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RESPONSES TO DL-IBOTENIC ACID AT LOCUST GLUTAMATERGIC NEUROMUSCULAR JUNCTIONS

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1 The responses of excitatory junctions on locust skeletal muscle fibres to iontophoretically applied L-glutamic acid and DL-ibotenic acid, a rigidly extended analogue of glutamate, were recorded by means of intracellular microelectrodes.

2 Iontophoresis of L-glutamate to junctional sites produced transient depolarizations. Ibotenate applied iontophoretically to these sites usually evoked small hyperpolarizations which probably resulted from the activation of glutamate H-receptors on the extrajunctional membrane surrounding the junctions. However, at a minority ($\sim 20\%$) of junctions, ibotenate iontophoresis evoked transient depolarizations.

3 Iontophoretically applied glutamate desensitized the ibotenate receptors, and *vice versa*. In experiments performed at junctional sites at which ibotenate depolarizations were absent, ibotenate had no effect on the responses to glutamate.

4 Glutamate and ibotenate junctional currents had similar reversal potentials, measured under voltage-clamp, suggesting that the ionic bases for these currents are identical.

5 It is proposed that the excitation caused by ibotenate results from the activation of receptors for extended L-glutamate and that these receptors co-exist on the post-junctional membranes of locust excitatory nerve-muscle synapses with ibotenate-insensitive glutamate receptors activated by glutamate in partially folded conformation.

Introduction

L-Glutamate applied iontophoretically to excitatory junctions on locust muscle fibres evokes transient depolarizations of the muscle membrane (Beranek & Miller, 1968; Usherwood & Machili, 1968). Application of glutamate to the extrajunctional muscle membrane produces biphasic responses which result from the simultaneous activation of two pharmacologically distinct types of receptors, designated D and H (Usherwood & Cull-Candy, 1974; Cull-Candy, 1976).

Lea & Usherwood (1973a) reported that ibotenic acid, a rigidly extended analogue of glutamic acid, produces a transient increase in the conductance of locust muscle fibres. The associated hyperpolarization is mediated mainly by an increase in Cl^- influx across the muscle fibre membrane (Lea & Usherwood, 1973b), with perhaps K^+ making a minor contribution (Clark, Gration & Usherwood, 1979). The effect of ibotenic acid results from the activation of L-glutamate H-receptors on the extrajunctional membrane (Lea & Usherwood, 1973a; Cull-Candy & Usherwood, 1973; Cull-Candy, 1976). Extrajunctional D-receptors for L-glutamate are not activated by ibotenate.

The effect of ibotenate on mammalian spinal neurones is mainly excitatory (Johnston, Curtis, De Groat & Duggan, 1968; Johnston, Curtis, Davies & McCulloch, 1974) but when applied to dorsal horn interneurones of the cat this compound both excites and depresses these cells (McDonald & Nistri, 1978).

Although it has been suggested that ibotenate does not activate postsynaptic receptors at insect excitatory nerve-muscle junctions, we have recently discovered receptors at some excitatory junctions on locust leg muscle fibres which are activated by this isoxazole and lead to a depolarization of the postsynaptic membrane. Some of the properties of these junctional ibotenate receptors are the subject of this paper.

Methods

All results described here were obtained from the metathoracic extensor tibiae muscle of the locust, *Schistocerca gregaria*. The muscle was exposed by removing the cuticle on the ventral side of the femur, followed by dissection of the overlying flexor tibiae

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muscle. Although a few experiments were done on muscle fibres which receive both excitatory and inhibitory innervation, most results were obtained from superficial fibres in the mid-region of the extensor tibiae muscle which receive only excitatory innervation (Cochrane, Elder & Usherwood, 1972; Hoyle, 1978). In some experiments denervated muscles were used. These were obtained by sectioning metathoracic nerves 3 and 5, which innervate the extensor muscle, using an aseptic technique (Usherwood, 1963). Operated animals were maintained in separate containers at 30°C and fed fresh grass daily.

Preparations were continuously superfused at room temperature (18° to 20°C) with either standard saline, which contained NaCl 180, KCl 10, $CaCl_2$ 2, Na_2HPO_4 6, NaH_2PO_4 4 mM, (pH 6.8) or Cl-free saline, $NaCH_3SO_4$ 180, KCH_3SO_4 10, $(C_2H_5COO)_2Ca$ 2, Na_2HPO_4 6, NaH_2PO_4 4 mM, (pH 6.8). Single- and double-barrelled micropipettes (resistance 100 to 200 MΩ in standard saline) were used for the iontophoresis of glutamate (0.1 M Na L-glutamate in distilled water, pH 7.0) and ibotenate (0.1 M DL-ibotenic acid in distilled water, pH 7.0.). When double-barrelled micropipettes were used they were checked for possible interactions between barrels during iontophoresis. Furthermore the results of experiments in which double-barrelled electrodes were used were checked with two single-barrelled electrodes. Backing currents in the range +3 to +20 nA between ejections were required to obtain optimum responses with both double- and single-barrelled pipettes. Intracellular recording electrodes were filled with 2 M tripotassium citrate and had resistances of 5 to 15 MΩ. Recordings were made by use of conventional electrophysiological techniques. A two-electrode clamp circuit was employed for voltage-clamp experiments (Anwyl, 1977).

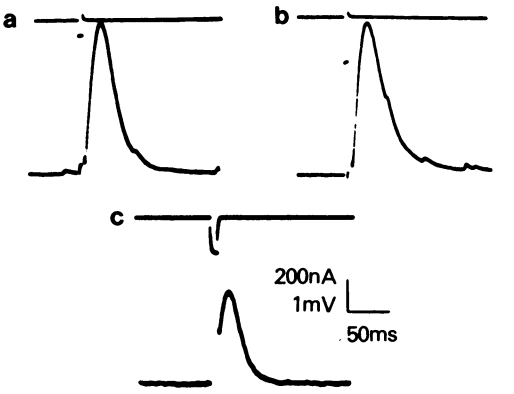

Figure 1 (a–b) Comparison of a glutamate depolarization (a) and an ibotenate depolarization (b) evoked by iontophoresis of these compounds onto the same area of excitatory junctional membrane on a locust muscle fibre (membrane potential, −56 mV). Note that a larger coulombic dose [upper traces in (a) and (b)] was used to evoke the ibotenate response. Miniature excitatory postsynaptic potentials can be seen on the intracellular records. (c) A depolarization evoked by iontophoresis of ibotenate to a localized site on a denervated muscle fibre. The fibre had been denervated for 18 days at 30°C. Note the absence of miniature excitatory potentials on the intracellular record, indicating that the nerve terminals had degenerated (Rees & Usherwood, 1972; Usherwood, 1973).

Results

Comparisons of the responses to iontophoretically applied glutamate and ibotenate

The iontophoresis of glutamate to junctional sites produced large transient depolarizations (see Figure 1a). A comparison was made of the responses to glutamate and ibotenate at 80 junctional sites involving 16 muscle fibres of 9 preparations. The iontophoresis of ibotenate to muscle fibres bathed in standard saline evoked hyperpolarizations from the majority of juctions, probably through the activation of surrounding extrajunctional H-receptors. However, depolarizations (Figure 1b) were recorded from a minority (~ 20%) of junctions during ibotenate iontophoresis. When the ibotenate-sensitivity at these sites was mapped, the area of peak ibotenate-sensitivity was found always to coincide with that for L-glutamate (Figure 2). A comparison of the distribution of ibotenate depolarizations with that of junctional glutamate responses showed that ibotenate-sensitive and -insensitive junctions occurred on the same muscle fibre. There was considerable variation in ibotenate-sensitivity between sites, the relative potency of glutamate and ibotenate ranging from 50:1 to 5:1. The lower potency of ibotenate does not necessarily imply a lower affinity for the receptors since the ibotenate used in this study was a racemic mixture of the D- and L-isomers. A further complication is that, unlike that of glutamate, the action of ibotenate may not be influenced by glutamate uptake sites (James, 1977). The iontophoresis of ibotenate onto denervated muscle fibres also evoked depolarizations, which were similar in amplitude to those recorded from junctions on innervated fibres. Figure 1c shows a depolarization evoked from an 18-day denervated muscle fibre. The response was obtained from a localized area of muscle membrane, which had a high sensitivity to glutamate and which was probably a former junctional site. We have no evidence for a denervation-induced 'spread' of ibotenate depolarizing receptors onto extrajunc-

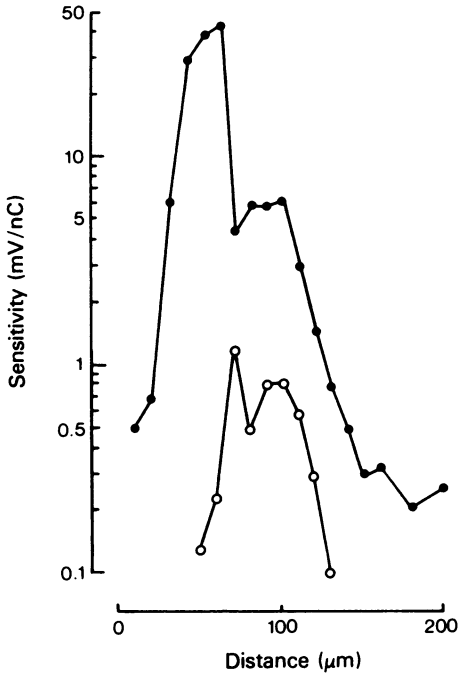

Figure 2 A comparison of the distributions of L-glutamate (●) and ibotenate (○) junctional sensitivities. Responses were obtained with a double-barrelled micropipette which was moved in 10 μM steps over the surface of the muscle fibre in the region of a nerve-muscle junction. The sensitivity at each point was measured in terms of peak depolarization per unit dose of ibotenate or glutamate (mV/nC). Note that the sensitivity scale is logarithmic. Note also the bimodal distribution of glutamate sensitivity over this restricted area of muscle membrane, suggesting that the iontophoretically applied ligands activated two junctions which were very close together.

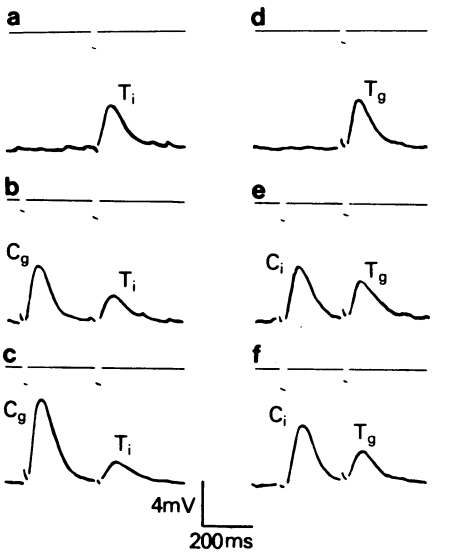

Figure 3 (a–c) The effect of a 'conditioning' dose of L-glutamate on the amplitude of a 'test' (T_i) ibotenate depolarization (b,c.). The glutamate response (C_g) was evoked 300 ms before that of ibotenate. Glutamate doses: (b) 3.2 nC; (c) 4.4 nC. The T_i response, in the absence of glutamate, is shown in (a); ibotenate dose, 5 nC. (d–f) The converse experiment showing the effect of a 'conditioning' dose of ibotenate on the 'test' glutamate depolarization (T_g) (d,e). The ibotenate response (C_i) was evoked 250 ms before that of glutamate. Ibotenate doses: (e) 6.0 nC; (f) 8.0 nC. The T_g response in the absence of ibotenate is shown in (d); glutamate dose, 3 nC.

tional membrane although we have not studied this thoroughly.

Cross-desensitization studies

The occurrence of mixed populations of ibotenate and glutamate receptors on the postjunctional membrane of locust muscle fibres raises the possibility that glutamate may also act upon the ibotenate receptor population. This possibility was tested by determining whether iontophoretically applied L-glutamate influenced, through desensitization, the depolarizations to DL-ibotenate and vice versa.

Ibotenate and glutamate were applied iontophoretically from either two single-barrelled micropipettes, positioned at the same site, or from double-barrelled micropipettes. The experiment illustrated in Figure 3a–c shows the effect of varying the dose of a 'conditioning' pulse of glutamate on the response of the same membrane site to the following 'test' pulse of ibotenate. The amplitude of the ibotenate response was reduced by the conditioning pulse of glutamate. The degree of desensitization was dose-dependent with a 4 nC dose of glutamate giving a 50% reduction in the amplitude of the ibotenate response at this site. The effect of a 'conditioning' dose of ibotenate on a following glutamate 'test' response is illustrated in Figure 3d–f. Dose-dependent cross-desensitization is again evident. In experiments performed at junctional sites at which ibotenate depolarizations were absent a large (8 nC) conditioning dose of ibotenate had no effect on test responses to glutamate. Similarly the amplitude of extrajunctional D-responses to a test dose of L-glutamate was unaffected by a conditioning dose of ibotenate.

Reversal potentials of junctional ibotenate and glutamate currents

The reversal potential of junctional ibotenate and glutamate currents was determined by voltage-clamp.

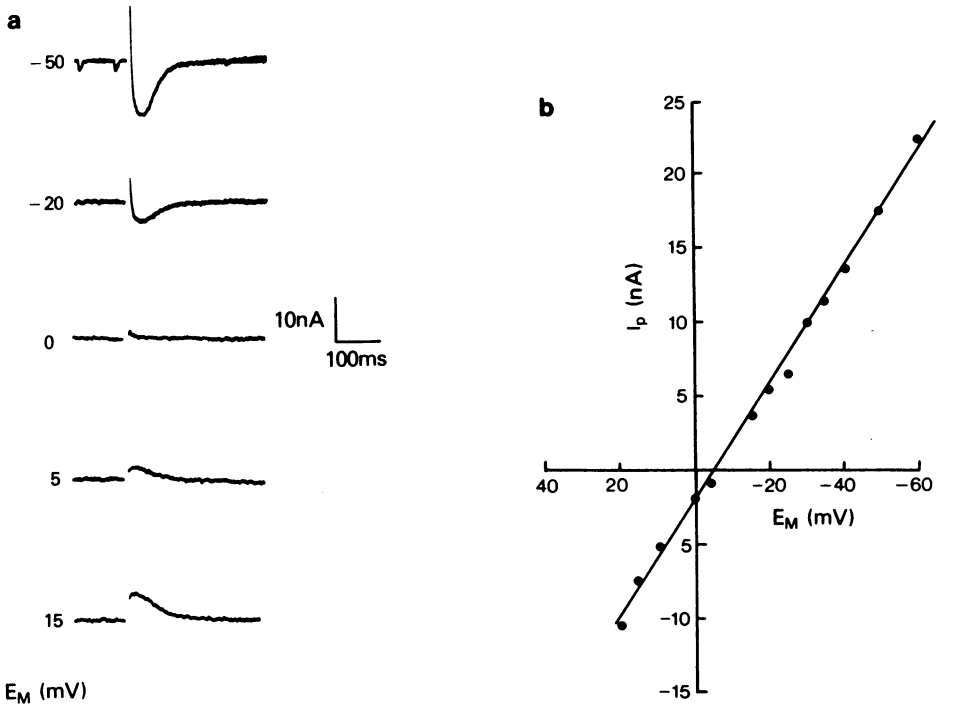

Figure 4 (a) The effect of membrane potential on the amplitude and polarity of junctional ibotenate currents recorded under voltage-clamp from a locust muscle fibre with a resting potential of −56 mV. The ibotenate dose was 3 nC. Note the miniature postsynaptic currents on the record obtained at a membrane potential of −50 mV. (Inward current shown by downward deflections on records.) (b) Relationship between membrane potential (E_m) and peak clamp current (I_p) of a junctional ibotenate response for a fibre with a resting potential of −57 mV. Inward currents are shown positive. The line is a linear regression fit to the data. The reversal potential of the ibotenate current (determined by linear interpolation) was −5 mV. The muscle fibre was bathed in Cl-free saline to abolish extrajunctional H-currents.

These studies were made in Cl-free saline to avoid involvement of extrajunctional H-currents which might have been activated by glutamate and ibotenate iontophoresis (Lea & Usherwood, 1973a). With the membrane potential clamped at the resting level (−50 to −60 mV), ibotenate iontophoresis produced peak inward currents of up to 40 nA for doses of 2 to 3 nC. When the membrane was clamped at more depolarized levels, the amplitude of ibotenate currents decreased, and at a clamped potential of near 0 mV the polarity of the current reversed (Figure 4a). Reversal potentials were determined from plots of peak current versus clamped membrane potential (Figure 4b). Within the range of potentials employed, the relationship between current and clamped potential was approximately linear and reversal potentials were determined by linear interpolation. The mean (±s.d.) reversal potential for junctional ibotenate inward currents was -1.6 ± 3.3 mV ($n = 7$, data from different fibres). For junctional glutamate responses the mean reversal potential was -1.7 ± 5.3 mV ($n = 7$, data from different fibres). Junctional responses to glutamate result from a membrane permeability increase to Na and K and possibly also Ca (Anwyl & Usherwood, 1974). The similar reversal potentials for the ibotenate and glutamate currents at junctional sites implies that the ionic bases for these currents are probably identical.

Coulomb dose–response relationship

Double logarithmic plots of the Coulomb dose-response relationships for the action of ibotenate and glutamate on extrajunctional H-receptors have mean slopes which differ significantly from those for the action of glutamate on junctional receptors (Cull-Candy, 1976). It was of interest therefore, to examine the Coulomb dose–response relationships for the interaction of ibotenate with receptors on the junctional membrane.

Typical ibotenate potentials, evoked by increasing iontophoretic doses applied to a junctional area on

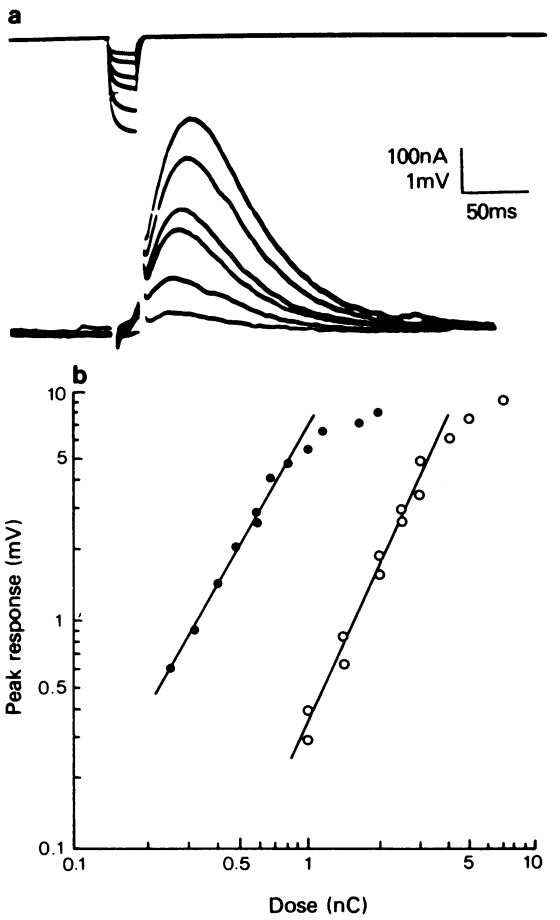

Figure 5 (a) Superimposed membrane depolarizations (lower trace) of a locust muscle fibre evoked by increasing doses (upper trace) of ibotenate delivered to an excitatory junctional site by an iontophoretic micropipette. Muscle fibre resting potential was −53 mV. The progressive increase in the time to peak probably results from the saturation of the receptor population nearest the micropipette tip and the increasing contribution, with dose, from more distant receptors (Del Castillo & Katz, 1955). (b) Double logarithmic coulombic dose–response relationships for a junctional ibotenate depolarization (○) and a junctional glutamate depolarization (●) recorded from the same site on a locust muscle fibre. The limiting slope (continuous line) is 2.3 for the ibotenate plot and 1.85 for the glutamate plot.

a muscle fibre are shown in Figure 5a. Plotted on double logarithmic co-ordinates, however, the dose–amplitude relationship was linear over most of the range of ibotenate doses used (Figure 5b). The mean value of the limiting slope of the double logarithmic plots was 2.3 ± 0.6 ($n = 6$, data obtained from six different muscle fibres). This is very similar to values obtained from double logarithmic plots for the interaction of glutamate with junctional receptors (see Figure 5b) which range from 1.8 to 3.0 (Walther & Usherwood, 1972; Anwyl, 1977).

Discussion

Previous structure-activity studies with glutamate and ibotenate, a conformationally restricted analogue of glutamic acid, have indicated the occurrence of two distinct types of glutamate receptor on locust skeletal muscle fibres (Lea & Usherwood, 1973a; Cull-Candy & Usherwood, 1973; Usherwood & Cull-Candy 1974; Cull-Candy, 1976). One type of receptor is activated by ibotenate but the other is insensitive to this drug. It has been suggested previously that the ibotenate-sensitive extrajunctional receptors on insect muscle are associated exclusively with Cl ionophores and that the excitatory junctional and extrajunctional receptors for glutamate are insensitive to ibotenate (Lea & Usherwood, 1973a; Cull-Candy, 1976). The results from the present study, while confirming that extrajunctional D-receptors on locust muscle fibres are insensitive to ibotenate, suggest the occurrence of excitatory ibotenate receptors at some excitatory junctional sites on these fibres. The similar reversal potentials of the junctional ibotenate and glutamate currents suggests that the ibotenate receptors at these sites are associated with ionophores for Na and possibly K, i.e. the changes in membrane permeability are similar to those caused by the activation of junctional glutamate receptors (Anwyl & Usherwood, 1974; Anwyl, 1977). An alternative explanation of our results is that the ibotenate depolarizations result from an ibotenate-induced release of endogenous glutamate, possibly from presynaptic sites on the excitatory nerve terminal. However, this is an unlikely explanation since depolarizations to ibotenate have been recorded from muscle fibres denervated for 18 days, at a time when the nerve terminals have degenerated (Rees & Usherwood, 1972). This does not, of course, exclude the possibility of ibotenate-induced release of glutamate from some store unaffected by degeneration of the excitatory input to the muscle. A study of ibotenate and glutamate 'noise' associated with the junctional depolarizations should resolve this point.

The junctional glutamate receptors which are ibotenate-sensitive apparently co-exist on the same muscle fibre with ibotenate-insensitive, junctional glutamate receptors. Although both receptor types seemingly cause identical changes in cation permeability, a comparison of the molecular structures of L-ibotenic acid and L-glutamic acid suggest that they may be activated by different conformations of L-glutamate. From the Dreiding molecular models of ibotenate

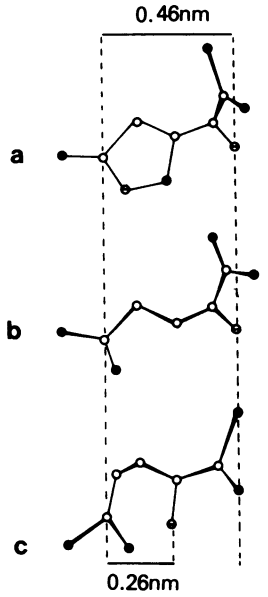

Figure 6 Carbon backbone models for L-ibotenic acid (a) and L-glutamic acid (b,c). Extended L-glutamate (b) complements the molecular conformation of ibotenate (a), but partially folded L-glutamate (c) complements neither (a) nor (b). Key: (●) oxygen; (○) carbon; (⊖) nitrogen.

and glutamate illustrated in Figure 6 it is clear that there is unique juxtaposition of the equivalent ionisable groups in ibotenate and the extended form of L-glutamate but that partly folded L-glutamate complements neither of these molecules. It is thus proposed that the excitation caused by ibotenate results from the activation of receptors for extended L-glutamate and in this respect, but not in respect of the ionophores that they gate, the excitatory junctional ibotenate receptors are similar to the extrajunctional H-receptors for L-glutamate found on locust muscle fibres which are also activated by ibotenate (Lea & Usherwood, 1973b).

Lea & Usherwood (1973a) showed that changes in the amplitude of the excitatory postsynaptic potential of locust leg muscle fibres seen during bath-application of ibotenate could be accounted for by the increased input conductance resulting from extrajunctional H-receptor activation. This suggests that the ibotenate receptors found at junctional sites are not involved in neuromuscular transmission. An alternative possibility is that ibotenate-sensitive receptors at junctional sites form only a small fraction of the total population of postjunctional receptors at the many excitatory junctions on a multiterminally innervated muscle fibre.

There is no obvious difference in the Coulomb dose-response relationship for junctional ibotenate and glutamate responses. However, both differ significantly from the relationships observed for the interaction of ibotenate and glutamate with extrajunctional H-receptors (Cull-Candy, 1976). This may arise partly from differences in population density between the junctional and extrajunctional ibotenate receptors.

The discovery that the post-junctional receptor populations at excitatory synapses on locust muscle fibres are not homogeneous with respect to their glutamate conformational requirements is surprising. Although the pharmacological data suggest that the ibotenate receptors which gate membrane depolarizations are restricted to a minority of excitatory junctions on locust muscle, further studies are required firmly to establish this point. Also, it would be worthwhile to determine whether the ibotenate-sensitive junctions are systematically distributed on fibres of the extensor tibiae and other muscles of the locust leg.

Pullman & Berthod (1975) predict that γ-aminobutyric acid (GABA), in solution, exists in a large number of conformations showing different degrees of folding. If glutamate behaves similarly at the locust nerve-muscle junctions, the presence of sub-populations of receptors with binding requirements ranging from partly folded to fully extended glutamate might maximize transmission efficiency at these sites. The efficiency of the system would be further enhanced by a receptor sub-population for the fully folded conformation of glutamate. Current studies of the effect of aspartate on locust excitatory nerve-muscle junctions should provide information on this point.

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