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FLUCTUATION ANALYSIS OF NEUTRAL AMINO ACID RESPONSES IN CULTURED MOUSE SPINAL NEURONES

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SUMMARY

1. Intracellular recordings using the voltage-clamp technique were made at room temperature ($24 \pm 1{\cdot}5$ °C) from mouse spinal and sensory neurones growing in dissociated cell culture.

2. Membrane current responses could be elicited by ionophoresis of the neutral amino acids, γ-aminobutyric acid (GABA), β-alanine (BALA) and glycine to the cell body and processes of these neurones.

3. All membrane current responses were associated with increases in current fluctuations. Most of the analysis presented here was applied to responses generated at the cell body.

4. Many of the fluctuations in membrane current occurring during the responses could be interpreted as reflecting the kinetic behaviour of a single population of two-state Cl^- ion-channels.

5. The properties of channels estimated during the desensitized phase of an amino acid-induced current response were not significantly different from those estimated during the peak of the response.

6. The properties of the amino acid-activated channels were relatively constant over the -40 to -90 mV range of membrane potential.

7. There was considerable variation in the estimated average conductance, γ, and duration, τ, of the elementary events evoked by the neutral amino acids on spinal cord neurones. The properties of the elementary channel events activated by one of the amino acids were significantly different from those activated on the same neurones by either of the other amino acids.

8. In sensory neurones the average γ and τ values for GABA-activated ion-channels were also determined and these values fell within the range of those for channels activated by GABA in spinal neurones.

9. The results indicate that different naturally occurring neutral amino acids activate channels with unique properties in cultured mouse spinal neurones. The relative charge transfer associated with these channels averages 1·00:0·74:0·32; GABA:glycine:β-alanine.

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INTRODUCTION

The elementary events underlying chemical excitability at synaptic junctions were first considered in experimental detail about 10 yr ago when Katz & Miledi (1970, 1971, 1972) observed small fluctuations in membrane voltage during a maintained response to prolonged application of synaptic transmitter substance. The fluctuations were analysed as if they reflected a statistical variation in the instantaneous frequency of elementary conductance events about a mean frequency of events. Based on this and several other assumptions Katz & Miledi (1970, 1971, 1972) and then Anderson & Stevens (1973) were able to estimate the properties of the average elementary conductance event. More recently experiments using techniques for directly recording the elementary events themselves have revealed close agreement between the estimates inferred from fluctuation analysis and the properties observed during patch clamp recordings (Neher & Sakmann, 1976; Jackson & Lecar, 1979).

Fluctuation analysis has been applied to agonist responses on a variety of chemically excitable membranes. The experiments have provided evidence to suggest that chemical excitability at different synaptic membranes involves a common form of molecular mechanism: activation of ion-channels of a singular and relatively constant conductance but of exponentially distributed duration (for review, see Neher & Stevens, 1977). It has also been demonstrated, at a number of different synapses under a variety of conditions, that the characteristics of the ion-channels, especially the kinetics of their closing, once opened, are responsible for shaping the synaptically evoked conductance change.

Studies into the molecular basis of transmitter actions at synapses within the central nervous system (C.N.S.) have been difficult owing to the relative complexity of the intact nervous system. Over the past 10 yr evidence has accumulated showing that monolayer cultures of C.N.S. neurones possess many of the properties characteristic of nerve cells studied *in vivo* including electrical and chemical excitability (for review, see Fischbach & Nelson, 1977). We have applied fluctuation analysis to membrane current responses evoked by naturally occurring neutral amino acids on cultured mouse spinal neurones and report here that (1) the amino acids appear to utilize channel-like mechanisms similar to those found elsewhere and, (2) the properties of the ion-channel events are unique for each ligand. Preliminary accounts of this study have been published previously (McBurney & Barker, 1978; Barker & McBurney, 1979*a*).

METHODS

Tissue culture

Nerve cells were dissociated from 13 day old mouse embryos (C57BL6) and grown in cell culture according to methods previously described (Ransom, Neale, Henkart, Bullock & Nelson, 1977; Barker & Ransom, 1978*a*). After about 6 weeks in culture the cell bodies were large enough (*ca.* 20–30 μm diameter) to allow stable intracellular recordings with two micro-electrodes (Fig. 1, inset). Two types of cell could be distinguished on visual inspection of mature cultures: (1) spinal cord (SC) neurones with an irregular cell body morphology and multiple, branching thick processes, and (2) dorsal root ganglion (DRG) cells which have smooth circular or elliptical cell bodies and relatively thin processes. Application of immunohistochemical techniques revealed that the DRG cells invariably stained less markedly than spinal cord cells for the presence of nerve-specific enolase, an enzyme marker of neuronal elements (Schmechel, Brightman & Barker, 1980).

Electrophysiological recording

Intracellular recordings were made from cultured spinal neurones at room temperature ($24 \pm 1{\cdot}5$ °C) using two micro-electrodes filled with 3 M-KCl (resistance 50–100 MΩ). The recording medium was the same as previously used (Barker & Ransom, 1978*a*). Under these conditions the membrane current responses to the neutral amino acids usually inverted at between −10 and −20 mV. Holding a cell's membrane potential in the range −50 to −100 mV was sufficient to maintain and stabilize the inversion potential at the relatively depolarized level. Recordings with K acetate-filled micro-electrodes were associated with a variable driving force since the inversion potential was markedly influenced by the potential at which the cell's membrane potential was held (Barker & Ransom, 1978). Therefore, KCl-filled micro-electrodes were used throughout the study.

Membrane potential was controlled using a voltage-clamp with two micro-electrodes (see Smith, Barker, Smith & Colburn, 1980) and membrane current was recorded by a virtual ground current-to-voltage converter. The voltage signal, proportional to membrane current, was recorded both as a DC signal, to allow measurement of the mean change in membrane current response to agonists, and as an amplified, band-limited signal, for subsequent analysis of current fluctuations. The amplified and filtered signal was fed in parallel: to a PDP11/10 computer for analogue-to-digital conversion; to a rectilinear pen recorder (band width DC-50 Hz); to an FM-tape recorder (band width DC-5 kHz); and, to a variance-to-voltage converter which continuously computed the instantaneous variance in membrane current and averaged the values over 1 sec intervals to produce a voltage level for display on another channel of the pen recorder (see Fig. 2).

Ionophoresis

Ionophoretic pipettes were freshly prepared for each experiment by back-filling theta glass micropipettes (1 μm tip diameter). The neutral amino acids γ-aminobutyric acid (GABA), glycine and β-alanine (BALA) (all from Sigma, St Louis) were ionophoresed with cationic current from 1 M solutions at pH 3·5. The most useful pipettes required less than 1 nA backing current. In some experiments amino acids were dissolved in recording medium (pH 7·4) to achieve a final concentration of 50 μM and were applied to neurones by pressure from micropipettes (3·5 μm tip diameter). The results obtained in those experiments agreed well with the results obtained from responses to ionophoretically applied agonist.

Data analysis

Data was acquired by digitizing continuous stretches of the amplified and filtered signal lasting from 6 to 30 sec (see Fig. 2), depending on the sampling frequency and the quality of the recording. The membrane current fluctuations occurring under control conditions or during responses to amino acid were usually digitized at either 1·0, 2·5 or 5·0 msec per point.

The filter bandpass was always set to encompass the spectral analysis band width determined by the sampling frequency and the number of points in the sub-sample (e.g. filter settings for 2048-point subsamples digitized at 1 msec/point were: high-pass cut-off frequency, 0·5 Hz; low-pass cut-off frequency, 500 Hz; roll-off in both cases was 24 db/octave). Analysis proceeded off-line in three stages using a PDP 11 computer. Each fluctuation analysis utilized records of digitized membrane current that contained multiples of 2048-point subsamples.

The variance of membrane current σ_i^2, was calculated directly from the digitized records by the formula

$$\sigma_i^2 = \frac{1}{(N-1)} \sum_{k=1}^{N} [i(k) - \bar{i}]^2$$

where N is the total number of points in the record (multiples of 2048 points), $i(k)$ are the individual data points and $\bar{i}$ is the mean value of the current record.

Power spectra of current fluctuations were calculated as the average of the fast fourier transform (Jenkins & Watts, 1968) of the subsamples of membrane current. Averaging techniques were used to smooth the shape of the power spectral density for a given record.

The second stage of analysis involved subtraction of the estimates of membrane current variance and power spectra obtained during base line recording conditions from corresponding values calculated during agonist-induced responses. The variance of membrane current fluctuations obtained during base line conditions depended on the quality of the recording. Base line variance

was relatively constant over the −50 to −100 mV range but usually increased as the membrane potential was clamped to more depolarized levels. Therefore, most of the analyses of amino acid-induced fluctuations were carried out on responses obtained over the range −40 to −100 mV since this range of membrane potential gave the best signal-to-noise ratio. The power spectra of base line fluctuations obtained in this range of membrane potential usually could be described by a relatively simple equation relating power to frequency, $S(f) = c.f.^{-1}$ where $S(f)$ is the spectral intensity at frequency f, and c is a constant (Fig. 4*A*, base line) (Mathers & Barker, 1980). Subtraction of base line values from those obtained during agonist responses gave an accurate reflexion of the variance due to the agonist and its associated spectrum (Fig. 4*B*).

The third stage of analysis involved fitting the power spectra of agonist-induced fluctuations with the most appropriate mathematical expression. When it became evident that many of the relatively smooth power spectra could be fitted by a single Lorentzian equation, a curve-fitting routine was used to obtain the best fit to this function. All spectra were subjected to this routine and a single Lorentzian curve representing the best fit of the data was superimposed on the spectrum and plotted. With the spectral points normalized by dividing each point, $S(f)$, by the zero frequency asymptote of the spectrum, $S(O)$, the single Lorentzian equation has the form $S(f)/S(O) = 1/[1+(f/f_c)^2]$, where f_c is the cut-off frequency at which the normalized spectra intensity has fallen to 0·5.

The agonist-induced membrane current fluctuations whose power spectra could be adequately described by a single Lorentzian curve were assumed to reflect the kinetic behaviour of a single population of two-state (open, closed) Cl^- ion channels. Upon this assumption and an assumption that during the responses there was a low probability of any single channel being in the open-state, the average duration of the open-state of the event was estimated using the equation $\tau = 1/(2\pi f_c)$. The associated agonist-induced variance was used to calculate the average conductance of a single channel, γ, using the equation $\gamma = \sigma_i^2/(\Delta I . V_D)$ where σ_i^2 is the agonist-induced current variance, ΔI is the macroscopic current change and V_D is the driving force underlying the current. The driving force is the difference between the membrane potential at which no net current flows and the potential at which a current response is evoked by an agonist. Approximately 1600 observations of agonist-induced fluctuations were made on spinal and sensory neurones and about half of these generated power spectra which were considered to reflect the kinetics of essentially one population of ion-channels (i.e., the spectrum could be fit by a single Lorentzian equation). These latter observations were used to estimate the properties of the elementary events.

RESULTS

The results of the present study are based on observations made in over fifty cultured mouse spinal neurones. Fluctuation analysis was applied to GABA responses elicited on thirty-two cells, to glycine responses evoked on twenty-three cells and to β-alanine responses on eleven cells. On a number of cells, two or three amino acid responses were compared. Fluctuation analysis was also applied to GABA responses induced in three cultured sensory neurones.

Localization of membrane responses to amino acids

All of the spinal neurones examined in the present study were sensitive to ionophoretic application of GABA, glycine or β-alanine. Responses could always be detected at the level of the cell body, while a non-uniform sensitivity to the amino acids was observed at sites on cell processes, as previously reported (Barker & Ransom, 1978*a*). Under the recording conditions used in the present study (two intrasomatic micro-electrodes filled with 3 M-KCl) ionophoresis of either amino acid usually evoked depolarizing responses (Fig. 1). The amplitude of these responses depended on the site at which the amino acids were applied. Responses generated at the cell body and on nearby process membranes were typically greater in amplitude than those evoked at distant processes (Fig. 1), although this was not invariable.

Analysis of membrane current responses to amino acids

With the cells voltage-clamped over the −50 to −100 mV range of membrane potential, ionophoretic applications of the amino acids at the level of the cell body evoked inwardly directed membrane current responses. These were always associated with a thickening of the membrane current trace and an increase in membrane current

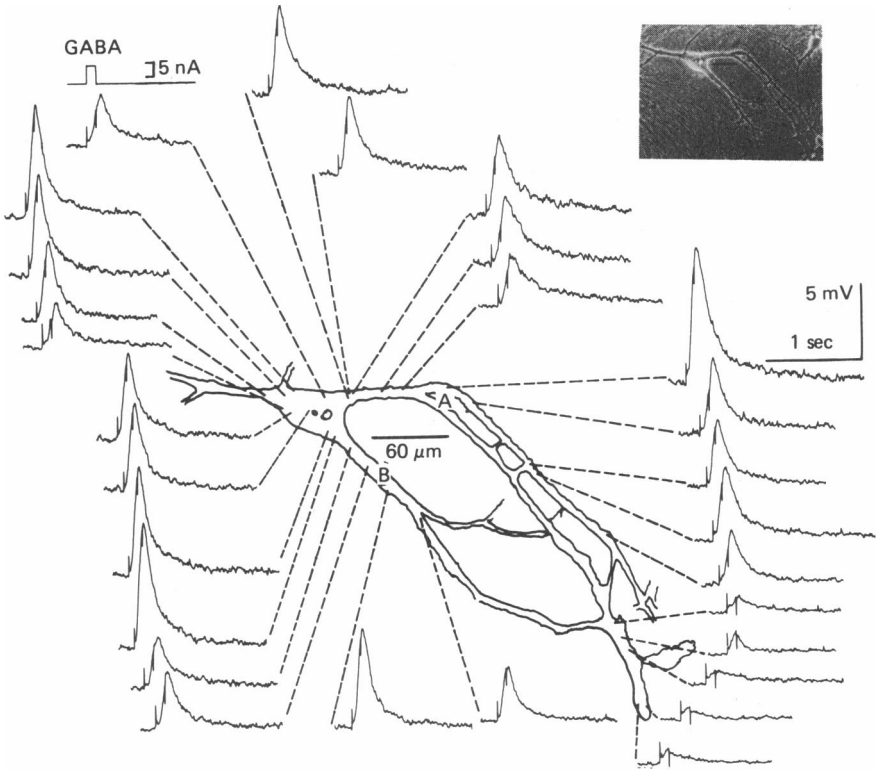

Fig. 1. Distribution of voltage responses to GABA in a cultured mouse spinal neurone. A phase contrast picture of the cell is shown in the upper right-hand corner. The schematic diagram in the centre shows the sites on the cell at which GABA was applied (indicated by the dashed lines). The two spots on the cell body mark the placement of the two 3 M-KCl micro-electrodes used to make the intracellular recording. The membrane potential arranged about the schematic diagram shows voltage responses to ionophoretic pulses (200 msec, 6 nA) of GABA. Two of the processes are labelled A and B for reference in Fig. 2. Resting membrane potential: −60 mV.

variance. Current responses elicited at process membranes within about 50 μm of the cell body were likewise associated with easily detectable increases in membrane current variance, while current responses evoked at more distant membrane sites were smaller in amplitude and associated with less increase in variance. Responses became practically negligible at distances greater than 140 μm from the cell body. Fig. 2 illustrates membrane current responses and accompanying variances evoked by the ionophoresis of identical pulses of GABA applied at ten different sites on the same cultured neurone. The results are representative of those obtained in eight cells

studied in the same way with the amino acids. We cannot localize precisely the portion of surface membrane active in the responses to the amino acids. However, membrane current responses were not evoked by applying amino acids at a distance of 40 μm or more from the cell body using ionophoretic currents similar to those employed to elicit responses when the ionophoretic pipettes were placed within 3 μm

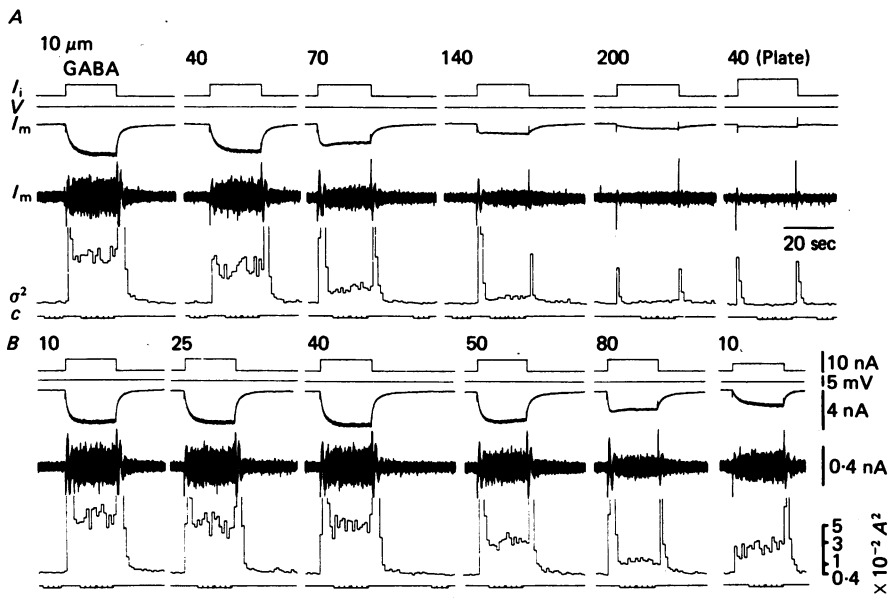

Fig. 2. Distribution of current responses to GABA in the same cultured mouse spinal neurone as shown in Fig. 1. *A* and *B* refer to the two processes labelled in Fig. 1. GABA was applied to different sites on the cell surface using identical current pulses (monitored in the topmost trace marked I_i). The distance between the site of application and the recording micro-electrodes in the cell body is indicated above the traces in μm. The membrane potential (V) of the cell was clamped to -80 mV. The upper of the two traces marked I_m is a DC recording of the membrane current and the lower is the current signal amplified 10 × and filtered at 0·5–500 Hz. Membrane current variance (σ^2) updated every second is displayed as a voltage. The lowest trace monitors data acquisition by computer, *C*. Each acquisition period is composed of three individual records. The rightmost traces in row *A* show an 8 nA application of GABA applied at a spot on the plate 40 μm from the cell body. The rightmost traces in row *B* show the response to a 3 nA application of GABA at the cell body 10 μm from recording micro-electrodes. GABA applications evoke inward current responses and changes in membrane current variance, both of which decrease as the distance between the application site and the recording electrodes increases. No detectable changes are observed when GABA is applied to the plate 40 μm away from the cell body.

of either the cell body or one of its processes (Fig. 2). This indicates that over a distance of 40 μm the agonist concentration is diluted to below that required to evoke detectable membrane responses. Therefore, for the range of ionophoretic currents used in this study to produce prolonged responses suitable for fluctuation analysis, we conclude that agonists applied to the cell body exert their actions on the membrane of the cell within 40 μm of the ionophoretic electrode.

On each of eight cells studied using ionophoretic applications at different sites on

the cell membrane, current responses were plotted as a function of the distance from the recording electrodes positioned in the cell body. Fig. 3 shows data obtained in the same cell as illustrated in Figs. 1 and 2. The data shown is representative of that observed in all of the cells studied in this way. It can be seen that membrane current responses decline in amplitude at distances greater than 50 μm. In all of the cells it

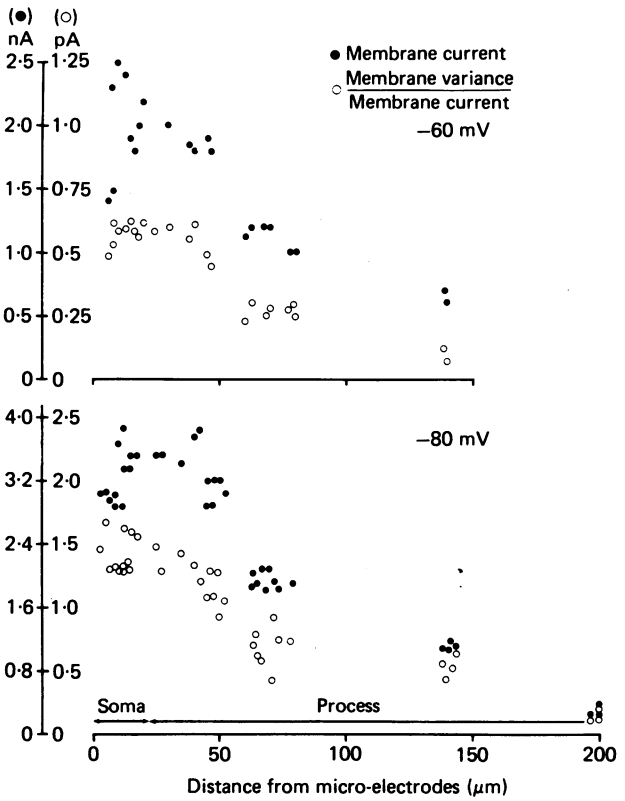

Fig. 3. Plot of membrane current and variance responses at different distances from the recording micro-electrodes in the cell body. The data were obtained on the same cell shown in Figs. 1 and 2. Each filled circle is the mean amplitude of a current response (ΔI) to identical pulses of GABA applied at various sites on the cell expressed in nA. Each open circle is the ratio of the membrane current variance (σ^2) to current response for each of the current responses, expressed in pA. Responses were obtained with the cell clamped to −60 and −80 mV. While there is considerable variability in the amplitudes of the current responses evoked within 50 μm of the recording micro-electrodes, the $\sigma^2/\Delta I$ ratio remains relatively constant. At distances beyond 50 μm both current responses and $\sigma^2/\Delta I$ decline.

was also evident that the membrane current variance associated with the responses decreased at distances greater than 50 μm. However, the variance and current did not decrease in parallel. Thus the $\sigma^2/\Delta I$ ratio did not remain constant for responses elicited at distances greater than about 40–50 μm (Fig. 3). Ratios for responses evoked at sites 70 μm or more from the cell body were significantly less than those calculated for responses at the cell body (Table 1).

Spectral analysis of membrane current variance

Spectral analysis was carried out on the membrane current variance associated with the membrane current responses to the amino acids as described in the Methods section. A difference spectrum obtained from analysis of current responses elicited at the cell body of the same cell used in Figs. 1–3 is shown in Fig. 4. The spectrum

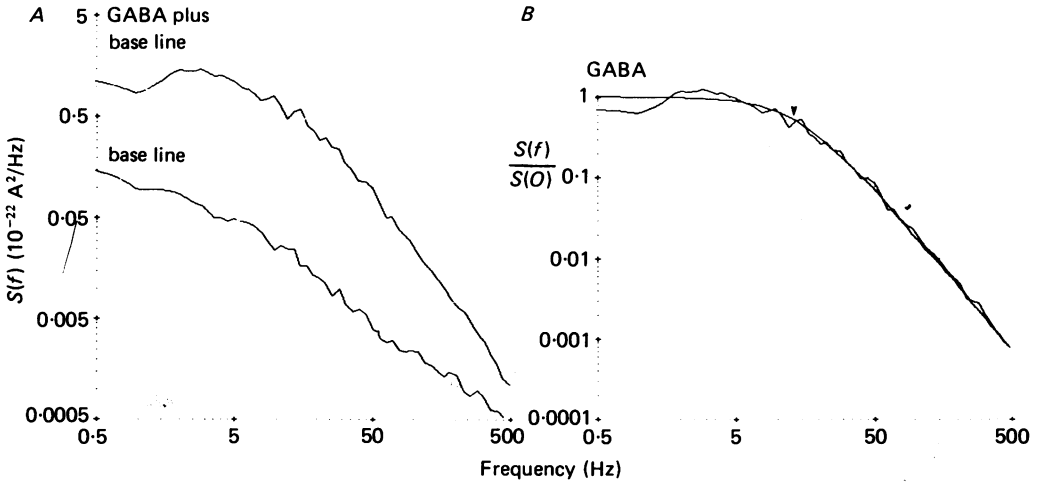

Fig. 4. Power spectral density plots of membrane current variance. The data were obtained on the same cell shown in Figs. 1–3. *A*, spectral density of base line variance declines in a relatively monotonic manner as frequency increases. During a 3·1 nA response evoked by GABA at the cell body spectral density shifts to higher powers at all frequencies, displays an asymptote over the lower frequency range and declines more rapidly than during base line conditions over the frequency range 5–500 Hz. *B*, power spectral density plot resulting from subtraction of the base line signal from that occurring during the application of GABA. The resulting 'difference' spectrum which resolves the variance due to GABA-activated ion channel activity has been normalized by dividing each spectral point, $S(f)$, by the zero frequency asymptote of the spectrum $S(O)$. The spectra in this and subsequent Figures have been subjected to a curve-fitting routine and fit by least-squares analysis with a Lorentzian equation (continuous line) of the form $S(f)/S(O) = 1/[1+(f/f_c)^2]$. The arrowhead indicates the corner-frequency (12·3 Hz), the frequency at which power spectral density falls by one half.

is well fitted by a single Lorentzian equation, having an f_c value of 12·3 Hz. Spectra calculated from current responses evoked on the same cell used in Figs. 1–4 at different distances from the recording electrodes are shown in Fig. 5. The spectra vary in their smoothness with those obtained from responses evoked at distances up to 80 μm being somewhat better fitted by single Lorentzian curves than those derived from more distant responses. Although there was some variability in the f_c values for spectra at each of the various sites, the difference between the average values at the different sites was not statistically significant (Table 1).

Since there were significant changes in the $\sigma^2/\Delta I$ ratio calculated for responses evoked at sites more than 50 μm from the cell body and since this ratio is used to estimate the conductance of a single channel, the amino acids were always applied to the cell body.

Neutral amino acids increase membrane current fluctuations

Membrane current responses to each of the naturally occurring neutral amino acids were associated with a thickening of the current trace (Figs. 6*B*, *C*, 8*A*) and an increase in membrane current variance (Figs. 6*D*, 8*A*). The increase in current variance induced by the amino acids was directly related to the amplitude of the current response in a linear manner at a given holding potential (Figs. 7*B*, 8*B*). On

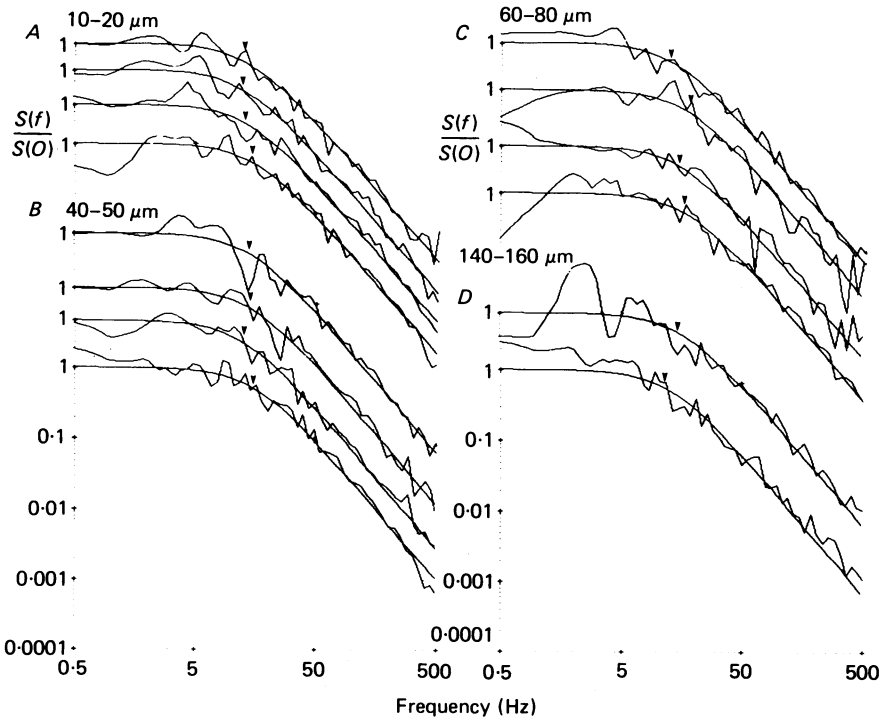

Fig. 5. Power spectral density plots of membrane current variance evoked by GABA at different distances from the recording electrodes in the cell body. These and all subsequent difference spectra have been normalized as in Fig. 4. Distances between the recording electrodes and ionophoretic pipette placement are indicated above each set of spectra. The upper two in each set of four spectra in *A–C* were obtained with the membrane potential clamped to −60 mV; the lower pair in *A–C* and that in *D* were obtained at −80 mV. The arrowheads indicate the f_c values. These are (Hz) 13·5, 12·5, 13·5, 15·5 (*A*); 15·0, 15·2, 12·5, 16·0 (*B*); 12·0, 17·0, 15·0, 16·5 (*C*); 15·0, 11·5 (*D*).

occasion relatively large membrane current responses were associated with less variance than would have been predicted from the linear relationship present over the lower range of membrane current responses (see Barker & McBurney, 1979*a*). Presumably larger responses were associated with an increased probability of an individual channel being open. Under these circumstances the variance recorded would lead to an underestimate of the elementary conductance (see Conti & Wanke, 1975). In many instances the membrane current during continued application of the agonist declined after initially plateauing (Fig. 12*A*). The variance associated with

the response also declined, but after analysing the fluctuations we found that neither the magnitude of the elementary conductance change (Fig. 12*B*) nor the average channel open-time (Fig. 12*C*) was altered during the densensitization to the agonist. These results suggest that desensitization is due to a decrease in the frequency of elementary conductance events rather than to a change in channel properties or the activation of other conductances.

TABLE 1. Estimated electrical properties of Cl^- ion permeable channels activated by GABA at different parts of a cultured mouse spinal neurone

Position* (μm)	$\sigma^2/\Delta I$ (pA)	n	P	τ(mS)	n	P
10–20	0·57±0·05 (−60 mV)	10	—	10·8±1·3	23	—
	1·33±0·30 (−80 mV)	13	—			
40–50	0·52±0·07 (−60 mV)	6	n.s.	10·7±0·6	13	n.s.
	1·15±0·14 (−80 mV)	7	n.s.			
70–80	0·26±0·03 (−60 mV)	6	0·01	9·8±1·9	14	n.s.
	0·61±0·10 (−80 mV)	8	0·01			
140	0·10±0·04 (−60 mV)	3	0·01	9·6±2·3	6	n.s.
	0·50±0·10 (−80 mV)	3	0·01			
200	0·26±0·10 (−80 mV)	3	0·01	†		

* Distance along processes from recording electrodes placed in the soma of the cell shown in Fig. 1.

σ^2: membrane current variance (pA^2).

ΔI: membrane current response (nA).

Average $\sigma^2/\Delta I$ ratio expressed in pA (±S.D.).

n: number of observations.

P: Student's t test of significance applied to values obtained at sites relatives to values observed at 10–20 μm.

n.s.: not significant.

τ: average single channel duration (msec) (±S.D.) obtained using all of the observations at −60 mV and −80 mV at each site.

† No interpretable spectrum.

The amplitude of the elementary current was linearly related to the membrane potential over an extensive range of membrane potential (Fig. 9). The slope of the relationship between the calculated amplitude of the single-channel current and the membrane potential was indistinguishable from the slope of the line relating agonist-induced current amplitude to membrane potential on every cell where this was examined closely. There was excellent agreement between the values of membrane potential at which we observed an inversion of the total membrane current and the estimated single-channel current. Similar observations were made with membrane current responses to all three amino acids.

Power spectra of amino acid-induced fluctuations

Power spectra calculated from fluctuations associated with membrane current responses to the different agonists could be fitted by single Lorentzian curves (Figs. 10–13, 15). The cut-off frequency of the spectrum for a given agonist was similar over a low-to-moderate range of membrane current (0·5–7 nA). This is most likely an indication that, during agonist-induced current responses of different amplitude,

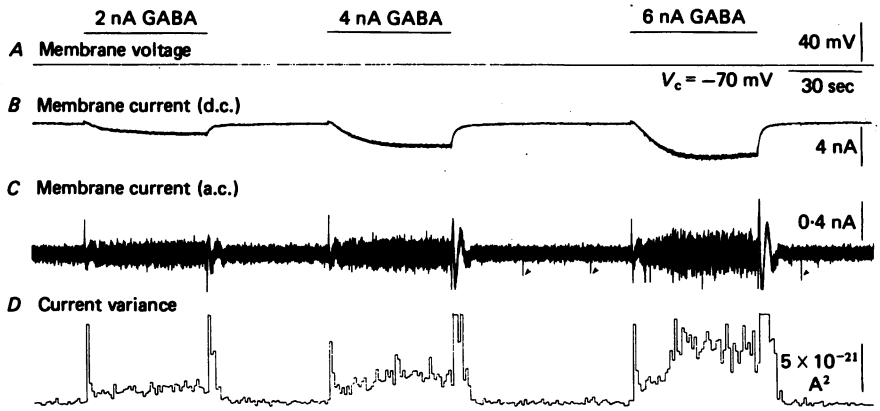

Fig. 6. Membrane current responses to ionophoretically applied GABA in a cultured mouse spinal neurone voltage-clamped at −70 mV. The membrane current is displayed unfiltered on the d.c. trace (*B*) and at 10× gain on the a.c. trace (*C*) filtered at 0·2–200 Hz. The variance associated with the filtered signal, updated at 1 sec intervals (see text), is displayed in *D*. Increasing ionophoretic currents cause inward current responses of increasing amplitude, each of which is associated with a thickening of the d.c. and a.c. current traces, and increases in membrane current variance. The largest changes in variance at the beginning and end of the current responses reflect the relatively rapid changes in membrane current occurring at these times due to a.c. coupling. The small arrowheads mark spontaneous inward current events which have a fast rise time and exponential decay, suggesting they are synaptic in origin.

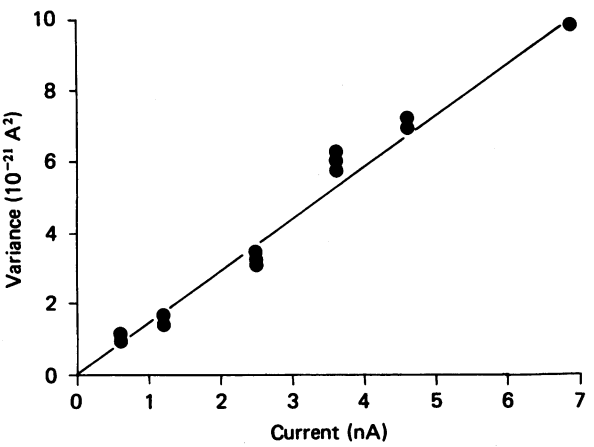

Fig. 7. The relationship between the mean current response and the variance of the underlying current fluctuations resulting from ionophoretic GABA applications to a cultured spinal neurone. Same cell shown in Fig. 6. The thirteen values were all obtained at −80 mV and 24 °C. A linear relationship between variance and mean current is evident. The slope of the line relating variance to current divided by the driving force gives an estimated single channel conductance of 20 pS.

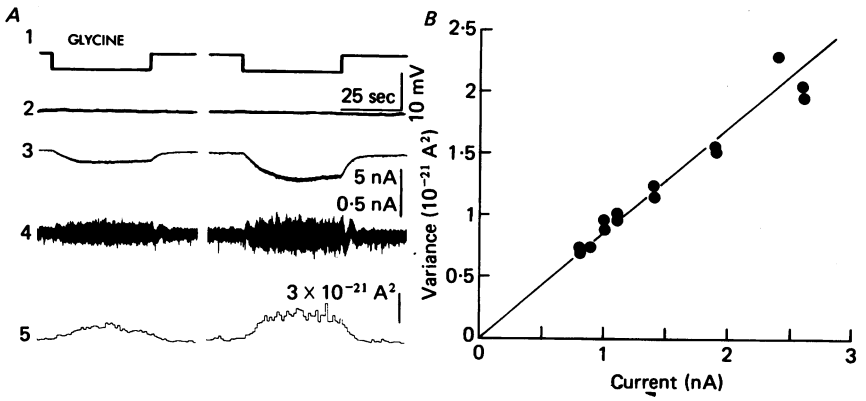

Fig. 8. Membrane current and variance responses to ionophoretically applied glycine in a cultured mouse spinal neurone voltage-clamped at −50 mV. *A*, glycine was applied ionophoretically during periods marked on trace monitoring ionophoretic current (not related to amount of ionophoretic current) (1). Membrane voltage has been clamped at −50 mV (2). Low-gain, d.c. traces (3) and high-gain, a.c. traces (4) of membrane current responses to glycine show a thickening of the traces during the responses which is coincident with increases in membrane current variance (5). *B*, plot of membrane variance as a function of membrane current response for fourteen glycine responses. The slope of the line divided by the driving force gives an estimated single channel conductance of 28 pS.

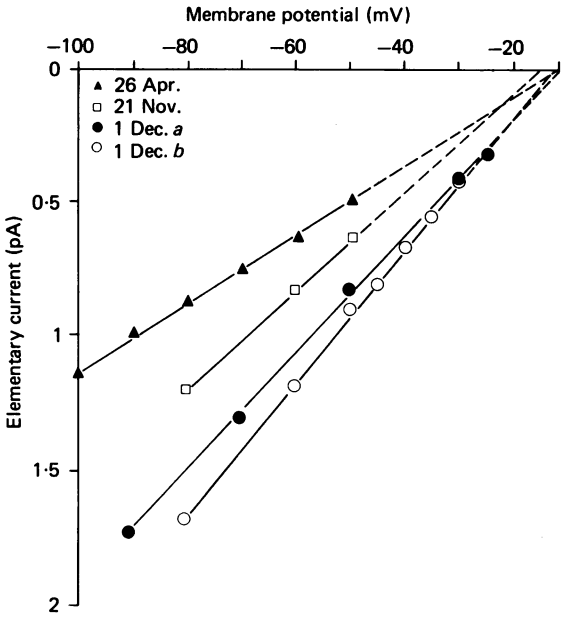

Fig. 9. Estimated elementary current amplitude underlying GABA responses plotted as a function of membrane potential for four different cultured spinal cord neurones. In each case there is a linear relationship between the elementary amplitude and the membrane potential, indicating that essentially the same null potential is associated with each of the values obtained on a given cell. The lines relating elementary current to membrane potential extrapolate to driving forces between −15 and −10 mV.

channels were recruited from a population with apparently similar kinetics (Figs. 10, 11). Similar results were derived from analysis of fluctuations induced by β-alanine. Power spectra obtained during the declining phase of GABA-induced current responses exhibited cut-off frequencies similar to those obtained at the peak of the response (Fig. 12*C*). Cut-off frequencies of spectra calculated from records taken during the declining phases of current responses to other amino acids were likewise

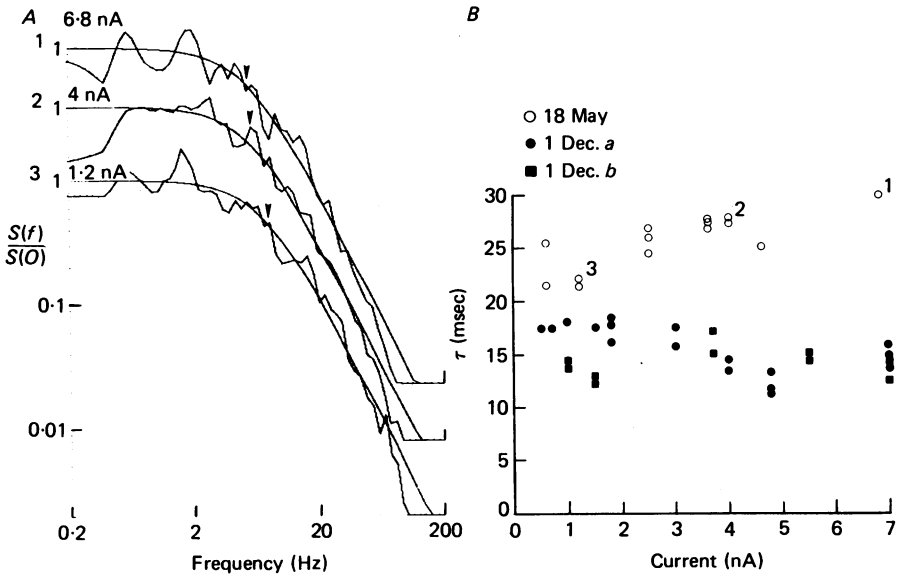

Fig. 10. Estimated durations for GABA-activated channels as a function of current response amplitude for three different spinal cord neurones. *A*, normalized power spectra of GABA-induced fluctuations occurring during membrane current responses of 1·2, 4·0 and 6·8 nA. The spectra have cut-off frequencies, f_c (marked by arrowheads) of 7·0 Hz (for the 1·2 nA response), 5·6 Hz (at 4 nA) and 5·3 Hz (at 6·8 nA). Since mean channel lifetime, $\tau = 1/(2\pi f_c)$, $\tau_{1\cdot2nA} = 22\cdot7$ msec, $\tau_{4nA} = 28\cdot4$ msec and $\tau_{6\cdot8nA} = 30\cdot0$ msec. These points are labelled 3, 2 and 1 in the plot of estimated channel duration as a function of membrane current response shown in *B*. Although channel duration tends to be longer at larger currents in the cell from which spectra were chosen for illustration, this was not so for the data taken from two other cells. All data was derived from membrane clamped at −70 mV at 24 °C.

similar to those associated with spectra calculated from records taken at the peak of the response (not shown). The cut-off frequencies of spectra calculated at different membrane potentials were not consistently nor significantly different from each other over the −50 to −100 mV range for GABA (Fig. 13) and the other amino acids (not shown). In some cells spectra obtained at −40 mV could not be fitted by a simple, single Lorentzian equation (Fig. 13). Thus, the estimated properties of the elementary events activated by the amino acids did not vary consistently with membrane potential for GABA (Fig. 14) or the other amino acids (not shown).

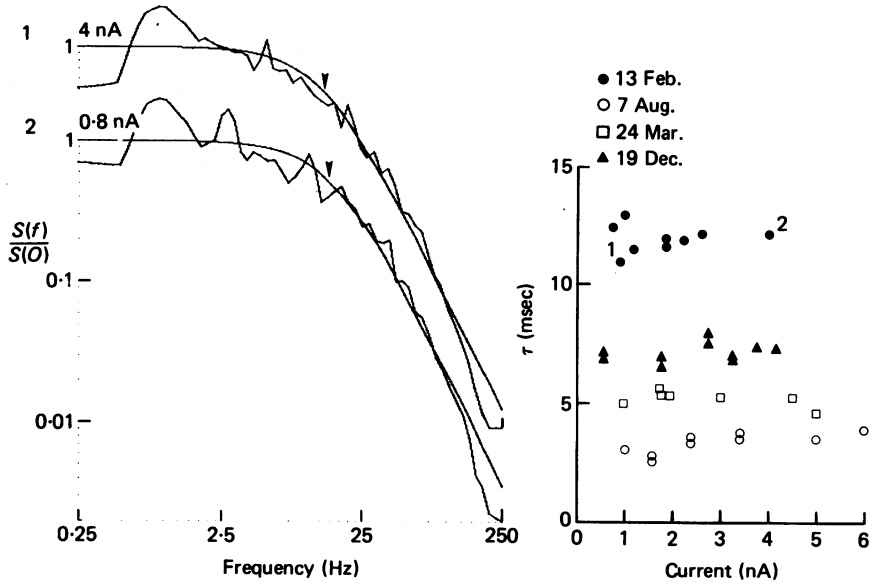

Fig. 11. Estimated durations for glycine-activated channels as a function of current response amplitude for four different spinal cord neurones. 1 and 2 are normalized power spectra of glycine-induced fluctuations occurring during membrane current responses of 0·8 and 4 nA. The spectra have been fitted by single Lorentzian equations whose f_cs are 14·9 Hz, (for the 0·8 nA response) and 12·9 Hz, (for the 4·0 nA response) giving estimated average channel durations of 10·7 msec and 12·3 msec respectively. These points are labelled '1' and '2' in the plot of channel duration as a function of membrane current amplitude at the right. There is no consistent or significant relationship between channel duration and current response over the range of currents studied.

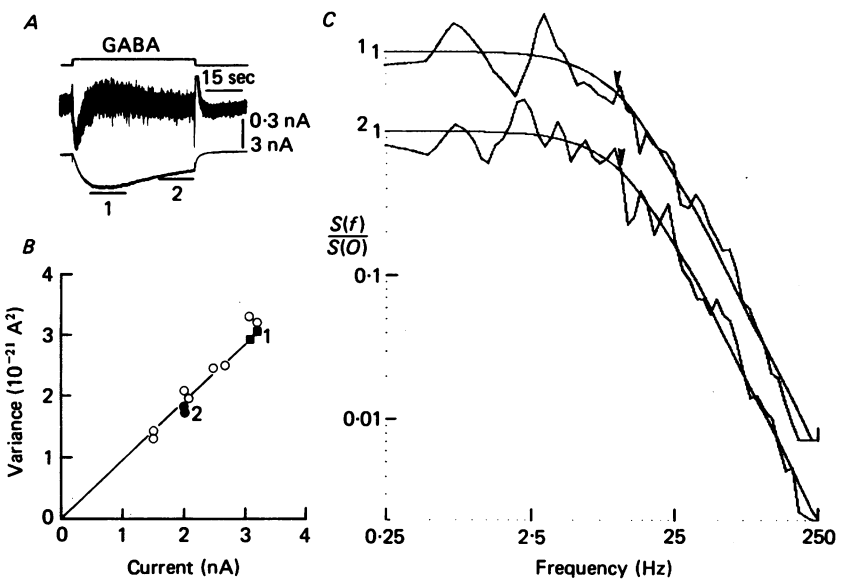

Fig. 12. For legend see opposite page.

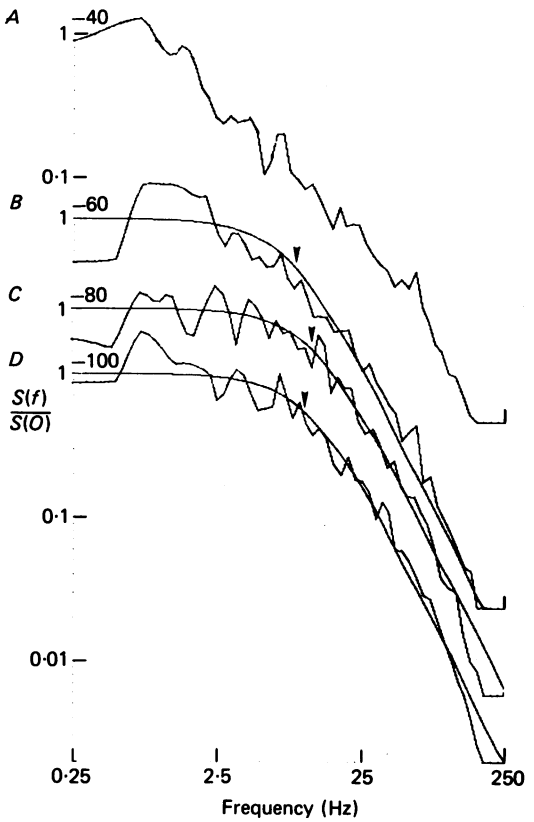

Fig. 13. Normalized power spectra of GABA-induced fluctuations at different membrane potentials. The spectra obtained at −60, −80 and −100 mV have been fitted by single Lorentzians while that calculated for fluctuations at −40 mV cannot be fitted by a single Lorentzian. The arrowheads indicate f_cs of 7·8 Hz (−60 mV), 11·8 Hz (−80 mV) and 10·9 Hz (−100 mV), corresponding to estimated channel durations of 20·4, 13·5 and 14·6 msec, respectively.

Fig. 12. Fluctuation analysis of the peak and plateau regions of a membrane current response exhibiting desensitization in a cultured mouse spinal neurone. *A*, sustained application of GABA caused a membrane current response and associated thickening of the current trace both of which faded during the course of the response. Samples of current fluctuations obtained during the peak and plateau phases of the response (indicated by bars) were taken for analysis. *B*, plot of the membrane variance associated with membrane current responses of different amplitude. The open circles indicate the variance associated with the peak responses. The filled symbols reflect the variance associated with the peak (squares) and plateau phase (circles) of desensitizing responses. The numbers refer to data derived from the current response shown in *A*. *C*, normalized power spectra of the fluctuations occurring at the peak of the response illustrated in *A*(1) and during the plateau phase (2). The arrowheads indicate the f_cs of 10·0 Hz (peak) and 10·8 Hz (plateau), giving estimated average channel durations of 15·9 and 14·7 msec.

Neutral amino acids activate channels with different properties

There was considerable variation in the estimated average conductance and duration of the elementary events evoked by the different neutral amino acids on different spinal cord neurones. For example, there was a 10-fold range in γ estimated for GABA-activated channels and about 3–5 fold variation for all the other channel properties estimated for the other amino acids (Fig. 15). The properties of channels

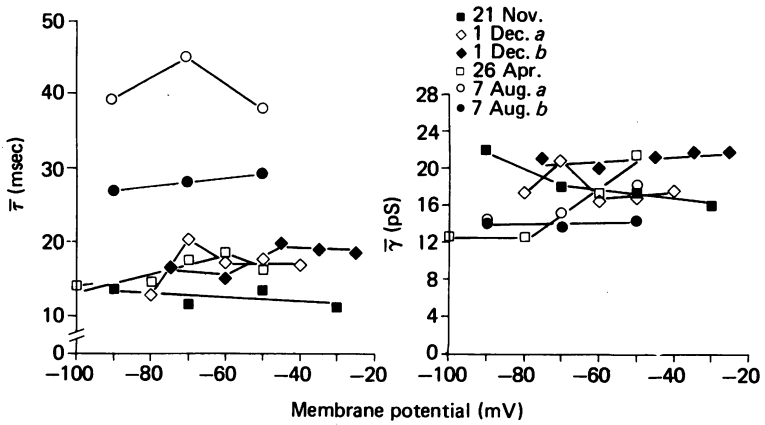

Fig. 14. Estimated properties of GABA-activated channels at a range of membrane potentials. Each point represents the average of at least three determinations made on six different cultured spinal cord neurones. Although a range of estimated channel durations (τ) and channel conductances (γ) is evident for the six cells studied, there is no consistent relationship between either τ or γ and membrane potential.

activated on three dorsal root ganglion (sensory) neurones by GABA showed less variability. They were for γ, $14{\cdot}4 \pm 0{\cdot}2$ pS (mean $\pm$S.D.), and for τ, $22{\cdot}6 \pm 2{\cdot}6$ msec (105 observations). Both the γ and τ values for GABA-activated channels on DRG neurones were within the range of the corresponding properties for GABA-activated channels on spinal neurones. Despite the considerable variation in the estimated properties of neutral amino acid-activated ion-channels, when estimated channel properties were compared on those spinal cord cells where a pair of agonists (or three agonists) were applied, the estimated properties were significantly different from each other at the 0·01 level or better except for four instances in forty-four comparisons (Fig. 16; Tables 2–4). From these results we suggest that the three amino acids activated channels with unique elementary properties.

DISCUSSION

From the results obtained by applying amino acids to different regions of the neuronal membane it is clear that the property $\sigma^2/\Delta I$ of the responses is not constant over the entire surface. The conductance, γ, of a single agonist-activated ion-channel calculated from this ratio would therefore have an apparently smaller value for

ion-channels on processes than for channels on the cell soma. However, such analysis would depend on: adequate control of the membrane potential of processes by the voltage clamp system; a similarity between the driving force at the cell body and at the process; and, the probability of channel activation by a particular agonist dose being low on processes.

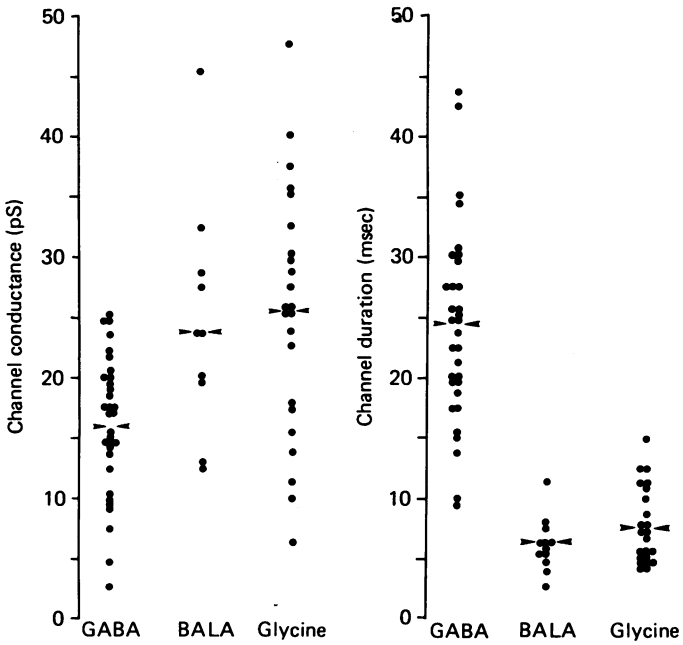

Fig. 15. Distribution of estimated values for single channel conductance, γ, and average channel duration, τ, activated by neutral amino acids in cultured spinal neurones. Each data point represents the average value obtained on an individual cell. The bars indicate the grand means of all the values in a particular column.

We have not further investigated the properties of amino acid-activated channels on processes. However, it is possible that adequate control of membrane potential exists over a significant fraction of the process membrane tree of these neurones. An estimate of the space constant of a 5 μm diameter process yields a value of about 900 μm for the d.c. space constant, $\lambda_{d.c.}$, and a value for the a.c. space constant at 1 kHz of about 100 μm (specific resistance and capacitance values used in the calculation were $R_i = 150\ \Omega$.cm, $R_m = 10\ \Omega$, cm^2, $C_m = 1\ \mu$F/cm^2). Ransom *et al.* (1977) estimated the average electrotonic length of all the processes of spinal cord neurones as 0·9 $\lambda_{d.c.}$. Furthermore, the similarity of the power spectra of agonist-induced current fluctuations at the cell soma and up to 160 μm from the recording electrodes (Fig. 5) is consistent with adequate voltage control over distances greater than 100 μm from the recording electrodes for fluctuations up to 500 Hz.

At present we have no information about the driving force for ion-movements across the process membrane nor of the relative distribution of possible different types of amino acid-activated ion-channels over the surface of a cultured spinal neurone.

TABLE 2. Paired comparison of the estimated properties of elementary events underlying membrane current fluctuations induced by GABA and glycine on cultured mouse spinal neurones

Cell	Property	GABA	n	Glycine	n	P
1	γ (±S.D.)	18·9±3·5	8	25·6±6·6	8	0·05
	τ (±S.D.)	19·1±2·1		4·5±1·6		0·01
2	γ	13·7±3·0	42	30·7±11·9	28	0·01
	τ	31·3±6·8		5·4±1·0		0·01
3	γ	4·5±1·1	18	6·7±1·2	37	0·01
	τ	43·3±10·0		4·5±0·5		0·01
4	γ	7·5±1·0	13	25·8±4·0	10	0·01
	τ	27·8±3·5		11·0±2·4		0·01
5	γ	24·2±3·4	6	35·6±6·3	11	0·01
	τ	30·4±2·5		12·2±3·8		0·01
6	γ	18·5±7·0	11	37·0±2·1	5	0·01
	τ	25·6±9·0		7·6±1·0		0·01
7	γ	14·7±2·7	21	28·9±4·3	9	0·01
	τ	19·5±3·2		3·7±0·6		0·01
8	γ	17·7±1·7	9	36·0±3·1	3	0·01
	τ	34·0±10·0		12·0±2·1		0·01
9	γ	12·6±3·1	6	29·4±4·8	4	0·01
	τ	19·9±2·5		7·1±1·6		0·01
10	γ	17·0±4·5	20	26·7±2·1	9	0·01
	τ	24·3±6·3		11·5±1·8		0·01

γ: mean conductance of elementary event (pS).
τ: mean duration of elementary event (msec).
n: number of observations.
P: significance level (Student's t test).

TABLE 3. Paired comparisons of the estimated properties of elementary events underlying membrane current fluctuations induced by GABA and BALA

Cell	Property	GABA	n	BALA	n	P
1	γ	8·6±3·0	8	15·6±3·1	17	0·01
	τ	9·9±1·0		5·2±1·2		0·01
2	γ	14·6±3·4	41	20·1±3·0	24	0·01
	τ	15·7±2·5		5·7±0·6		0·01
3	γ	7·5±1·0	13	12·6±3·3	11	0·01
	τ	27·8±3·5		4·2±0·4		0·01
4	γ	24·2±3·4	6	19·9±3·9	6	0·05*
	τ	30·4±2·5		7·8±1·0		0·01
5	γ	18·5±7·0	11	32·0±3·0	8	0·01
	τ	25·6±9·0		8·1±1·2		0·01
6	γ	12·6±3·1	6	23·2±3·0	9	0·01
	τ	19·9±2·5		2·1±0·5		0·01
7	γ	17·6±4·5	20	27·3±1·4	8	0·01
	τ	24·3±6·3		5·8±0·5		0·01

* Mean conductance of GABA significantly greater than that of BALA.

TABLE 4. Paired comparisons of the estimated properties of elementary events underlying membrane current fluctuations induced by glycine and BALA

Cell	Property	Glycine	*n*	BALA	*n*	*P*
1	γ (±S.D.)	25·8±4·0	10	12·6±3·3	11	0·01
	τ(±S.D.)	11·0±2·4		4·2±0·4		0·01
2	γ	35·6±6·3	11	19·9±3·9	6	0·01
	τ	12·2±3·8		7·8±1·0		0·01
3	γ	37·0±2·1	5	32·0±3·0	8	0·01
	τ	7·6±1·0		8·1±1·2		n.s.
4	γ	29·4±4·8	5	23·2±3·0	9	0·01
	τ	7·1±1·6		2·1±0·5		0·01
5	γ	26·7±2·1	9	27·3±1·4	8	n.s.
	τ	11·5±1·8		5·8±0·5		0·01

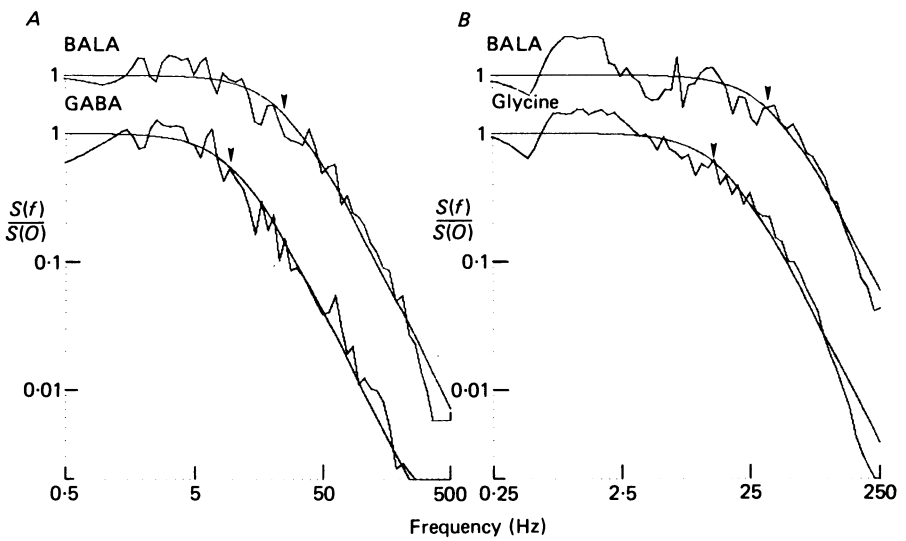

Fig. 16. Normalized power spectra of fluctuations produced by pairs of amino acids on two different spinal cord neurones both clamped to −70 mV. *A*, the f_cs for spectra derived from BALA and GABA fluctuations in this cell are 25·0 and 10·3 Hz, giving estimated average channel durations of 6·4 and 15·5 msec, respectively. For a complete statistical analysis of the observations made in this cell see 'cell 2' of Table 3. *B*, the f_cs for spectra derived from BALA and glycine fluctuations in this cell are 36·1 and 15·3 Hz, respectively. This gives estimated average channel durations of 4·4 and 10·4 msec. For a complete statistical analysis of the observations made in the cells see 'cell 1' of Table 4.

We have therefore restricted our analysis to Cl^- ion-dependent current responses evoked at the cell body of cultured mouse spinal cord and sensory neurones by naturally occurring neutral amino acids. The principal findings are that (1) the fluctuations in anionic membrane current can often be interpreted as reflecting the kinetic behaviour of a single population of two-state ion-channels, (2) the properties

of the elementary channel events activated by one of the amino acids are significantly different from those activated by either of the other amino acids, and (3) these properties each appear to be relatively constant over the −40 to −90 mV range of membrane potential.

Fluctuation analysis of GABA responses in crayfish muscle membranes reveals an elementary event with a conductance of about 9 pS and an average open-state lifetime of about 5 msec at −100 mV and 23 °C (Dudel, Finger & Stettmeier, 1977, 1980). Furthermore channel kinetics at the muscle membrane are clearly voltage-sensitive. Thus, the channels activated by GABA at synapses in crayfish muscle membranes appear to have properties which are different from those studied in cultured mammalian spinal neurones. Fluctuation analysis of GABA and glycine voltage responses in the Mauthner cell of the goldfish shows that both amino acids produce elementary events of about 7 msec in duration (Faber & Korn, 1980). It is not yet clear how these channels are related to the channels activated by amino acids in muscle or spinal cord cell membranes.

We have recently applied the patch clamp technique to record directly the properties of the elementary channel events activated by GABA in microscopic patches on cultured neuronal membranes (Mathers, Jackson, Lecar & Barker, 1981). Preliminary analysis shows that the single elementary events have properties similar to those estimated from fluctuation analysis (unpublished observations). These preliminary results support the basic assumptions necessary for fluctuation analysis and, due to the comparability of the two methods, we suggest that the single ion-channel events were recorded from essentially the same population of channels as that operated upon by the agonist to produce the membrane current responses used in our analysis for this study.

For each agonist used there was a wide distribution of the mean values of single channel properties estimated among the cells studied (Fig. 15). This variation in channel properties recorded among individual cells may be due to technical difficulties inherent in making exact estimates. Certainly the use of unidentified cultured spinal cord neurones makes it impossible to know to what extent, if any, the distribution in mean values among individual cells reflects a naturally occurring variation. Such variation could arise if channel properties alter during the development and maintenance of neurones in culture, or if channel properties are different on functionally distinct cell types, or if synaptic (or clustered) and extrasynaptic (unclustered) channel properties vary. There is less variation in the estimates of channel properties activated by GABA in DRG cells than in those made for GABA-activated properties in spinal cord cells. Although we have not yet determined the ionic mechanism underlying the depolarizing effects of GABA in cultured DRG cell bodies, depolarizing responses to GABA recorded in DRG cell bodies in situ are thought to involve an increase in membrane conductance to Cl^- ions with the Cl^--ion distribution across the DRG cell membrane different from that across spinal cord cells (for review, see Nistri & Constanti, 1979). If we assume that a Cl^- ion conductance underlies the depolarizing responses to GABA in cultured DRG cells, then the values for the properties of Cl^- ion channels activated by GABA in DRG cells lie within the range of values for the properties of GABA-activated channels on spinal cord neurones. Since there is no morphological evidence for synaptic investment of DRG

cell bodies in cultured mammalian DRGs (Peacock, Nelson & Goldstone, 1973; Ransom *et al.* 1977) the channels activated in DRG somal membranes are presumably non-synaptic. We do not know whether the channels activated in spinal cord cells are synaptic and/or extrasynaptic. It will be necessary first to establish whether the non-uniform distribution of amino acid responses in spinal cord neurones (Fig. 1: see also Barker & Ransom, 1978 or, in the case of GABA, the presence of glutamic acid decarboxylase positive structures investing this cell type (Barker, MacDonald, Mathers, McBurney & Oertel, 1981) reflects the presence of functional synaptic input.

In some spinal cord neurones under voltage-clamp conditions we recorded spontaneous, randomly occurring currents with a rapid growth phase (< 1 msec) to a peak (< 1 nA) followed by an exponential decay phase (Barker & McBurney, 1979*b*). We presume, since we normally take steps to eliminate evoked synaptic transmission between neurones during recording, that these currents result from the post-synaptic action of single packets of neurotransmitter release from presynaptic nerve terminals. In a few cells the time constant of the decay phase of these synaptic currents was similar to the average lifetime of GABA-activated ion-channels in the same cell. Moreover in the presence of phenobarbitone both the time constant of the decay of the synaptic currents and the average lifetime of GABA-activated channels were prolonged (Barker & McBurney, 1979*b*). While this result does indicate a similarity in a few cells between the properties of putative-neurotransmitter-activated ion-channels and natural-transmitter-activated synaptic channels, we cannot definitely relate our agonist-activated channel properties to physiologically relevant synaptic events mediated by GABA or either of the other neutral amino acids.

When the estimated channel properties activated on individual cells by different amino acids were compared statistically, it was evident that each amino acid activated elementary events with different properties (Tables 2–4). Thus, three neutral amino acids with closely related structures can activate Cl^- ion channels with uniquely different properties. If these channels are closely related to synaptic channels, then the three amino acids may mediate synaptic events in cultured mouse spinal neurones with different characteristics.

Conductance and duration were not significantly related to each other for channels activated by a particular amino acid but there appeared to be a slight correlation ($r = -0{\cdot}45$) which was not significant ($P = 0{\cdot}10$) when conductance was related to duration for fifteen pairs of properties on five cells where all three amino acids were studied. More observations are necessary to evaluate a possible relationship between conductance and duration for these three amino acids.

The present findings do not indicate whether one channel can be activated by more than one amino acid, since the mutual antagonism of responses in spinal cord neurones exhibited by the three amino acids could occur at either the receptor or the channel level (Barker & McBurney, 1979*a*). It is possible that several receptor sites may share the same channel, and that engagement of different sites generates different electrical properties and different transfers of ionic charge through the common channel over different periods of time. Alternatively, the amino acids may all bind at the same site, coupled to a common channel, or they may bind to physically distinct receptor channel complexes. The relative charge transfer during the three

types of elementary ion channel event is on average 1·0:0·74:0·32; GABA:glycine:β-alanine.

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