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THE SYNERGISTIC ACTION OF L-GLUTAMATE AND L-ASPARTATE AT CRUSTACEAN EXCITATORY NEUROMUSCULAR JUNCTIONS

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SUMMARY

1. When L-glutamate and L-aspartate are simultaneously applied to the excitatory neuromuscular junctions of *Maia squinado*, they produce an increase in the conductance of the post-synaptic membrane much larger than the sum of conductance effects produced by the individual amino acids alone.

2. An examination of the synaptic noise occurring during this synergistic action reveals that the elementary conductance events produced by aspartate are suppressed and that normal elementary conductance events produced by glutamate are occurring at an enormously increased rate.

3. It is suggested that aspartate causes this potentiation by inhibiting a system for transmitter inactivation in the region of the post-synaptic receptors and that this system, under normal conditions, prevents the access of externally applied glutamate to the synaptic receptors.

INTRODUCTION

L-Glutamate and L-aspartate both produce conductance changes at the excitatory neuromuscular junctions of crustacea (Takeuchi & Takeuchi, 1964; Kravitz, Slater, Takahashi, Bownds & Grossfeld, 1970). When applied together to the excitatory membrane, these substances produce a much larger conductance change than the sum of their individual conductance effects (Kravitz *et al.* 1970; Shank, Freeman, McBride & Aprison, 1975; Shank & Freeman, 1975). It is not clear whether the synergistic action of these two amino acids represents a potentiation of the glutamate response by aspartate, a potentiation of the aspartate response by glutamate, or

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whether the conducting mechanism is quite different when both amino acids are present.

The synergistic action of glutamate and aspartate has prompted several authors to propose that both amino acids are synchronously released by the excitatory nerves of crustacea during normal transmission, and that the generation of the excitatory junctional potential depends upon this potentiating effect (reviewed by Freeman, 1976).

We have recently shown (Crawford & McBurney, 1976*a*) that the elementary conductance events produced by aspartate and glutamate at the excitatory neuromuscular junctions of *Maia squinado* have different mean lifetimes, and it therefore seemed that an examination of the synaptic noise occurring during the synergistic action of these amino acids might reveal something of the underlying mechanism.

METHODS

Experiments were performed on the flexor musculature of the carpopodite in the walking legs of the spider crab, *Maia squinado*. The preparation was bathed in crab saline (composition in mM: 510 NaCl, 10 KCl, 11 $CaCl_2$, 25 $MgCl_2$, 2·5 $NaHCO_3$) at room temperature (19–23° C) and recordings of intracellular and extracellular potential changes at the excitatory neuromuscular junctions made as described previously (Crawford & McBurney, 1976*a*, *b*). Amino acids were obtained from Sigma Chemical Co., and added to the crab saline before each experiment. Amino acid concentrations given in the text are the total concentrations: both glutamate and aspartate chelate divalent cations; and it is not clear whether the free (ionized) forms of the amino acids have the same activating effect at the excitatory membrane as the complexes of the amino acids with calcium and magnesium. Formally, the fraction of the total glutamate in the ionized form is given by

$$\frac{[\mathrm{Glu}]_{\mathrm{free}}}{[\mathrm{Glu}]_{\mathrm{total}}} = \frac{1}{1+K_{\mathrm{Ca}}.[\mathrm{Ca}^{2+}]+K_{\mathrm{Mg}}.[\mathrm{Mg}^{2+}]},$$

where K_{Ca} and K_{Mg} are the stability constants of the calcium and magnesium complexes of L-glutamate respectively, and $[Ca^{2+}]$ and $[Mg^{2+}]$ are the free concentrations of the divalent cations. In these experiments the concentrations of the divalent cations greatly exceeded the concentrations of the amino acids and thus the total divalent cation concentrations approximated to the concentrations of these ions in the free form. Using the stability constants of Dawson, Elliott, Elliott & Jones (1969) one can show that about 31 % of the glutamate and 13 % of the aspartate will be in the ionized form in our crab saline.

Samples of transmitter noise were 'captured', examined and subjected to spectral analysis as described previously (Crawford & McBurney, 1976*a*, *b*). In most experiments the variance of noise signals was continuously monitored using an especially constructed variance meter. This device performed the operation described by the following equation

$$V_{\mathrm{out}} = \frac{1}{T}\int_0^T [V_{\mathrm{in}}(t)]^2.\mathrm{d}t,$$

where V_{out} is the output voltage of the device, T is the integration time (usually 1 sec) and V_{in} is the noise signal after amplification and removal of any mean changes in the signal. Removal of the mean component was achieved using a high-pass filter (roll-off 12 db/octave) with variable 3 db point. Since changes in the focally recorded potentials occurred over periods of 30 sec to 1 min when junctions were superfused with excitatory amino acids, the 3 db point was usually set at $\frac{1}{2}\pi$ Hz. This filtering process removes less than 1 % of the variance of the signal because the synaptic noise is distributed evenly up to frequencies of at least 50 Hz. After removal of the mean the signal was squared (using a 1 sec multiplier type XR-2308) and integrated over a period (integrator time constant 1 sec) using a switching circuit timed by a type 555 timer (R. S. Components). The integrator was re-set to zero at the end of each integration. Examples of the output of the variance meter led directly to a penwriter are given in Figs. 1 and 2. Variances obtained in this way were checked against values obtained by standard computations on digitized samples of the noise. All spectral densities were calculated as single-sided spectral densities and normalized to the zero frequency spectral density ($G(0)$) for ease of comparison.

RESULTS

The synergistic action of L*-glutamate and* L*-aspartate*

The currents flowing at an excitatory neuromuscular junction during the application of excitatory amino acids can be followed by a glass micropipette placed at the focus of the convergent currents. Focally recorded extracellular potentials appear as in Figs. 1 and 2. The synergistic action of glutamate and aspartate described in lobster preparations by Kravitz *et al.* (1970) and Shank & Freeman (1975) can be demonstrated in a number of ways. For instance, in Fig. 1 the application of 20 μM glutamate to a spot produced little change in the extracellular potential, whereas the application of 0·5 mM aspartate caused a potential change of about 125 μV and the simultaneous presence of these amino acids caused a potential change of about 250 μV.

The focal recorded potentials are unlikely to be distorted by a reduction of the driving force on ion movement caused by depolarization of the muscle because in *Maia* giant muscle fibres superfusion of junctions with amino acids at these concentrations produces only a few millivolts reduction in the membrane potential (Crawford & McBurney, 1976 *b*). Essentially similar results can be obtained by intracellular recording with a KCl filled micropipette impaling the muscle fibre, although the time courses of the potential changes are distorted by lateral diffusion of amino acids to adjacent excitatory junctions on the muscle fibre. In this preparation the 'electrode artifact' described by Katz & Miledi (1973) at frog neuromuscular junctions does not seem to be present; miniature excitatory junctional currents (min.e.j.c.s) are not prolonged by pressure of the electrode on the surface of the muscle, probably because crustacean excitatory neuromuscular junctions lie within the cleft system of the muscle (Jahromi & Atwood, 1974) at some distance from the focal electrode.

One question that immediately arises is whether the order in which the amino acids are applied affects the extent of the synergistic action. The experiments illustrated in Fig. 2, both from the same neuromuscular junction, indicate that this does not seem to be the case. In Fig. 2*A*

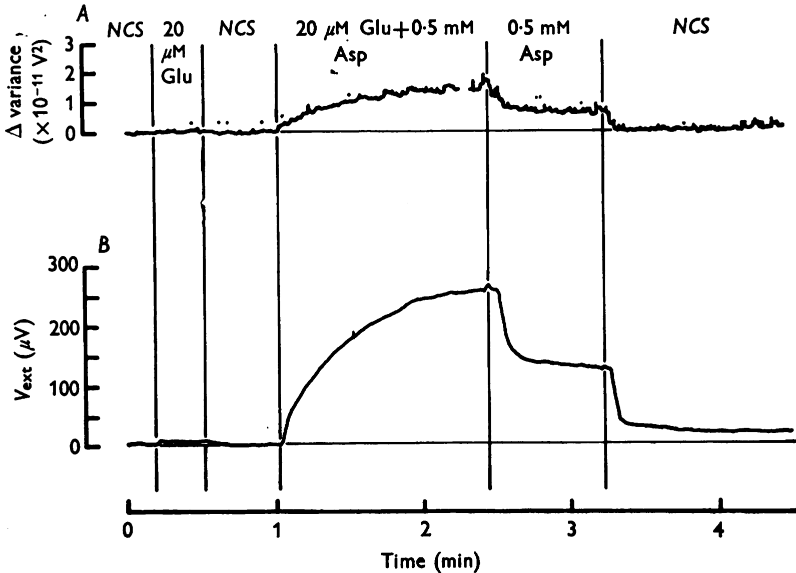

Fig. 1. Responses of an excitatory neuromuscular junction to the application of L-glutamate and L-aspartate. Amino acids were applied by microsuperfusion of the recording spot. *A*, shows the change in variance of the extracellular potential (Δ variance, $\times 10^{-11}$ V^2) as a function of time (min). Changes in variance are measured by the variance meter with a 1 sec integration time (see Methods). Dots above the record indicate points at which miniature excitatory junction at potentials occurred. Measurements of the noise variance have been rejected at these places. *B*, the extracellular potential (V_{ext}) measured simultaneously with the variance as a function of time (min). Vertical lines indicate timing of solution changes. *NCS*, normal crab solution. Note that 20 μM glutamate alone produces little change in the extracellular potential or its variance but when combined with 0·5 mM aspartate a potentiated response occurs. Temperature 22° C.

glutamate is applied before aspartate and in Fig. 2*B* aspartate before glutamate, yet the final levels of extracellular potential are similar in the two cases, when both amino acids are present. Similar observations were made in seven other experiments at three different excitatory junctions.

The extent of the synergistic action can be expressed in a number of ways that may, or may not, be meaningful depending on the mechanism of synergism. For instance, one can define a potentiation of the glutamate response (P_G) as

$$P_G = V_{G+A}/V_G, \quad (1)$$

the inverse ratio of the extracellular potential change produced by a concentration of glutamate (V_G) to the potential change that occurs when the same concentration of glutamate is applied in the presence of aspartate (V_{G+A}). On this basis the synergistic effect depends on the concentration of the amino acids and P_G ranged up to a value of 135 (50 μM) glutamate, 1 mM aspartate). In seven measurements on four junctional spots using 20 μM glutamate and 0·5 mM aspartate, P_G assumed a mean value of

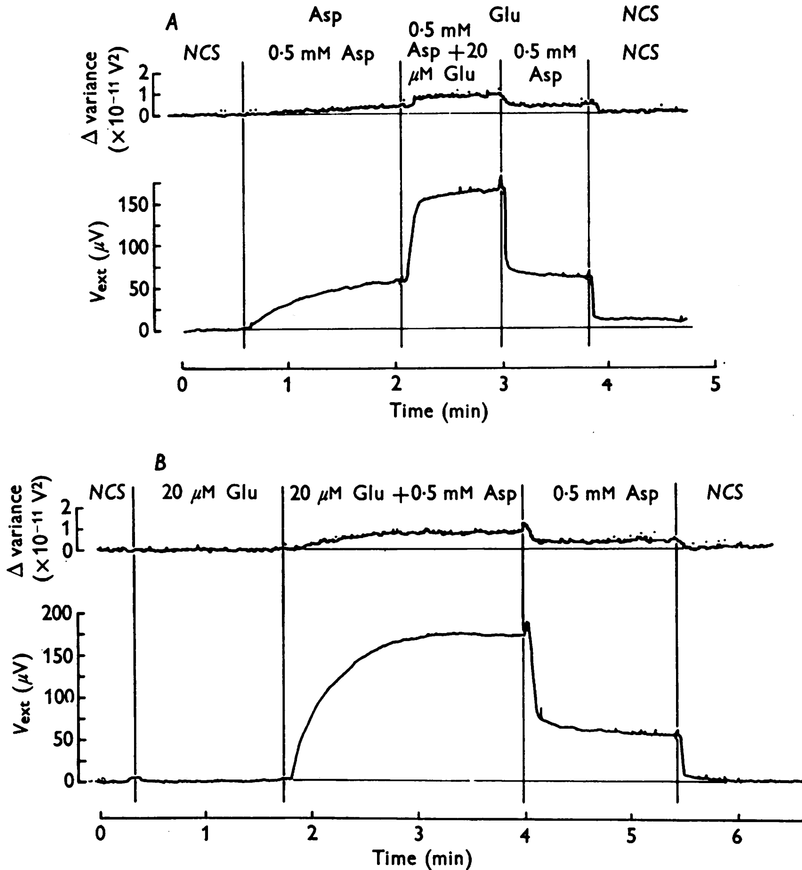

Fig. 2. Synergistic action of L-glutamate and L-aspartate. In both *A* and *B* the upper trace gives the variance of the focally recorded extracellular potential ($\times 10^{-11}$ V^2) while the lower trace is the extracellular potential (V_{ext}) both as a function of time (min). All records from the same spot at 22° C. Solution changes are indicated by the vertical lines. *NCS*, normal crab solution. Note that changes in the variance parallel changes in the extracellular potential and that the order in which aspartate and glutamate are applied does not affect the final level of the potential in the presence of 20 μM-glutamate and 0·5 mM-aspartate.

23·0 ± 1·9 (S.E. of mean). In the presence of aspartate (1 mM) current could be detected at excitatory junctions to concentrations of glutamate as low as 2 μM. Below this the records contained too much aspartate 'noise' (Crawford & McBurney, 1976*a*) to detect changes in the extracellular potential. In the absence of aspartate the smallest response to glutamate that could be detected was about 5 μV: in the presence of aspartate the minimum detectable response to glutamate was after an order of magnitude larger than this. These results are consistent with those found by Shank & Freeman (1975) and Shank *et al.* (1975) in lobster.

Noise during the synergistic action of the amino acids

Whenever aspartate or glutamate are present at *Maia* excitatory neuromuscular junctions, fluctuations of the focally recorded extracellular potential about its mean value can be recorded. This 'noise' probably arises from the discontinuous nature of the conductance change in a way similar to acetylcholine noise at frog neuromuscular junctions (Katz & Miledi, 1972; Anderson & Stevens, 1973).

Changes in the variance of the signal are given in Figs. 1 and 2 as the continuous output of the variance meter (see Methods).

It is clear that the variance of the noise recorded by a focal electrode follows the same time course as the change in the mean extracellular potential. Indeed at all times during the application of glutamate and aspartate there exists a linear relationship between the extra variance induced by the amino acids and the magnitude of the extracellular potential change. This has been demonstrated previously for glutamate or aspartate alone (Crawford & McBurney, 1976*a*).

The characteristics of the noise occurring during the synergistic action of aspartate and glutamate are summarized in Tables 1 and 2 and in the power spectra of Fig. 3.

We have restricted our attention to those ratios of glutamate and aspartate that gave large potentiations. This proved rather difficult to arrange because the access of the amino acids to the excitatory membrane can be restricted by the placement of the focal electrode on the neuromuscular junction and thus the concentrations needed to obtain maximum potentiation were rather variable from experiment to experiment. The spectral properties of the noise occurring during large potentiations were however quite consistent. Spectra were obtained for samples of noise occurring during the application of the two amino acids. The spectrum of the base-line noise in the recording system was subtracted as described previously (Crawford & McBurney, 1976*a, b*).

Fig. 3*A* shows the spectrum of the noise produced in the presence of the

two amino acids during an experiment of the type shown in Fig. 2*A*. When the potentiation was large, the spectra were always well described by a single Lorentzian of the form

$$G(f) = \frac{G(0)}{1+(f/f_{\frac{1}{2}})^2},$$

where $G(f)$ is the power of the focally recorded voltage noise (V^2/Hz) at frequency f, $G(0)$ the power at zero frequency, and $f_{\frac{1}{2}}$ is the half-power

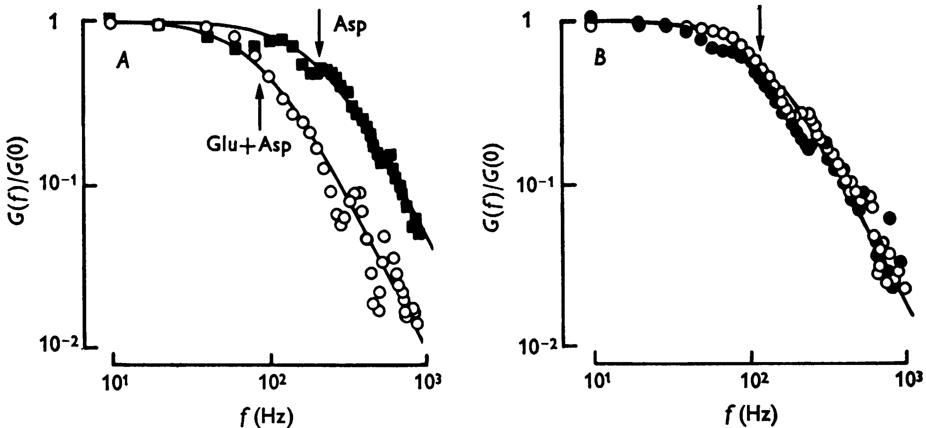

Fig. 3. Power spectra of synaptic noise. Ordinates; power of the extracellular voltage noise at frequency, f ($G(f)$) normalized to the power at zero frequency ($G(0)$). Abscissae; frequency (Hz). *A*, spectra obtained in 10 mM aspartate alone (filled squares) and in 10 mM aspartate plus 50 μM glutamate (open circles) at the same synaptic spot. *B*, spectra obtained in 20 μM glutamate plus 0·5 mM aspartate (open circles) and in 0·5 mM glutamate alone (filled circles) again at the same synaptic spot. Lines drawn through the points are Lorentzians of the form $G(f)/G(0) = [1+(f/f_{\frac{1}{2}})^2]^{-1}$ with half-power frequencies ($f_{\frac{1}{2}}$) as indicated by the arrows. Values of $G(0)$ and total variance are respectively: *A*, open circles $1{\cdot}44 \times 10^{-13}$ V² sec and $3{\cdot}78 \times 10^{-11}$ V²; filled squares $4{\cdot}17 \times 10^{-14}$ V² sec and $1{\cdot}23 \times 10^{-11}$ V²; *B*, open circles $3{\cdot}51 \times 10^{-14}$ V² sec and $5{\cdot}48 \times 10^{-12}$ V²; filled circles $6{\cdot}57 \times 10^{-14}$ V² sec and $1{\cdot}26 \times 10^{-11}$ V². Temperature 22° C throughout.

frequency (Hz) of the Lorentzian. In the spectra illustrated the values of $G(f)$ have been normalized to the values of $G(0)$ in order to facilitate comparison of spectra with different values of $f_{\frac{1}{2}}$. $G(0)$ values and total variances are given in the legends to the Figures.

One major point emerged immediately; during large potentiations the spectral properties of the noise are those of glutamate *alone* despite the presence of an aspartate concentration that alone gave noise with its characteristic spectrum. This point is illustrated in Fig. 3*A*, which shows

TABLE 1. Half-power frequencies of power spectra of the noise produced by aspartate alone (f_A), of aspartate plus glutamate (f_{A+G}) and of glutamate alone (f_G) at the concentrations indicated

Expt.	[Asp] (mM)	[Glu] (μM)	f_A (Hz)	f_{A+G} (Hz)	f_G (Hz)
1	10	50	205	100	—
	10	50	205	85	—
	10	—	240	—	—
2	1	100	210	115	—
	1	100	—	130	—
	1	20	—	110	—
3	5	50	—	102	—
	5	—	210	—	—
	5	—	195	—	—
4	0·5	20	170	85	—
	0·5	20	160	110	—
5	0·5	20	190	105	—
	0·5	20	190	110	—
6	0·5	20	160	105	120
	0·5	20	—	105	—
7	0·5	20	195	105	110
	0·5	20	170	110	105
	0·5	20	190	—	92
					105
8	0·5	20	—	97	125
9	0·5	20	210	125	—
	0·5	20	195	110	—
	0·5	20	—	95	—
		Mean	193·4	105·8	109·5
		S.E. of mean	± 5·3	± 2·7	± 4·8
			(n = 16)	(n = 18)	(n = 6)

the spectrum of the noise in the presence of aspartate, and the spectrum of the noise at the same spot a few minutes later when 50 μM L-glutamate had been added. Note that the spectra are both single Lorentzians with different half-power frequencies. The aspartate noise has a value of $f_{\frac{1}{2}}$ of about 200 Hz as described previously (Crawford & McBurney, 1976*a*) yet on adding glutamate the $f_{\frac{1}{2}}$ changes to about 100 Hz, a value similar to that for the spectrum of glutamate alone (Crawford & McBurney, 1976*b*).

Table 1 summarizes the results of nine similar experiments all using concentrations of glutamate and aspartate that gave large potentiations. Fig. 3*B* shows that the noise occurring during the synergistic action of the amino acids has a spectrum that coincides with that of L-glutamate alone;

the two spectra are obtained from the same spot within an excitatory junction.

It thus seems clear that the current produced by simultaneous application of glutamate and aspartate arises from elementary conductance events with the same average duration as those produced by glutamate since the mean duration of the elementary current event largely determines the power spectrum of focally recorded transmitter noise (Katz & Miledi, 1972).

TABLE 2. Amplitudes of the extracellular voltage events in aspartate (γ_A), aspartate plus glutamate (γ_{A+G}) and in glutamate (γ_G) at the concentrations indicated. Relative amplitudes are compared only for data from the same synaptic spot

Expt. no.	[Asp] (mM)	[Glu] (μM)	γ_A ($\times 10^{-7}$ V)	γ_{A+G} ($\times 10^{-7}$ V)	γ_G ($\times 10^{-7}$ V)	$\frac{\gamma_{A+G}}{\gamma_G}$	$\frac{\gamma_{A+G}}{\gamma_A}$
1	10	50	0·565 ($n = 3$)	0·386 ($n = 2$)	—	—	0·68
2	1	100	0·472 ($n = 4$)	0·542 ($n = 1$)	—	—	1·15
3	5	50	1·877 ($n = 3$)	2·18 ($n = 1$)	—	—	1·16
4	0·5	20	0·998 ($n = 1$)	1·016 ($n = 1$)	1·116 ($n = 1$)	0·910	1·018
5	0·5	20	—	1·612 ($n = 1$)	1·58 ($n = 1$)	1·020	—
6	0·5	20	0·395 ($n = 3$)	0·389 ($n = 2$)	0·549 ($n = 4$)	0·692	0·984
7	0·5	20	0·479 ($n = 2$)	0·611 ($n = 3$)	—	—	1·224
				Mean		0·874	1·036
				S.E. of mean		±0·096 ($n = 3$)	±0·080 ($n = 6$)

Values of half-power frequencies of spectra of aspartate noise, glutamate noise, and the noise caused by the two amino acids, are given in Table 1. The mean value of the half-power frequency (105·8 ± 2·7 (S.E. of mean) Hz) of the noise in the presence of the two amino acids corresponds well with the value for glutamate noise shown in Table 2 and described by us previously (Crawford & McBurney, 1976*b*).

If the potentiated currents do arise from extra glutamate events occurring in the synaptic membrane, then the size of the extracellular potential event should be the same as that of glutamate. Unfortunately the method of focal recording does not allow an absolute measurement of the size of

the current event but it does allow us to compare amplitudes of extracellular glutamate and aspartate events with the size of the new events occurring when both amino acids act together. Table 2 summarizes the results of seven experiments. Within the accuracy of our data there is no difference between the amplitudes of these three types of conductance events.

It seems then, that in the presence of aspartate a given concentration of glutamate is able: (i) to suppress the occurrence of elementary conductance events normally occurring in that concentration of aspartate; and (ii) produce *normal* glutamate events at a far higher rate than occurred at that glutamate concentration previously.

DISCUSSION

The mean conductance change (Δg_s) caused by any agonist of the post-synaptic receptors can be expressed as a product of the rate of occurrence (n) of successful agonist–receptor interactions and the average effect that is produced by each successful interaction. This can be stated as follows:

$$\Delta g_s = n.\gamma.\tau,$$

where γ is the average conductance of a single open channel, and τ is the average lifetime of the 'open state'. The product $\gamma.\tau$ can be termed the 'efficiency' of a single channel.

The measurement of extracellular voltage fluctuations in solutions containing both glutamate and aspartate has revealed that the potential responses are not due to an altered conductance event but are due to an increase in the rate of occurrence of events. Moreover the fact that the lifetime (τ) of the ionic channel when activated by glutamate + aspartate is the same as when activated by glutamate alone, but not by aspartate alone, suggests that the potentiation is due to an increased rate of successful glutamate-receptor interactions. Our definition of the potentiation produced by the two amino acids (P_G) given on page 700 was chosen because it reveals directly the multiplication of the number of glutamate events occurring per second during the synergistic action. This change in the rate of the glutamate–receptor interactions can be expressed so simply only because aspartate conductance events are suppressed during the simultaneous action of the amino acids.

An increase in the number of events per second could arise either by an increase in the available receptor population, or by an increase in the concentration of glutamate reaching the receptor region, or by an increase in the affinity of the receptors for glutamate. There is little evidence which helps to decide between these three possibilities.

Changes in the total population of available receptor-ionophores seems unlikely as any increase in the number of receptors would be reflected in the maximum level of the dose–response curves which presumably reflects receptor saturation. In these experiments no change was seen in the saturation level of the dose–response curves for the different conditions of activation nor was it seen in similar experiments performed by Shank & Freeman (1975).

There is a possibility that some non-activating binding of aspartate to the receptors can cause an increase in the affinity of the receptors for glutamate in a similar way to the mechanism of allosteric modification of enzyme function. Although this type of co-operative interaction has been suggested by other workers (Shank & Freeman, 1975) there is no direct evidence at present to support or reject this hypothesis.

In our view the most likely explanation is that aspartate allows an increase in the glutamate concentration in the region of the synaptic receptors. It is known that aspartate, at the concentrations used in these experiments, is a potent blocker of the glutamate uptake system in *Maia* nerve (Baker & Potashner, 1971) and it may be the case that such an uptake system prevents the ready access of externally applied glutamate to the synaptic regions especially for those junctions occurring deep within infoldings in the surface membrane of these muscle fibres. In the presence of a blocked uptake system, a higher concentration of glutamate might be achieved in the region of the receptors. This would result in an apparent shift to the left of the dose–response curve for the glutamate–receptor interaction (as found by Shank & Freeman, 1975) but would merely represent a shift in the concentration of glutamate in the receptor regions towards the applied concentration. An indication that there might be a barrier to the access of glutamate to the synaptic region is that the onset of responses to glutamate alone is considerably slower than the onset of responses to glutamate in aspartate (A. C. Crawford and R. N. McBurney, unpublished).

The suppression of the occurrence of aspartate events by glutamate is perhaps not surprising. Several authors have reported that much higher concentrations of aspartate than glutamate are necessary to produce conductance changes in the excitatory junctional membrane of crustacea (Takeuchi & Takeuchi, 1964; Shank & Freeman, 1975), and these results have been interpreted as reflecting different affinities of amino acids for the excitatory receptor. If this view is correct then the reduction in the rate of aspartate conductance events produced by glutamate might simply reflect the successful competition of a strong agonist for the receptor over an agonist with a lower binding constant. Such a simple competition would of course be dose-dependent and we have noticed that when aspartate and

glutamate are applied at concentrations that produce a small potentiating effect, spectra of the noise in the presence of both amino acids do show extra power at high frequencies that can be accounted for by adding a small Lorentzian component with the characteristics of aspartate noise.

In a previous paper (Crawford & McBurney, 1976*b*) we have shown, for the junctional regions on *Maia* muscle fibres, that the time constant of the decay phase of miniature excitatory junctional currents can be predicted from the duration of the elementary event produced by L-glutamate. This observation implies that the transmitter concentration achieved in the synaptic cleft by the release of a quantum of glutamate, decays very quickly compared to the average lifetime of the elementary glutamate event (about 1·5 msec). It seems possible that the glutamate removal system that we think underlies the synergistic action of aspartate and glutamate may actually be a mechanism for the rapid inactivation of quantally released transmitter, performing a similar role to the acetylcholinesterase at vertebrate neuromuscular junctions. In the following paper evidence is presented to support this view.

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