence of kainate. In this patch kainate gave a noise increase that was accompanied by few if any discrete single-channel currents, although in the same patch 10 μM-aspartate gave 49, 38, 19 and 9 pS openings (not shown). However, in some patches the noise increase to kainate (10–50 μM) was accompanied by a relatively low frequency of directly resolvable openings to conductance levels that were similar to those produced by the other glutamate receptor agonists. An example of this is shown in Fig. 7*C* (and see also histogram in Fig. 2*D*); a single 48 pS opening is present, as well as several well-resolved 18 and 8 pS events. In order to obtain the properties of the channels that underly the small kainate noise increase, spectral analysis was applied to the currents in outside-out patches. The spectra were invariably described by the sum of two components, an example of which is illustrated in Fig. 7*B*. For this spectrum the estimated single-channel conductance is 1·6 pS (at $V_m = -70$ mV).

The mean single-channel conductance, obtained from spectral analysis of kainate currents in excised patches, was $\gamma = 1{\cdot}03 \pm 0{\cdot}2$ pS (mean ± S.E.M., fourteen patches). This value is likely to be an overestimate of the conductance of the channels underlying the kainate noise, since discrete openings were not edited from the records during this analysis. Nevertheless, this is less than the smallest directly resolved levels which we regularly observed in response to the various agonists. The estimate of γ from kainate noise in patches is also smaller that the estimate from whole-cell kainate noise ($\gamma = 3{\cdot}6$ pS, see Cull-Candy & Usowicz, 1989). Both these methods of

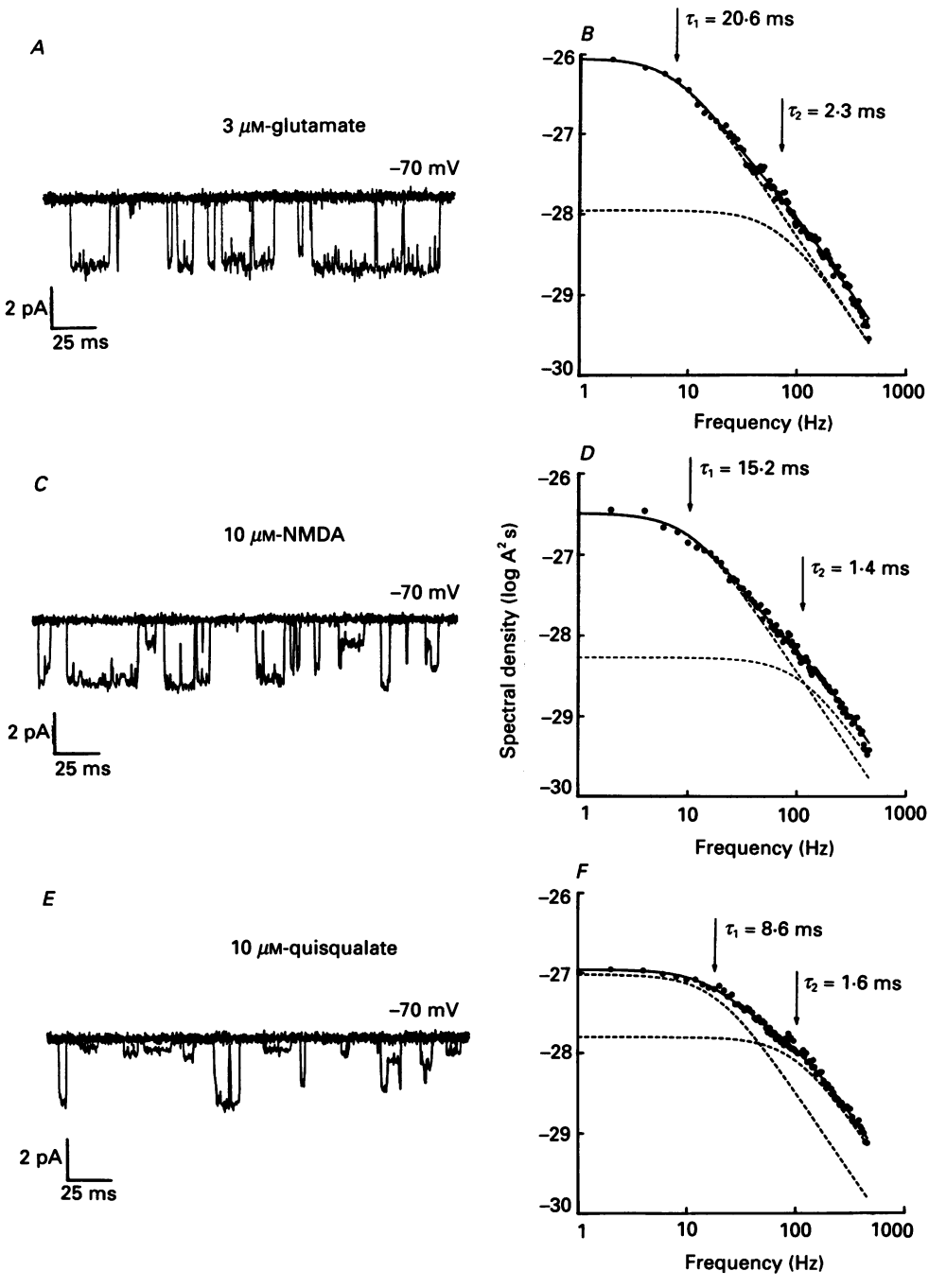

Fig. 6. For legend see facing page.

estimating γ (i.e. analysis of whole-cell noise or analysis of noise in patches) have some inherent inaccuracies: the low amplitude of kainate noise is likely to limit the frequency resolution that is attainable in whole-cell responses, while in isolated patches it is less easy to measure accurately the mean membrane current change elicited by kainate. Nevertheless, it is apparent that kainate preferentially activates small channel conductances, with a maximum conductance of about 1 pS, and possibly also some smaller channel conductances.

The mean time constants for kainate noise spectra obtained in patches were: $\tau_1 = 16{\cdot}4 \pm 2{\cdot}4$ ms and $\tau_2 = 0{\cdot}9 \pm 0{\cdot}1$ ms (mean $\pm$ S.E.M., -70 mV, fourteen patches). The high-frequency component (τ_2) was slightly briefer than the value estimated with whole-cell recording ($2{\cdot}0 \pm 0{\cdot}2$ ms, Cull-Candy & Usowicz, 1989), possibly because of low-pass filtering by the series resistance present in whole-cell currents. The mean value of $G_2(0)f_2/G_1(0)f_1$ was $1{\cdot}87 \pm 0{\cdot}3$, which is close to the mean value of 2 obtained for whole-cell spectra (see Cull-Candy & Usowicz, 1989).

The slow time constant of kainate spectra in outside-out patches is similar to that obtained from spectra of single-channel currents produced by glutamate, aspartate, NMDA, and quisqualate, while the fast time constant is slightly shorter. $G_2(0)f_2/G_1(0)f_1$ followed the sequence: kainate > quisqualate > glutamate > aspartate > NMDA, suggesting that the fast component contributed most power to spectra of kainate and quisqualate single channels ($P < 0{\cdot}01$, one-way ANOVA).

Comparison of spectral parameters and burst lengths

The two mean time constants (pooled for all agonists), obtained from spectra of *single-channel currents* in excised patches, are in good agreement with the two time constants from the burst-length distributions that represent the mean durations of long and short bursts. The mean time constants of single-channel current spectra were $17{\cdot}2 \pm 1{\cdot}4$ and $1{\cdot}6 \pm 0{\cdot}13$ ms (mean $\pm$ S.E.M., forty-five spectra, pooled for all agonists including kainate) while the mean burst-length time constants were $13{\cdot}5 \pm 1{\cdot}8$ and $1{\cdot}7 \pm 0{\cdot}2$ ms (pooled for all agonists, except that burst-length distributions were not constructed for kainate channels). This similarity suggests that the two spectral time constants give a reasonable reflection of the mean channel burst lengths.

The mean values of $G_2(0)f_2/G_1(0)f_1$ from spectral analysis of single-channel

Fig. 6. Single-channel currents activated in outside-out patches and their corresponding spectral density functions. *A*, single-channel currents evoked by 3 μM-glutamate (at -70 mV). *B*, power spectrum of glutamate currents (from same patch as data in *A*) fitted with the sum of two Lorentzian components. The time constants of the individual components (indicated by dashed lines) are $\tau_1 = 20{\cdot}6$ ms and $\tau_2 = 2{\cdot}3$ ms (indicated by arrows). The area of the second component, relative to the first, $G_2(0)f_2/G_1(0)f_1$ is 0·11. *C*, single-channel currents evoked by 10 μM-NMDA (at -70 mV). *D*, spectral density of NMDA currents (from same patch as data in *C*) with time constants of $\tau_1 = 15{\cdot}2$ ms and $\tau_2 = 1{\cdot}4$ ms, and $G_2(0)f_2/G_1(0)f_1$ *is* 0·17. *E*, single-channel currents evoked by 10 μM-quisqualate (at -70 mV). *F*, power spectrum of quisqualate currents (from same patch as data in *E*) with time constants of $\tau_1 = 8{\cdot}6$ ms and $\tau_2 = 1{\cdot}6$ ms; $G_2(0)f_2/G_1(0)f_1$ is 0·89. Each trace in *A*, *C* and *E* consists of several superimposed recordings. Points in the spectra above 60 Hz have been averaged.

currents, are smaller than the values from whole-cell noise produced by glutamate, aspartate and NMDA (see Cull-Candy & Usowicz, 1989), although the difference is significant only for NMDA ($P < 0.05$, two-tailed t-test). The fast component contributed most power to the whole-cell noise induced by NMDA ($G_2(0)f_2/G_1(0)f_1 = 1.6$),

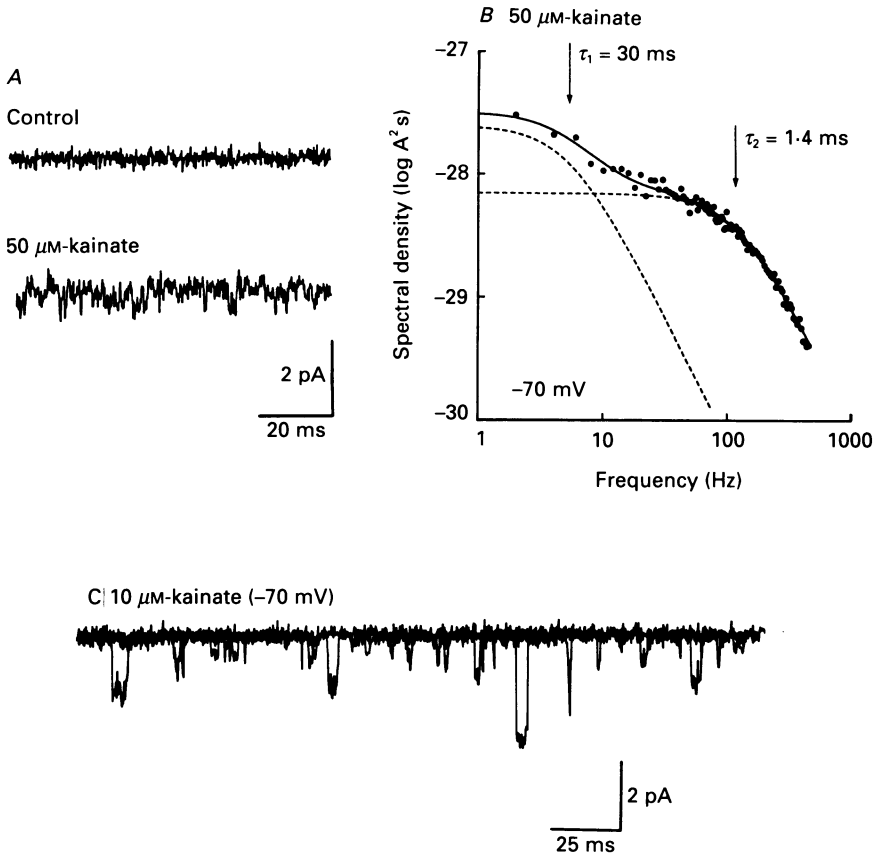

Fig. 7. Noise and single-channel currents produced by kainate in isolated patches. *A*, current noise produced by kainate in an outside-out patch at a potential of −70 mV. Upper trace: control noise level; lower trace: increase in noise during the presence of 50 μM-kainate. Single-channel currents are not evident in this patch. Calibration, 2 pA and 20 ms. *B*, spectral density of kainate-evoked noise, fitted with the sum of two Lorentzian components (continuous curve). The time constants of the individual components (indicated by dashed lines) are $\tau_1 = 30$ ms and $\tau_2 = 1.4$ ms. The single-channel conductance calculated from the fitted curve is $\gamma = 1.6$ pS (for a reversal potential of −4 mV). *C*, discrete single-channel currents evoked by 10 μM-kainate to multiple conductance levels (at −70 mV). The trace consists of several superimposed recordings and was obtained from a different cell from the recording in *A*. Calibration, 2 pA and 25 ms.

while in the single-channel spectra a greater contribution was made by the slow component ($G_2(0)f_2/G_1(0)f_1 = 0.37$). This may perhaps suggest that the relative density of NMDA channels that underly the slow spectral component is greater in the cell soma than in the rest of the cell, or that the channel kinetics are different in different regions, or that the kinetics of the NMDA channels are altered in excised patches. By contrast, there is much closer agreement between the mean relative

variances of the whole-cell spectra (see Cull-Candy & Usowicz, 1989) and single-channel spectra produced by quisqualate (1·3 and 1·18, respectively) or by kainate (2 and 1·87, respectively).

DISCUSSION

As previously reported, glutamate receptor channels in outside-out patches isolated from the cell soma of cultured large cerebellar neurones give openings with more than one conductance level (Cull-Candy & Usowicz, 1987). The multiple conductances activated by a variety of excitatory amino acids can be classified into a minimum of five discrete groups, with mean values of 48, 38, 28, 18 and 8 pS. In addition, a lower conductance level of ~ 1 pS is activated by kainate (and perhaps also by quisqualate), although this may represent more than one type of channel conductance with an 'average' conductance of ~ 1 pS. The relative frequency of occurrence of the multiple levels differs significantly between the three receptor-selective agonists NMDA, quisqualate and kainate. The presence of multiple-conductance glutamate receptor channels has also been described in cultured hippocampal neurones (Jahr & Stevens, 1987), freshly dissociated or cultured cerebellar granule cells from young rats (Cull-Candy, Howe & Ogden, 1987, 1988; Bertolino, Vincini, Mazzetta & Costa, 1988), embryonic mouse midbrain neurones (Ascher *et al.* 1988; Ascher & Nowak, 1988*a*), dissociated adult rat hippocampal cells (Gibb, 1988), and in type-2 cerebellar astrocytes (Usowicz *et al.* 1989). The maximum level of about 48 pS, found in the present study, corresponds to the 50 pS conductance previously reported for NMDA channels in other types of mammalian neurones (Nowak *et al.* 1984; Cull-Candy & Ogden, 1985).

Comparison with multiple-conductance glutamate channels in other neurones

There appear to be some important differences between different types of mammalian central neurones in the properties of the multiple conductance levels activated by the various agonists. NMDA preferentially activates > 30 pS openings in all mammalian neurones so far examined (see Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987; Ascher *et al.* 1988; Cull-Candy *et al.* 1988). However, in some patches from large cerebellar neurones a majority of these > 30 pS openings were to the submaximal 38 pS level, whereas in other mammalian neurones the majority were to the maximum (48–50 pS) level. Furthermore, the relative frequency with which NMDA and the putative transmitters glutamate and aspartate activated lower conductance (< 20 pS) levels in large cerebellar neurones appears to be considerably higher than in hippocampal neurones (Jahr & Stevens, 1987), mouse neurones (Ascher *et al.* 1988) or cerebellar granule neurones (Cull-Candy *et al.* 1988). Indeed, the lowest conductance (8 pS) is rarely, if ever, observed in mouse neurones exposed to NMDA (Ascher *et al.* 1988).

In the present experiments (and in hippocampal and some granule cells) quisqualate activated mainly small conductances with a mean of 8 pS, with some higher levels. All of these levels appear to be similar in amplitude to those activated by NMDA, although they may differ in duration (Cull-Candy & Usowicz, 1987). In contrast, in mouse central neurones only the 40–50 pS openings produced by

quisqualate are thought to be the same as those activated by NMDA (Ascher & Nowak, 1988*a*). There are also differences between cell types in their response to kainate. This preferentially gives small levels (< 20 pS) in all neurones, but in all except mouse central neurones it can also activate large multiple conductances that are similar in amplitude to some of those activated by NMDA. In most of the different types of neurone (except rat hippocampal neurones, Jahr & Stevens, 1987) kainate also activates very small-conductance channels of 1–4 pS, seen as a noise increase in outside-out patches.

Transitions between levels

There are further differences between glutamate receptor channels described here and in other neurones, both in the types of direct transition that can occur between the various open levels, and in the relative numbers of these transitions. In large cerebellar neurones, direct transitions can be readily identified between 48 and 38 pS, and between 38 and 18 pS. Similar direct transitions have been clearly observed (albeit with a lower frequency) in rat hippocampal neurones (Jahr & Stevens, 1987) and mouse central neurones (Ascher *et al.* 1988). The transition between 38 and 18 pS is particularly common in the present experiments (comprising up to 20% of transitions in a given patch). However, in cerebellar granule neurones the equivalent transitions (i.e. between levels above and below 20 pS) rarely, if ever, occur (Cull-Candy *et al.* 1988).

The various cells may also differ on the question of whether direct transitions occur between the smallest level (~ 8 pS in large cerebellar neurones, cerebellar granule cells and mouse central neurones) and other conductance levels. Clear transitions of this sort are described in hippocampal neurones (Jahr & Stevens, 1987), although they probably do not occur in cerebellar granule neurones (Cull-Candy *et al.* 1988) or mouse neurones (Ascher *et al.* 1988). We have observed such transitions in large cerebellar neurones but much less frequently than other transitions. Also, we cannot exclude the possibility that some of these transitions may have arisen from the incomplete resolution of sequential openings and shuttings.

In summary, the evidence so far suggests that all the levels observed in rat hippocampal neurones and large cerebellar neurones (in response to all the agonists) may be substates of a *common type* of glutamate receptor channel, i.e. may arise from several channels with any number of similar subconductances, or as substates of a channel that can show all multiple conductances. This does *not* imply that all three receptors are simultaneously coupled to a single channel. In mouse central neurones, it is suggested that the conductance of 15–18 pS, but *not* the 8 pS conductance, may arise from the same *type* of channel as the larger > 30 pS conductances. On the other hand, in cerebellar granule cells direct transitions rarely occur between events above and below 20 pS, suggesting that neither the 8 nor the 15–17 pS level originate from the 50 pS channels. It is not known how the small-conductance openings (1–4 pS) activated by kainate relate to the larger discernible conductance levels.

Possible interpretations of multiple conductance levels

The single-channel records have allowed five (or six, including the ~ 1 pS conductance) levels to be identified in isolated patches in these cells. While this is

probably an underestimate of the actual number of discrete open levels it exceeds the proposed number of types of glutamate receptors (three) present in mammalian neurones (Watkins & Olverman, 1987). Therefore some, or all, of these three receptor types can presumably activate more than one conductance level. Furthermore, the presence of direct transitions between certain levels argues against the idea that each conductance represents a different channel. Moreover, it seems reasonable to assume that single openings of a given conductance, and openings of the same conductance that show direct transitions to another level, represent the same channel.

It has recently been neatly demonstrated that the binding sites for NMDA and kainate are on different receptor channel molecules: the dissociative anaesthetic ketamine has been used to block the NMDA-activated channel selectively in hippocampal neurones (MacDonald, Miljkovic & Pennefather, 1987). Ketamine becomes trapped within the channel when it closes, but in cells where the NMDA channel had been blocked by trapped ketamine, kainate produced normal responses. Further evidence that NMDA and non-NMDA receptors do not occur as part of the same channel molecule is that some outside-out patches from central neurones responded to NMDA but not to quisqualate or kainate; conversely, some patches were sensitive to quisqualate or kainate, but not to NMDA (Ascher & Nowak, 1988*b*; Cull-Candy *et al.* 1988).

Various possible schemes can be put forward to explain why the three selective agonists all activate similar multiple conductance levels. Briefly, the two simplest possible models that could explain our observations are as follows. One possibility is that NMDA, quisqualate and kainate receptors are associated with similar types of channel (i.e. each receptor is associated with a separate channel, but the amplitudes of some or all of the multiple open levels are similar for the three different receptor channels). The type of receptor associated with each channel would then determine the most frequently visited (i.e. main conductance) level and the different receptor channels would not necessarily all display the same number of conductances. A similar model has been proposed to account for the fact that the multiple-conductance channels activated by GABA and glycine have similar conductance levels (Hamill, Bormann & Sakmann, 1983; Bormann, Hamill & Sakmann, 1987). GABA and glycine receptors are coupled to separate channel molecules (Schofield *et al.* 1987; Grenningloh *et al.* 1987), and there is about 50% amino acid sequence identity and homology between the 48 kD glycine receptor subunit and the $GABA_A$ receptor α-subunit (see Stephenson, 1988). Similarity in the multiple conductance levels activated by NMDA, quisqualate and kainate may, therefore, also reflect structural similarities in the receptor channels (at least in their membrane spanning regions), as expected for a family of related glutamate receptor channels (Cull-Candy & Usowicz, 1987).

Another possibility is that the different glutamate receptors are associated with separate and dissimilar channels, not all necessarily displaying subconductances. For example, the relative frequency with which these levels are activated by the various selective agonists in large cerebellar neurones, and the types of direct open–open level transitions most commonly observed, could perhaps be taken to suggest that 18, 38, and 48 pS openings arise from NMDA receptor channels, and the 8 pS openings originate from quisqualate receptor channels, while the small conductance ($\leqslant 1$ pS) channels detected by noise analysis may be associated with the kainate receptor. For

all the conductances to be activated by a given agonist, the patch would have to contain all the receptor types and the agonist would have to activate all three. The relative frequency of occurrence of the multiple levels would then reflect the relative selectivity and potency of the agonists for each type of receptor.

Both of the models described above are too simple to explain all the observations. They cannot explain why, for example, in some patches NMDA produces more 38 than 48 pS openings, while in others it produces more 48 than 38 pS openings. It could be that the NMDA channel has multiple binding sites for NMDA or is associated with several subtypes of NMDA receptor, and the number of agonist molecules bound determines the type and number of subconductance states entered by the channel. Furthermore, there may be several types of NMDA receptor channel with different affinities for NMDA and showing multiple conductances in slightly different proportions. Both of these predict a dependence of the relative frequencies of the multiple levels on agonist concentration. However, a non-selective action of the agonists is also likely to be concentration dependent. Furthermore, the relative proportions of the levels may also vary if some levels desensitize more rapidly than others. For instance, if the NMDA-receptor channel can show all the multiple conductance levels, then the *decrease* in the relative proportion of 48 pS openings with increasing NMDA concentration (or the increase in the relative proportion of 38 pS and lower levels) may be a form of desensitization.

Kinetics of glutamate receptor channels

Many of the openings caused by the activation of glutamate receptor channels occur in bursts in large cerebellar neurones, mouse central neurones (Ascher *et al.* 1988; Ascher & Nowak, 1988*a*) and cerebellar granule cells (Howe *et al.* 1988). As suggested for acetylcholine receptor channels (Dionne & Liebowitz, 1982; Colquhoun & Sakmann, 1985) and glutamate receptor channels at peripheral synapses (Cull-Candy & Parker, 1982), the gaps or brief shuttings, within single activations of the channels, may result from multiple openings during a single receptor occupation. It seems unlikely that the gaps are caused by transient blockages of the channel by residual Mg^{2+} (see Ascher *et al.* 1988; Howe *et al.* 1988).

In the present experiments the burst-length distributions could be fitted with two (or three) exponential components, with time constants and relative areas similar to those reported for the large-conductance (50, 40, 30 pS) glutamate openings in rat cerebellar granule cells (Howe *et al.* 1988). The mean *slow burst lengths* (τ_3) of NMDA channels (10–13 ms), estimated in large cerebellar neurones and in granule cells, appear equivalent to the mean *burst lengths* (single-exponential fit) obtained for NMDA channels in mouse central neurones (Ascher *et al.* 1988; Ascher & Nowak, 1988*b*). The origin of the briefest component (τ_1) in our distribution is unclear at present. The fact that every patch yielded burst-length distributions clearly showing the two slower components is consistent with idea that these components reflect the existence of two kinetically distinct open states, with time constants (τ_2 and τ_3) that are equivalent to the mean durations of the short and long bursts respectively. The ratios of the relative areas indicated that the relative *frequency* (but apparently, not the duration, in these cells) of short and long bursts may be determined by the nature of the agonists, but this requires further investigation. It remains to be seen whether

the burst length, or the conductance of the open levels within a burst, is determined by the number agonist molecules bound.

The burst-length time constants (1·7 and 13·5 ms) were similar to the time constants from single-channel spectra (1·6 and 17·2 ms). This suggests that the two components of the single-channel spectra reflect two open states of glutamate receptor channels, with spectral time constants that are reasonably good estimates of the mean burst lengths. Indeed, the spectral time constants may be the more accurate estimates, since the time constants from the burst-length distributions will be affected by any error in the calculation of the critical gap duration, t_c, used to classify directly measured openings into bursts.

The time constant of decay of a synaptic current gives an estimate of the duration of the 'elementary event', (i.e. the burst length of the transmitter-activated channel) if the transmitter concentration in the synaptic cleft declines rapidly, compared with the lifetime of the elementary event (Anderson & Stevens, 1973; Katz & Miledi, 1973). A comparison of the burst length with the time constant of the synaptic current decay has previously provided a means of testing whether an agonist is a likely transmitter candidate at, for example, glutamate synapses in locust and crayfish muscle (Anderson, Cull-Candy & Miledi, 1976; Crawford & McBurney, 1976). However, given our findings that the burst lengths of glutamate channels in large cerebellar neurones are similar for all agonists (although the short burst length in cerebellar granule cells may show agonist dependence, Howe *et al.* 1988), central glutamate synapses may not be amenable to this approach. A comparison of the conductances activated or the relative proportions of the different bursts lengths (which appear to be agonist dependent) may well be more useful for identifying transmitters or the types of receptor channels involved in transmission.

The decay of excitatory post synaptic currents (EPSCs), recorded from mouse hippocampal neurones or from mouse spinal neurones in Mg^{2+}-free solutions, can be described by the sum of two exponentials, the slower of which has a time constant of 85 ms (Forsythe & Westbrook, 1988). The fast and slow components are thought to be mediated mainly by non-NMDA and NMDA receptor channels, respectively. In the presence of extracellular Mg^{2+} (i.e. when NMDA receptor channels are blocked) EPSCs recorded from spinal neurones in culture (Nelson, Pun & Westbrook 1986) and from synapses between Ia afferents and motoneurones *in vivo* (Finkel & Redman, 1983) decay monoexponentially, with mean time constants of $\sim$ 1 ms. The fast time constant of the EPSC decay ($\sim$ 1 ms) is briefer than the mean lifetimes of NMDA-activated 40–50 pS conductances (Nowak *et al.* 1984; Cull-Candy & Usowicz, 1987; Ascher & Nowak 1988*b*; Cull-Candy, Howe & Usowicz, 1988) but falls in the range of lifetimes of some of the lower conductances. Further, from the ratios of the variance ($G_2(0)f_2/G_1(0)f_1$) of our single channel spectra, a majority of the charge is carried by channels with a mean burst length of $\sim$ 1·0–1·5 ms when kainate and quisqualate receptor channels are opened. This corresponds well to the time constant of decay of the EPSC mediated by non-NMDA receptors (Finkel & Redman, 1983; Nelson *et al.* 1986), and clearly supports the involvement of these channels in synaptic transmission.

It is apparent that the slow time constant of the synaptic current decay (85 ms) greatly exceeds the mean slow burst length (τ_3 = 13·5 ms) measured in the present

and in previous studies (~ 10 ms, Howe *et al.* 1988; 10–17 ms, Ascher *et al.* 1988; Ascher & Nowak, 1988*b*). However, > 30 pS openings activated by glutamate, aspartate and NMDA in rat cerebellar granule cells occur in clusters up to 100 ms in duration (Howe *et al.* 1988). Such clusters may conceivably explain the extremely slow component of the EPSC in cultured mouse central neurones (Forsythe & Westbrook, 1988), and of the excitatory postsynaptic potentials (EPSPS) recorded at various central synapses (Dale & Roberts, 1985; Herron, Lester, Coan & Collingridge, 1985; Dale & Grillner, 1986; Thomson, 1986; Collingridge, Herron & Lester, 1988).

Given that in the present experiments the burst-length distributions, and the power spectra of single channels activated by NMDA, quisqualate or kainate display at least two components (see also Howe *et al.* 1988; Cull-Candy & Usowicz, 1989), one would predict that EPSCs mediated *either* by NMDA *or* by non-NMDA receptors would also display two components. However, fast components of EPSCs mediated by NMDA receptor channels, and slow components of EPSCs mediated by non-NMDA receptor channels have not yet been reported, and the reasons for this apparent discrepancy between single-channel currents and synaptic currents remain unclear.

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