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Agonists, antagonists and modulators of excitatory amino acid receptors in the guinea-pig myenteric plexus

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1 The receptors for glutamic acid (L-Glu) present in the guinea-pig myenteric plexus-ileal longitudinal muscle preparation have been studied by measuring the muscle contraction induced by numerous putative endogenous agonists acting at these receptors. Furthermore, the actions of different concentrations of antagonists, glycine, Mg^{2+} and Ca^{2+} on the ileal contractions induced by L-Glu have been evaluated.

2 The EC_{50} values of the most common putative endogenous agonists of these receptors were: L-Glu 1.9×10^{-5} M; L-aspartate 8×10^{-5} M; quinolinate 5×10^{-4} M; L-homocysteate 1.4×10^{-4} M; the dipeptide aspartyl-glutamate 8×10^{-5} M, while N-acetyl-aspartyl-glutamate was inactive. Among the molecules used to classify excitatory amino acid receptors, N-methyl-D-aspartate (NMDA) was the most potent (EC_{50} 5×10^{-4} M). Kainic and quisqualic acids were almost completely inactive.

3 The responses to L-Glu were competitively antagonized by 2-amino-5-phosphonovaleric acid. They were, also, prevented by hyoscine (10^{-7} M) and by tetrodotoxin (3×10^{-7} M), suggesting that the L-Glu-induced ileal contraction was in some way dependent upon an action on the myenteric cholinergic neurones. Kynurenic acid was a non-competitive antagonist, γ-D-glutamyl-taurine (10^{-4} M) and aminophosphonobutyric acid (10^{-4} M) did not modify the L-Glu-induced contractions.

4 Glycine (10^{-5} M) significantly potentiated the effects of glutamate especially when the ionic composition of the superfusion medium contained concentrations of Ca^{2+} in the range of 0.6–1.2 mM. Strychnine 3×10^{-5} M did not modify the actions of glycine.

5 The data presented here confirm the presence of NMDA receptors in the guinea-pig myenteric plexus, and show that these receptors, similar to those present in primary neuronal cultures may be modulated by glycine.

Introduction

The guinea-pig ileum-myenteric plexus preparation *in vitro* has been extremely useful in the pharmacological characterization of receptors for neurotransmitters with agonists and antagonists (Ash & Schild, 1966; Rang, 1966; Gyang & Kosterlitz, 1966; Cook, 1971; Black *et al.*, 1972; Couture *et al.*, 1979; Giotti *et al.*, 1983). This knowledge prompted us to investigate whether or not the guinea-pig myenteric plexus contains excitatory amino acid receptors. In preliminary experiments, we showed that this preparation is sensitive to glutamate (L-Glu) and that the receptors involved are probably of the N-methyl-D-aspartate (NMDA) type. Agonist-induced stimulation of these receptors caused a contraction of the longitudinal ileal muscle which appeared to be mediated by the myenteric cholinergic neurones (Moroni *et al.*, 1986). In the present studies we further investigated the actions of numerous putative endogenous agonists and antagonists possibly acting at the NMDA receptor level. Furthermore, since it has been clearly shown that glycine significantly potentiates the electrophysiological actions of L-Glu at the level of the NMDA receptor-ion-channel complex (Johnson & Ascher, 1987; Ascher & Nowak, 1987), we studied the effects of glycine on the actions of L-Glu .

Methods

Male guinea-pigs weighing 300–500 g were used for the study. The longitudinal muscle of their ilea with

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the myenteric plexus attached was prepared according to Paton & Zar (1968) with minor modifications (Giotti et al., 1983). Strips, about 3 cm in length, weighing approximately 20 mg, were prepared from the terminal portion of the ileum and placed in a modified Krebs solution of the following composition (mM): NaCl 135, $CaCl_2$ 2.4, KH_2PO_4 1.3, $NaHCO_3$ 16.3 and glucose 7.7. Both ends of the strips were tied with cotton thread, mounted in an organ bath and the solution bubbled with a mixture of O_2 (95%) and CO_2 (5%) at 37°C. One end of the strip was attached to the bath and the other was connected to an isometric transducer under a resting tension of 0.5 g. The volume of the bath was 4 ml. During the stabilization time the tissue was continuously superfused at an approximate rate of 2 ml min^{-1}. The agonists were added to the bath dissolved in small volumes (20 or 40 μl) after temporarily closing the superfusion system. Approximately 1 min later the preparation was washed with 20–30 ml of Ringer solution by completely opening the tubing connecting the bath to a reservoir. Afterwards, the preparation was continously superfused at a rate of 2 ml min^{-1} and the flow was interrupted only shortly before adding the agonists. The antagonists were added to the superfusing medium and maintained in contact with the preparation for 15–20 min before starting the dose-response curves. When the interaction between glycine or other amino acids with L-Glu had to be studied, these compounds were added to the bath dissolved in small volumes, approximately 30 s before different concentrations of L-Glu . The responses were recorded with a polygraph.

Substances

The following substances were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.): L-glutamic acid (L-Glu), D-glutamic acid, L-aspartic acid (L-Asp), D-aspartic acid, L-glycine, N-methyl-D-aspartic acid (NMDA), quisqualic acid, quinolinic acid (Quin), kainic acid, DL-2-amino-4-phosphono-butyric acid (AP4), D-homocysteic acid, L-homocysteic acid, kynurenic acid, L-tyrosyl-glutamic acid, γ-D-glutamyl-taurine, phenylalanyl-glutamic acid, L-cysteine sulfinic acid, L-methionyl-glutamic acid, L-glutamic acid-monohydroxamate, pyroglutamic acid, γ-L-glutamyl-glutamic acid, α-L-glutamyl-alanine, γ-L-glutamyl-histidine, γ-L-glutamyl-glutamine, N-(-L-glutamyl)-phenylalanine, γ-L-glutamyl-leucine, L-α-aspartyl-glycine, L-α-aspartyl-alanine, L-tyrosyl-glutamic acid, L-tryptophyl-glutamic acid, dihydrokainic acid, ibotenic acid, hyoscine hydrobromide, acetylcholine chloride (ACh), histamine dihydrochloride, 5-hydroxytryptamine (5-HT), γ-aminobutyric acid (GABA), mepyramine, bacitracin, tetrodotoxin. DL-2-amino-5-phosphono-valeric acid (AP5) was obtained from Cambridge Research Biochemicals (Harston, U.K.). DL-2-amino-7-phosphono-heptanoic acid (AP7) and γ-D-glutamyl-glycine were obtained from Tocris Chemicals (Essex, U.K.), γ-Carboxyglutamic acid was obtained from Calbiochem-Behring (San Diego, U.S.A.). Bicuculline methiodide was obtained from Research Biochemicals Inc. (Wayland, MA, U.S.A.). Methysergide was obtained from Sandoz (Milano, Italy). N-Acetylaspartyl-glutamic (NAAG) acid was synthesized by Dr Borin (C.N.R. Padova). Aspartyl-glutamic acid was a gift from Dr Biziére (Sanofi, France); it contained less than 0.5% of both free Glu and free Asp. This was checked using GC/MS after derivatization of the amino acids (Moroni, unpublished observations).

Statistical evaluation of the data

At least four concentrations of each agonist were tested. The EC_{50} values were calculated from dose-response curves utilizing a computer programme as described by Tallarida & Murray (1984). Each response was referred to the maximal contraction evoked by Glu (3×10^{-4} M). This dose of L-Glu was applied to the preparations only at the end of each experiment. Usually when antagonists or glycine had to be tested, the first dose-response curve was terminated at a concentration of L-Glu of 10^{-4} M because higher doses of the amino acid desensitized the preparations for at least 1 h. After 10^{-4} M L-Glu, the preparations were washed for 30 min and another dose-response curve in the presence of the compounds to be tested was obtained. When a response very similar to that obtained in the first test by L-Glu 10^{-4} M was observed, the preparation was again carefully washed and 30 min later the maximal concentration (3×10^{-4} M) of L-Glu was applied. The response obtained was used for calculation purposes.

The pA_2 calculations were performed using the Schild plot (Arunlakshana & Schild, 1959) and the above mentioned computer programme. Four concentrations of the antagonists in the range 10^{-6}–10^{-4} M were routinely used.

Results

The effects of Mg^{2+} on the L-Glu-evoked responses

When the guinea-pig myenteric plexus-longitudinal ileal muscle was mounted in normal Krebs (containing $MgSO_4$ 0.6–1 mM), the addition to the bath of L-Glu 10^{-4} M caused no effect. However, after 3 h of superfusion of the preparation at a rate of

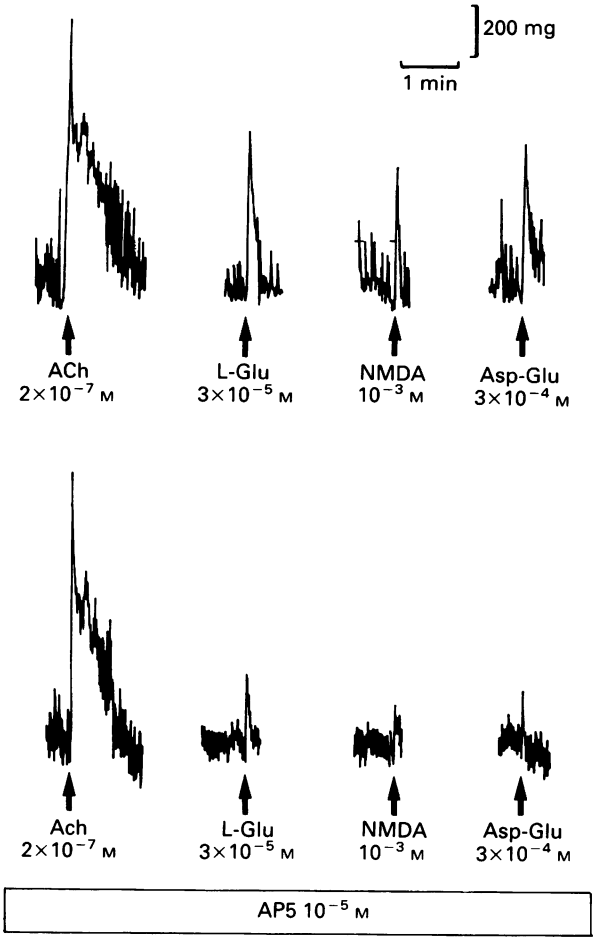

Figure 1 The differential effect of 2-amino-5-phosphonovaleric acid (AP5), on the acetylcholine (ACh), L-glutamic acid (L-Glu), N-methyl-D-aspartate (NMDA) or aspartyl-glutamate (Asp-Glu)-induced contraction of the myenteric-plexus-longitudinal muscle preparation of the guinea-pig incubated for 3 h in a Mg^{2+}-free medium. Note the difference in the concentration of the agonists tested. Calibration: tension in mg; time in min.

$2\,ml\,min^{-1}$, with a Mg^{2+}-free Krebs solution, the addition to the bath of L-Glu or other L-Glu agonists caused a dose-dependent contraction of the ileal muscle (Figures 1 and 3). The responses were reproducible and stable for at least four hours, provided the addition of agonists was not of supramaximal concentration, and was evoked not more frequently than every 15 min. This procedure, namely a preincubation for 3 h in a Mg^{2+}-free Krebs solution and a 15 min recovery after each test dose of agonist, has been used throughout. If the ileum, after the preincubation period, was exposed again to a medium con-

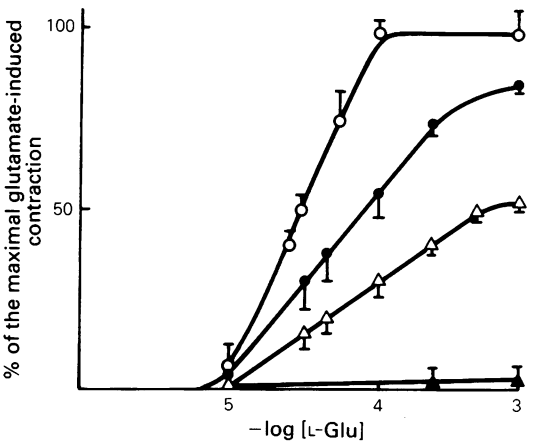

Figure 2 The effect of different concentrations of Mg^{2+} ions on the contraction of the myenteric plexus-longitudinal muscle induced by L-glutamic acid (L-Glu). The experiments shown in the figure were performed in preparations first exposed for at least 3 h to a Mg^{2+}-free Ringer solution and subsequently, for 30 min, to the following Mg^{2+} concentrations. (○) control, (●) 10^{-5} M; (△) 10^{-4} M; (▲) 10^{-3} M. Each preparation was used for two dose-response curves, one to L-Glu up to a concentration of 10^{-4} M in the absence of Mg^{2+} and the other at the given Mg^{2+} concentration (see text for details). The value for 3×10^{-4} M L-Glu was measured at the end of the experiment in Mg^{2+}-free medium. Each point is the mean of at least 5 preparations, vertical lines show s.e.mean.

taining different concentrations of Mg^{2+}, the actions of L-Glu were antagonized in a non-competitive manner (Figure 2). The maximal intensity of the ileal contraction evoked by L-Glu (3×10^{-4} M) and by the same concentration of GABA were apparently very similar. These maximal contractions reached approximately 50% those induced by histamine (3×10^{-6} M) or by ACh (10^{-6} M).

Actions of other putative agonists of excitatory amino acid receptors

In order to characterize the receptors responsible for the L-Glu-induced ileal contraction, several putative agonists of excitatory amino acid receptors were used (Table 1 and Figure 3). Among the three compounds generally used to classify these receptors in the CNS, NMDA was the most active in our test system, while kainate and quisqualate were weak or inactive. Interestingly, NMDA itself was less active than L-Glu (Figure 3). The responses to L-Glu seemed to be stereospecific, in that D-Glu (10^{-4} M) was not active. This stereospecificity was not present in the case of aspartate but was found when the two isomers of homocysteic acid were studied. Quin, another putative endogenous agonist of NMDA

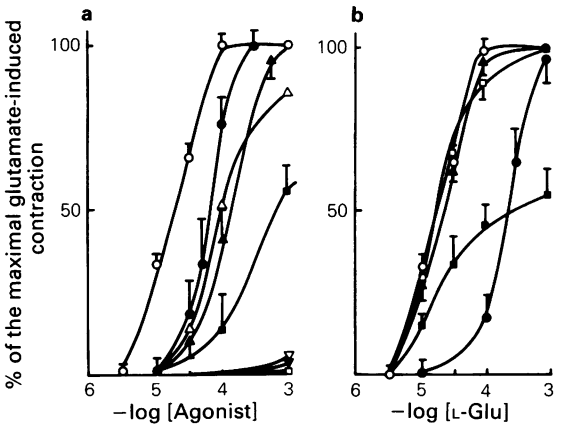

Figure 3 (a) Dose-response curves for several agonists of L-glutamic acid (L-Glu) receptors: (○) L-Glu; (●) L-aspartate; (△) aspartyl-glutamate; (▲) L-homocysteic acid; (■) *N*-methyl-D-aspartate; (▽) N-acetyl-aspartyl-glutamate; (▼) quisqualate; (□) kainate. Each preparation was used for a dose-response curve to L-Glu and to another agonist. The maximal response (100% value) to L-Glu 3×10^{-4} M was obtained at the end of each experiment. (b) Dose-response curves to L-Glu in the absence and presence of various antagonists. The antagonists were added to the superfusion medium 15 min before starting the second dose-response curve (see Methods section for details). (○) Control dose-response curve to L-Glu; (▲) DL-2-amino-4-phosphono-butyric acid 10^{-4} M; (□) γ-D-glutamyl-taurine 10^{-4} M; (●) DL-2-amino-5-phosphonovaleric acid 10^{-4} M; (■) kynurenate 10^{-4} M. Vertical lines show s.e.mean of at least 5 preparations in (a) and (b).

receptors (Stone & Perkins, 1981; Moroni *et al.*, 1984), was slightly more active than NMDA (Table 1).

A series of dipeptides containing L-Glu such as N-acetylaspartyl-glutamate, phenylalanyl-glutamate and tyrosyl-glutamate, which have been proposed as agonists or as modulators of excitatory amino acid receptors (Zaczek *et al.*, 1983; Ferkany *et al.*, 1984), did not affect the spontaneous motility or the L-Glu (3×10^{-5} M)-induced ileal contraction. On the other hand, the dipeptide aspartyl-glutamate was among the most active compounds tested (see Table 1). The time course of the ileal contraction it caused was identical to that of L-Glu and AP5 antagonized its action (Figure 1). When the preparation was pretreated with bacitracin (3×10^{-5} M) in order to attempt to inhibit the peptidases, the EC_{50} of aspartyl-glutamate was not changed.

Actions of antagonists of excitatory amino acid receptors

Several proposed antagonists of excitatory amino acid receptors were tested with the aim of achieving a better characterization of the L-Glu-induced responses. The NMDA receptor antagonists AP5 and AP7 antagonized the L-Glu-induced responses in an apparently competitive manner (Figure 3b and 4). Figure 4 shows the Schild plots for AP5 and for AP7 when tested against L-Glu or NMDA. Other antagonists were also used: kynurenic acid displayed a non-competitive type of antagonism whereas the dipeptide γ-D-glutamyl-taurine (10^{-4} M) did not change the dose-response curve to L-Glu (Figure 3b). Similarly, AP4 (10^{-4} M) did not evoke a contraction and did not modify the actions of L-Glu.

Table 1 The EC_{50} of several agonists of L-glutamic acid receptors in the guinea-pig myenteric plexus

Compound	EC_{50}
L-Glutamic acid	$1.9 \pm 1.2 \times 10^{-5}$ M
L-Aspartic acid	$6.8 \pm 1.0 \times 10^{-5}$ M
D-Aspartic acid	$7.4 \pm 1.3 \times 10^{-5}$ M
Aspartyl-glutamic acid	$7.9 \pm 1.0 \times 10^{-5}$ M
L-Homocysteic acid	$1.4 \pm 1.2 \times 10^{-4}$ M
L-Cysteine sulfinic acid	$2.9 \pm 1.5 \times 10^{-4}$ M
Quinolinic acid	$5.6 \pm 1.2 \times 10^{-4}$ M
N-methyl-D-aspartic acid	$6.8 \pm 1.1 \times 10^{-4}$ M

The following compounds were almost inactive at 5×10^{-4} M: L-methionyl-glutamic acid, ibotenic acid, D-homocysteic acid, L-glutamic acid-monohydroxamate, D-glutamic acid, pyro-glutamic acid, γ-carboxyglutamic acid, N-acetyl-aspartyl-glutamic acid, γ-L-glutamyl-glutamic acid, γ-D-glutamyl-glycine, γ-L-glutamyl-histidine, γ-L-glutamyl-glutamine, N-(L-glutamyl)-phenylalanine, α-L-glutamyl-alanine, γ-L-glutamyl-leucine, L-α-aspartyl glycine, L-α-aspartyl-alanine, L-tyrosyl-glutamic acid, N-glutamyl-phenylalanine, L-tryptophyl-glutamic acid, dihydrokainic acid.

Interactions between glycine and L-Glu

Submicromolar concentrations of glycine have been shown to potentiate NMDA responses in primary neuronal cultures (Johnson & Ascher, 1987). These concentrations of the amino acid did not affect the L-Glu (3×10^{-5} M)-induced contraction of the guinea-pig myenteric plexus longitudinal-muscle preparation incubated in a medium containing 0 Mg^{2+} and 2.4 mM Ca^{2+}. However, a higher concentration (10^{-5} M) of glycine significantly potentiated the L-Glu responses in most of the preparations (9 out of 16). When the concentration of Ca^{2+} in the bathing medium was lowered to 1.2 mM the variability of the responses apparently decreased, while the amplitude of the L-Glu-induced contraction was not significantly reduced. Under those conditions, 10^{-5} M glycine reproducibly potentiated the actions of L-Glu (Figure 5). The effects of glycine were not affected by strychnine (3×10^{-5} M)

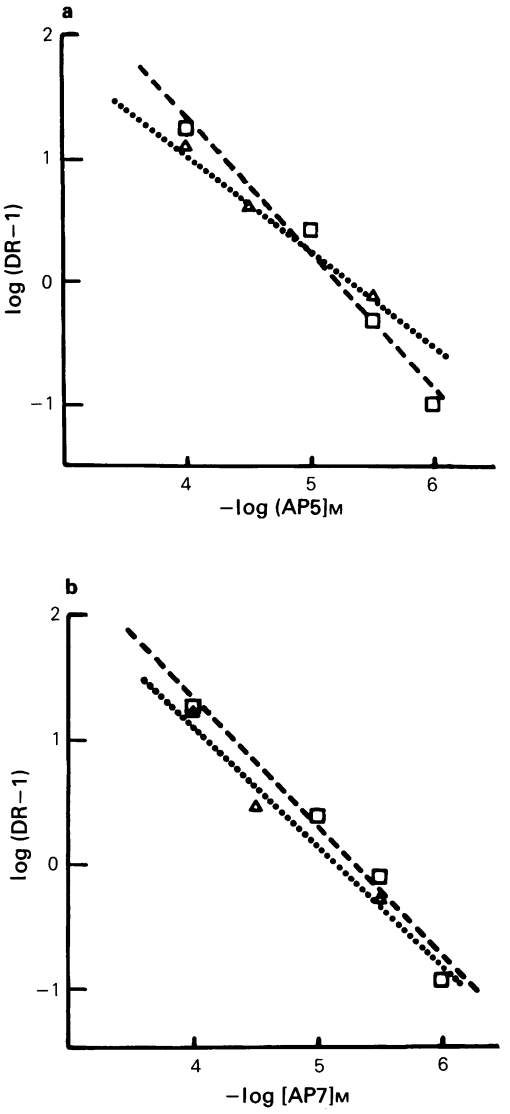

Figure 4 Schild plots of (a) DL-2-amino-5-phosphonovaleric acid (AP5) and (b) DL-2-amino-7-phosphonoheptanoic acid (AP7) when constructed against L-glutamic acid (L-Glu; □) or N-methyl-D-aspartate (NMDA; △). The pA_2 values of AP5 were 5.2 ± 0.084 for L-Glu (slope 1.1 ± 0.12; correlation coefficient 0.98) and 5.3 ± 0.93 for NMDA (slope 0.82 ± 0.8; correlation coefficient 0.99). The pA_2 values of AP7 were 5.27 ± 0.12 for L-Glu (slope 1.02 ± 0.13; correlation coefficient 0.98), 5.16 ± 0.13 for NMDA (slope 0.97 ± 0.16; correlation coefficient 0.98) and 5.48 ± 0.80 for L-aspartate (slope 0.89 ± 0.10; correlation coefficient 0.99). The calculations were performed using a computer programme according to Tallarida & Murray (1984). The slopes of the Schild plot were not significantly different from 1.

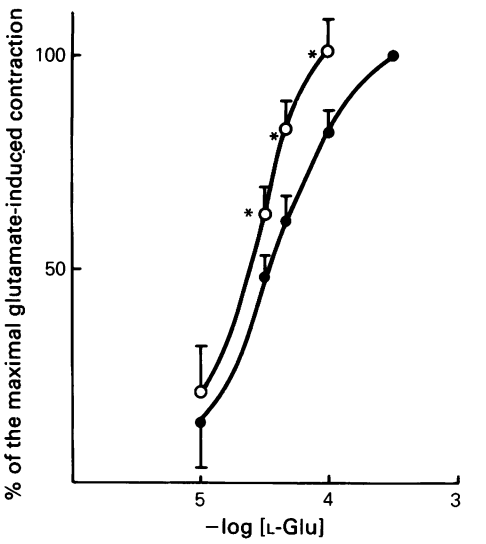

Figure 5 Dose-response curves for L-glutamic acid (L-Glu) in the presence (○) and in the absence (●) of added glycine (10^{-5} M) in the bathing solutions. * Significantly higher ($P < 0.01$) in the presence than in the absence of glycine. Each point represents the mean of at least 7 preparations (see text and Figure 6 for details); vertical lines show s.e.mean.

and were specific for L-Glu: the responses to GABA (10^{-5} M) were not potentiated (Figure 6).

When glycine was added to the bath in concentrations higher than 10^{-5} M, the basal motility of the preparations significantly increased and it was difficult to evaluate the effects of L-Glu.

Discussion

The data presented in this paper further confirm the presence of functional receptors for excitatory amino acids outside the central nervous system of mammals (Moroni *et al.*, 1986). In the guinea-pig myenteric

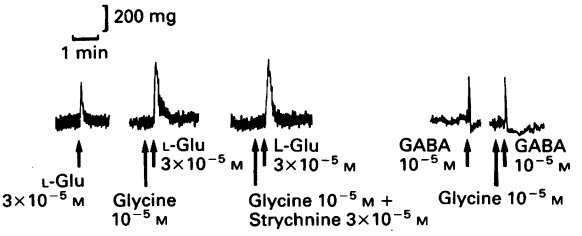

Figure 6 The effects of glycine (10^{-5} M), in the presence and absence of strychnine (3×10^{-5} M) on the ileal contraction induced by L-glutamic acid (L-Glu, 3×10^{-5} M), or by GABA (10^{-5} M). The Ca^{2+} concentration in the medium was 1.2 mM. Calibration: tension in mg; time in min.

plexus these receptors appear to be of the NMDA or A_1 type (Foster & Fagg, 1984; Fagg, 1985). There are, however, apparent differences between the NMDA receptors present in the guinea-pig myenteric plexus longitudinal muscle preparation and those described in the cerebral cortex (Harrison & Simmonds, 1985; Thomson & Lodge, 1985), the hippocampus (Collinridge *et al.*, 1983; Lanthorn & Cotman, 1983) and other models commonly used for neurophysiological and neuropharmacological investigations (Watkins & Evans, 1981; Perkins & Stone, 1982; Scatton & Lehmann, 1982; Garthwaite, 1985). Among these is the higher relative potency of L-Glu and to a lesser extent of L-Asp in comparison with NMDA (Table 1). This could be explained by considering that the high affinity uptake system for these amino acids is probably not very efficient in the myenteric plexus, while it is extremely important in brain slices (Garthwaite, 1985). In support of this concept is the observation that not only in our model, but also in binding studies and in isolated cells, L-Glu is more potent than NMDA (Olverman *et al.*, 1984). Differences between the NMDA receptors in the CNS have previously been suggested on the basis of differential actions of quinolinic acid in various brain areas (Perkins & Stone, 1983). Our data are in line with these observations and further indicate that quinolinate is quite active at the NMDA sites we describe. The dipeptide aspartyl-glutamate, while being less potent than other molecules, seems to be able to activate these NMDA receptors. This is supported by the time course of its action which is identical to that of all the other receptor agonists. Furthermore, the actions of aspartyl-glutamate were antagonized by AP5.

The observation that glycine potentiates the actions of L-Glu at these sites suggests that their properties are similar to those described by Johnson & Ascher (1987) in primary neuronal culture. The potentiating effect of glycine is apparently better observed when Ca^{2+} concentrations in the medium are in the range of 0.6–1.2 mM. It is widely accepted that the NMDA receptors are coupled to channels permeable to Ca^{2+} which are gated by Mg^{2+} (Nowak *et al.*, 1984; MacDermott *et al.*, 1986). Our observations indicate that under standard incubation conditions (2.4 mM Ca^{2+}; 0 Mg^{2+}) it is difficult to increase further the L-Glu-induced Ca^{2+} influx in the myenteric neurones which are sensitive to the excitatory amino acids. When the extracellular Ca^{2+} concentration was decreased it became easier to show this phenomenon.

Thus, our data confirm and extend to mature neurones the observation of Johnson & Ascher (1987) indicating that the NMDA receptors possess a site which recognizes glycine and which is able to potentiate the actions of L-Glu. This site is insensitive to strychnine and it is therefore different from the commonly recognized glycine receptor. Strychnine-insensitive glycine-binding sites have been described in several areas of the rat CNS (Bristow *et al.*, 1986; Bowery, 1987) and they could be associated with NMDA receptors.

Where are the Glu receptors in the myenteric plexus-ileal muscle preparation located? Since tetrodotoxin (3×10^{-7} M) and hyoscine (10^{-7} M) completely prevented L-Glu-induced contraction of the preparation, it appears that cholinergic neurones of the myenteric plexus are necessary for L-Glu-induced contractions of the ileum. It has previously been shown that $GABA_A$- and $GABA_B$-receptors are directly present on the cholinergic neurones of the myenteric plexus (Giotti *et al.*, 1983; Cherubini & North, 1984). The possibility therefore exists that L-Glu receptors are also located on the cholinergic neurones.

In conclusion, receptors for excitatory amino acids of the NMDA type have been demonstrated in the guinea-pig myenteric plexus. These receptors modulate the activity of cholinergic neurones in this tissue. We have not studied their physiological role, but in view of the peculiar voltage-sensitivity of the ion channels coupled to these receptors (Mayer *et al.*, 1984; Nowak *et al.*, 1984) it is possible that they could affect the intestinal function both in physiological and in pathological situations.

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