A comparison of the actions of agonists and antagonists at non-NMDA receptors of C fibres and motoneurones of the immature rat spinal cord *in vitro*

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1 The shift in d.c. potential in dorsal roots (EC_{50} $8.0 \mu\text{M} \pm 0.9$ s.e.mean, $n = 5$) or depression of the C elevation of the compound action potential (EC_{50} $3.0 \mu\text{M} \pm 0.3$, $n = 7$) have been used to measure the depolarizing action of kainate on dorsal root C fibres of immature (3 to 5 day old) rats. Depolarization of motoneurones was measured from the shift in d.c. potential in ventral roots.

2 6-Cyano-7-nitroquinoxaline,2,3-dione (CNQX) (pA_2 5.78 ± 0.06 , $n = 8$) and 6-nitro-7-sulphamobenzo(f)quinoxaline-2,3-dione (NBQX) (pA_2 5.75 ± 0.04 , $n = 7$) had similar potencies as antagonists of kainate at dorsal root fibres. The potency of NBQX as a kainate antagonist was similar also at motoneurones (pA_2 5.72 ± 0.07 , $n = 3$). At motoneurones, NBQX was less potent as an antagonist of domoate (pA_2 5.29 ± 0.05) and more potent as an antagonist of S- α -3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (pA_2 6.80 ± 0.09) than as an antagonist of kainate.

3 Application of L-glutamate, quisqualate and RS-AMPA to dorsal roots produced only short lasting depolarizations but kainate concentration-effect plots were shifted to the right in the presence of these three agonists (pA_2 5.08 ± 0.08 , ($n = 3$), 5.59 ± 0.04 , ($n = 4$) and 4.46 ± 0.04 ($n = 4$) respectively). Slopes of dose-ratio against concentration were significantly less than one for the latter antagonism.

4 The amplitude of depolarizations induced by L-glutamate, AMPA and quisqualate were increased up to ten fold and those induced by kainate up to two fold following treatment of dorsal roots with concanavalin A. The duration of the responses was increased also by the latter treatment. Following 85 s applications of glutamate, quisqualate, AMPA and kainate the mean respective times ($s \pm$ s.e.mean (n)) taken for responses to decay to half the peak amplitude were increased from 63 ± 7 (10), 86 ± 17 (4), 95 ± 19 (4) and 135 ± 3 (12) to 202 ± 49 (10), 147 ± 7 (4), 160 ± 13 (6) and 163 ± 10 (10). Under similar conditions the mean decay time of γ -aminobutyric acid-induced responses was 145 ± 7 (10). This was not significantly altered by concanavalin A treatment.

5 Application to dorsal roots of L-aspartate at concentrations up to 5 mM (with or without concanavalin A treatment), the selective metabotropic agonist 1S,3R-*trans*-1-aminocyclopentane-1,3-dicarboxylate (1 mM) and D-serine (20 μM) in the presence or absence of N-methyl-D-aspartate (NMDA, 500 μM) neither depolarized the preparations nor shifted the kainate concentration-effect plot.

6 It is concluded that primary afferent C fibres possess only one type of non-NMDA receptor which is activated strongly by domoate or kainate but only weakly by AMPA. This receptor is readily desensitized by glutamate, quisqualate or AMPA and it is less readily desensitized by kainate.

Keywords: Kainate; C fibres; motoneurones; quinoxalinediones

Introduction

Non-N-methyl-D-aspartate (NMDA) ionotropic excitatory amino acid receptors can be activated by the selective agonists α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), quisqualate or kainate. The non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline,2,3-dione (CNQX) (Honoré *et al.*, 1988) has been shown to be five times more effective at displacing bound AMPA than bound kainate from rat brain membranes (Honoré *et al.*, 1988) and a higher selectivity (30 fold) in the same direction has been reported for 6-nitro-7-sulphamobenzo(f)quinoxaline-2,3-dione (NBQX) (Sheardown *et al.*, 1990). In a functional test at neonate rat motoneurones little selectivity was found between the antagonism of quisqualate or kainate by CNQX (Long *et al.*, 1990; Watkins *et al.*, 1990). Therefore it appeared likely that the depolarizations of motoneurones produced by kainate and quisqualate are mediated mainly by the same population of non-NMDA receptors.

Somatic primary afferent C fibres are selectively sensitive to kainate, apparently lacking sensitivity to quisqualate, AMPA or NMDA (Agrawal & Evans, 1986). This kainate-sensitive site is suitable therefore to examine the antagonist selectivity of the quinoxalinediones in a functional test. In the present study the isolated dorsal roots of immature rats have been used for this purpose. Potencies of the antagonists at primary afferent C fibres have been compared with measurements from motoneurones, recorded in ventral roots. The results suggest that two subtypes of non-NMDA receptor are present on motoneurones.

A voltage clamp study of primary afferent neurones (Huettnet, 1990) revealed a population which showed sensitivity to kainate analogous to that observed previously in dorsal root C fibres (Agrawal & Evans, 1986). In these neurones AMPA evoked inward current responses which desensitized rapidly and the desensitization was diminished by concanavalin A treatment of the neurones (Huettnet, 1990). The effects of concanavalin A have been investigated on dorsal root C fibres in the present study and the findings are consistent with the previous observations on dorsal root ganglion neurones.

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Methods

Dorsal roots and hemisected spinal cords were isolated from rats of 2 to 5 days post partum age. Depolarizing responses of C fibres were measured either from depression of the C fibre component of the compound action potential recorded via a suction electrode or from the d.c. shift in a dorsal root placed across a grease gap (Agrawal & Evans, 1986). The compound action potential was evoked by 0.5 ms pulses applied at 0.1 Hz at eight times threshold. Depolarization of motoneurons was measured from d.c. shifts in ventral root polarity (Long *et al.*, 1990). Dose-ratios for antagonism and equipotent molar ratios for agonists were measured as described by Evans *et al.* (1987). Dose-ratios for the non-parallel shifts as occurred with agonist-induced desensitization were estimated at the mid-point of the control concentration-effect range (at two to five times threshold concentration).

The bathing medium for both preparations contained (mM): NaCl 118, KCl 3, CaCl₂ 1.5, MgSO₄ 0.75, NaHCO₃ 24 and dextrose 12. The medium and recording site were maintained at 25°C and the medium was superfused over the preparations at a rate of 2 ml min⁻¹. For the recordings of motoneuronal depolarization the medium contained 0.1 µM tetrodotoxin.

Concanavalin A type III was applied to isolated dorsal roots dissolved (1 to 10 mg ml⁻¹) in bathing medium containing no dextrose. The normal dextrose containing medium was restored following up to 2 h incubation in concanavalin A.

Concanavalin A (Type III) was obtained from Sigma (Poole, U.K.) and CNQX, (S)-AMPA and (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) from Tocris Neuramin (Bristol, U.K.). (RS)-AMPA and NBQX were kindly provided by Prof. P. Krogsgaard-Larsen and Dr T. Honoré respectively.

Values are given as mean ± s.e.mean (*n*).

Results

Since it was reported that kainate-sensitive C fibres are insensitive to NMDA (Agrawal & Evans, 1986), it has been determined that glycine has an obligatory role at the NMDA receptor/ionophore (Henderson *et al.*, 1990). Also NMDA has been reported to depolarize neurones of the nodose ganglion (Lovinger & Weight, 1988). Thus it was possible that the failure to observe any effect of NMDA in the previous study may have been due to a requirement for co-agonism at the NMDA-linked glycine site (see Thomson, 1990). Therefore in the present experiments, NMDA was tested on dorsal root C fibres in the presence and absence of D-serine which is a selective agonist for this glycine site (see Thomson, 1990). As previously observed (Agrawal & Evans, 1986) kainate (EC₅₀ 3.0 ± 0.3 µM, *n* = 7) reversibly depressed the C wave of the compound action potential but NMDA at concentrations up to 1 mM failed to depolarize dorsal root C fibres in the presence or absence of D-serine (20 µM) (Figure 1).

Figure 1 Failure of 100 µM N-methyl-D-aspartate (NMDA) and/or 20 µM D-serine (Ser) to modify the C fibre compound action potential recorded from an immature dorsal root. The potential was depressed by 25 µM kainate (Ka). Traces are shown 8 min following application of agonists. Scale bar 10 ms, 1 mV.

Also as reported previously (Agrawal & Evans, 1986) L-glutamate depolarized C fibres and depolarizations induced by concentrations higher than 50 µM contained an initial fast-decaying component (see Figure 4). AMPA and quis-

Figure 2 Effect of excitatory amino acid agonists on kainate-induced depression of the C-fibre compound action potential. The twelve oscillographic records in (A) (scale bar 0.2 mV, 10 ms) were made at the points indicated on the time-course plotted in (B). The points on the time course are the means of four consecutive recordings, standard errors of the mean all fall within the width of the symbol. Kainate (25 µM) was present during the periods indicated by the hatched bar above. S-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA, 0.2 mM), quisqualate (0.2 mM) (QA), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), D-serine (Ser, 20 µM) + N-methyl-D-aspartate (NMDA, 1 mM), L-aspartate (Asp, 2.5 mM) and L-glutamate (Glu, 0.2 mM) were introduced as indicated by the solid bars. It can be seen that introduction of kainate depressed the peak amplitude and this depression was reversed by AMPA, quisqualate and glutamate but not by ACPD, NMDA or aspartate. The large depression near the end of the experiment was due to introduction of capsaicin (4 µM) which abolished the C-fibre potential (trace l). (C) Gaddum-Schild plot for the antagonism of kainate produced by 6-cyano-7-nitroquinoxaline,2,3-dione (CNQX) (○), AMPA (□) and quisqualate (●).

qualate at appropriate concentrations also produced fast decaying depolarizations similar to those produced by glutamate. At the lower range of concentration, responses induced by AMPA, although smaller in amplitude, were similar in profile to those produced by kainate (see Figure 5). L-Glutamate (pA_2 5.06 ± 0.09 , $n = 3$), AMPA (pA_2 4.53 ± 0.05 , $n = 4$) and quisqualate (pA_2 5.38 ± 0.08 , $n = 4$), but not ACPD or NMDA or aspartate, were able to antagonize the depolarizing action of kainate. This is illustrated by the reversal of the depressant action of kainate on the C fibre compound action potential shown in Figure 2. Preparations showed a slow loss of sensitivity to kainate which developed over a period of several hours. This is illustrated in Figure 2 by the progressive reduction in the depressant action of kainate which occurred over a period of almost 8 h application of $25 \mu M$ kainate (cf. recordings (b) and (j)).

It appears likely that, as described for dorsal root ganglion neurones (Huettner, 1990), the antagonism of kainate produced by glutamate, AMPA and quisqualate represents receptor desensitization rather than competitive antagonism. The slopes of the plots in Figure 2C illustrate the non-competitive nature of the antagonism produced by quisqualate and AMPA. CNQX at concentrations from 3 to $50 \mu M$ (pA_2 5.78 ± 0.06 , $n = 8$) however, produced a plot of dose-ratio versus concentration consistent with competitive antagonism of kainate. NBQX at concentrations of 1 to $10 \mu M$ gave a mean pA_2 of 5.75 ± 0.04 ($n = 7$) for antagonism of the kainate-induced depolarization of C fibres. NBQX was tested also against non-NMDA agonist-induced depolarization of motoneurones. The pA_2 (n) values obtained with domoate, kainate, quisqualate and (S)-AMPA were respectively 5.29 ± 0.05 (3), 5.72 ± 0.07 (3), 6.12 ± 0.08 (3) and 6.80 ± 0.09 (4). Thus for this series of four agonists a

differential of NBQX on motoneurones was most evident when domoate (least affected) and AMPA (most affected) were tested.

Concanavalin A prevented desensitization of AMPA-induced responses of dorsal root ganglion neurones (Huettner, 1990). In the present study concanavalin A (1 to 10 mg ml^{-1}) was applied to dorsal roots for 15 to 20 min in the absence of glucose. Concanavalin A-containing and glucose-free medium produced reversible depolarization of grease-gap mounted dorsal root preparations. In four preparations the mean concanavalin A-induced depolarization was $1.62 \pm 0.24 \text{ mV}$. The depolarizing effect of glucose-free medium developed slowly and became clearly evident because of the hyperpolarization which developed over a few minutes on reintroduction of glucose. In four preparations the mean hyperpolarizing effect of reintroduction of glucose was $1.06 \pm 0.32 \text{ mV}$. Following treatment of dorsal roots with concanavalin A the effect of L-glutamate was enhanced considerably such that the C fibre compound action potential was permanently depressed in the presence of glutamate (Figure 3). Figure 4a illustrates the effect, on responses to glutamate, of a 15 min application of each concentration of the lectin. It can be seen that 10 mg ml^{-1} was the most effective concentration and that the effect once established persisted for long after the tissue was returned to lectin-free medium. In a similar manner to glutamate-induced depolarizations, depolarizations of dorsal root C fibres induced by AMPA, kainate or quisqualate were also increased in amplitude relative to control responses. As illustrated in Figure 4b, L-aspartate was still found to be inactive on dorsal roots following concanavalin A treatment. In concanavalin A-treated nerves, mean equipotent molar potency ratios for depolarizations induced by L-glutamate, quisqualate and AMPA (relative to kainate = 1) were 0.065 ± 0.01 (5), 0.061 ± 0.01 (4) and 0.029 ± 0.004 (6) respectively. The responses induced by kainate were less affected by concanavalin A treatment than those induced by the other three agonists.

In eight preparations the leftward shift of the kainate concentration-effect plot induced by concanavalin A was equivalent to a mean dose-ratio of 2.69 ± 0.36 . The corres-

Figure 3 Effect of concanavalin A (Con A) on glutamate-induced depression of C-fibre compound action potential. The oscillographic traces (scale bar 0.25 mV , 10 ms) (a) to (f) were recorded at the points indicated below the time-course. L-Glutamate (Glu, 0.2 mM) and concanavalin A (1 mg ml^{-1}) were applied as indicated above the time-course. The preparation was superfused with glucose-free medium for 15 min before application of concanavalin A which was applied in glucose-free medium. The glutamate-induced depression was very brief but following concanavalin A treatment, glutamate caused a persistent depression.

Figure 4 Effect of concanavalin A (Con A) treatment on the grease-gap recorded agonist-induced depolarization of dorsal root fibres. (A) Glutamate ($200 \mu M$) was applied as indicated by the bars below the traces. The lectin was applied at 1 mg ml^{-1} during the interval between the first and second trace and at 10 mg ml^{-1} between the second and third trace. The last trace was recorded 4 h after the third trace. (B) Glutamate (Glu) and aspartate (Asp) were applied as indicated (μM) below the traces. The second and third row of traces were obtained following 15 min incubation of the dorsal root in concanavalin A (10 mg ml^{-1}) containing medium. Fading responses to glutamate can be seen before the lectin was applied and the lectin-treated nerve failed to respond to aspartate but was still sensitive to low concentrations of kainate. Vertical scale bar 0.1 mV , horizontal scale bar 2 min .

ponding value for GABA-induced responses was 0.77 ± 0.15 . The concentration-effect plots for AMPA, glutamate and quisqualate were shifted in a non-parallel manner following concanavalin A treatment. Consequently it was not possible to estimate mean values for the leftward shift. However, the effect of concanavalin A (illustrated in Figures 4 and 5) is characterized by the mean times taken for the responses to decay to half the peak depolarization from the start of an 85 s application of agonist. These values (s) for glutamate, quisqualate, AMPA and kainate were 63 ± 7 (10), 86 ± 17 (4), 95 ± 19 (4) and 135 ± 3 (12) s respectively. Following concanavalin A treatment these were increased to 202 ± 49 (10), 147 ± 7 (4), 160 ± 13 (6) and 163 ± 10 (10) respectively. As illustrated in Figure 5 depolarizations induced by γ -aminobutyric acid (GABA) in ten preparations were not significantly affected by concanavalin A treatment with the

time to half decay being 145 ± 7 s under control conditions and 149 ± 12 s following treatment with concanavalin A.

It is possible that the potentiating effect of concanavalin A is produced through unmasking of fast desensitizing AMPA sites on C fibres. CNQX shows a five fold higher affinity for AMPA- than for kainate-binding sites (Sheardown *et al.*, 1990). Thus it is possible that a changed proportion of AMPA/kainate sites could be reflected by an alteration in potency of CNQX. Thus dose-ratios for the antagonism by CNQX ($10 \mu\text{M}$) of kainate-induced responses were measured before and after concanavalin A treatment. In four preparations the mean pA_2 value following concanavalin A treatment was 5.69 ± 0.10 . This is not significantly different ($P > 0.3$) from the value above obtained in the absence of concanavalin A.

Figure 5 Effect of concanavalin A (Con A) treatment on agonist-induced depolarization of dorsal root fibres. The second row of traces was obtained following concanavalin A treatment. Integrated areas of the response profiles (mV s) are plotted in (B) against μmolar concentration of agonist. All except S- α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) are from the same preparation; (●) after concanavalin A treatment. Vertical bars are standard errors of three or four responses. In (A) the vertical scale bar 0.1 mV, horizontal scale bar 2 min. Other details as for Figure 4.

Discussion

The EC_{50} of $8\text{ }\mu\text{M}$ for kainate-induced depolarization of C fibres is consistent with previous studies (Agrawal & Evans, 1986; Evans *et al.*, 1987). The EC_{50} of $3\text{ }\mu\text{M}$ obtained for depression of the C elevation of the compound action potential in the present study is significantly lower than the former value, presumably because the action potential of individual C fibres is fully depressed at submaximal levels of depolarization.

It has been established in the present study that the lack of functional receptors for NMDA on primary afferent C neurones (Evans & Agrawal, 1986; Huettner, 1990) is unlikely to be due to absence of a co-agonist for the NMDA-linked glycine receptor. The absence of any effect of ACPD suggests also that C fibres either lack metabotropic glutamate receptors or that their action on the membrane electrical properties of C fibres induces no measurable change under the present experimental conditions.

The antagonism of kainate produced by CNQX, and by analogy that produced by NBQX, was probably competitive as suggested by the slope of Figure 2c. The plots of dose-ratio against concentration for the antagonism produced by AMPA, glutamate and quisqualate (Figure 2c) suggest a non-competitive antagonism of kainate by these ligands. The antagonism produced by quisqualate, AMPA and glutamate was likely to have been caused by receptor desensitization (Huettner, 1990). The potencies of quisqualate, AMPA and glutamate for occluding kainate-induced whole cell current responses at cultured striatal neurones (Tse *et al.*, 1991) were similar to those of the present study on C fibres. The desensitization induced by the non-NMDA receptor agonists was prevented following concanavalin A treatment (Huettner, 1990). Thus these agonists might be expected to lose their antagonistic properties following concanavalin A treatment. This possibility was not tested during the present experiments because of interference by the persistent depolarizing action which the agonists had after concanavalin A treatment (Figure 3).

There was, as illustrated in Figure 2 and as previously observed (Agrawal & Evans, 1986; Evans *et al.*, 1987), little evidence of fast desensitization caused by kainate on the present preparation. However, there was a very slow decrease in sensitivity to kainate which developed over periods of exposure greater than 60 min. Concanavalin A treatment produced a consistent two fold displacement of the kainate-induced concentration-effect plot which was independent of the period of exposure to kainate. Thus it is unlikely that reversal of the slow loss in sensitivity to kainate was responsible for the effect of concanavalin A on kainate-induced responses. This very slowly developing loss in sensitivity may have been due to rectification of the membrane current induced by kainate. The depolarizing effect of kainate and the other agonists on C fibres measured in the present study is likely to correspond to the steady state currents described by Huettner (1990) at dorsal root ganglion neurones. The rank order of potency of the agonists used in the present study is similar to that obtained in Huettner's study. However it can be estimated that glutamate and quisqualate were only four times less active than kainate in Huettner's experiments on dorsal root ganglion somata whereas these two compounds and AMPA were more than thirty fold less active than kainate in the present study on dorsal root fibres. The present values correspond closely to previous molar potency ratios estimated at immature dorsal roots (Agrawal & Evans, 1986; Ishida & Shinozaki, 1991). The latter studies were on preparations untreated with concanavalin A. Thus the more than twenty fold lower potency of AMPA relative to kainate at this site is unlikely to be a consequence of AMPA-induced desensitization.

The present effects of concanavalin A were consistent with the two to four fold increase in agonist-induced responses of cultured hippocampal neurones (Mayer & Vycklicky, 1989)

and a two fold increase at dorsal root ganglion cells (Huettner, 1990). In the present study concanavalin A treatment distinguished between responses induced by glutamate, quisqualate or AMPA and induced by kainate because, following such treatment, the time-course of responses to the former three agonists was prolonged significantly. This effect may correspond to an effect of concanavalin A on the slower phase of desensitization described by Huettner (1990) since the present method of application of agonists did not allow resolution of processes with time constants in the millisecond range. It is possible that desensitization involves interaction with a binding site which is separate from that which mediates the depolarizing action of kainate. Or it may be, as others have proposed (Mayer & Vycklicky, 1989; Huettner, 1990), that AMP, quisqualate and glutamate have higher affinities for a desensitized form of the receptor analogous to the model proposed for desensitization at the nicotinic receptor (Katz & Thesleff, 1957). Presumably in concanavalin A-treated nerves, both forms of the receptor are expressed in the depolarizing response.

The present pA_2 of 5.84 for the antagonism of kainate-induced depolarization of C fibres by CNQX is similar to values obtained previously at motoneurones (5.8 from Long *et al.* (1990) and 5.7 calculated from the data of Ishida & Shinozaki (1991)). In the present study NBQX gave pA_2 values not significantly different from those above for antagonism of kainate-induced depolarization at motoneurones and C fibres. A similar value was obtained for the antagonism of kainate-induced responses of cerebrocortical neurones (Lodge *et al.*, 1991). Thus in these functional tests the two quinoxalinediones appear to have similar affinities for the kainate receptor. This contrasts with the six fold greater potency of NBQX relative to CNQX for the displacement of AMPA from cerebrocortical membranes (Sheardown *et al.*, 1990; Lodge *et al.*, 1991). Thus the present C fibre site represents a 'kainate' rather than AMPA subtype of non-NMDA receptor.

The selectivity of CNQX or NBQX for AMPA/kainate binding sites should allow the dose-ratio for antagonism to be used as an index for the relative proportions of AMPA and kainate sites involved in generation of responses. CNQX has a five fold higher affinity for AMPA- compared to kainate-binding sites (Sheardown *et al.*, 1990). In the present study, due to availability, CNQX rather than NBQX was used for this purpose in order to show if concanavalin A treatment caused any significant change in the receptors which generate the kainate-induced depolarization of C fibres. There was no significant difference between pA_2 values obtained before and after treatment with concanavalin A. This suggests that concanavalin A did not affect the interaction between CNQX and the non-NMDA receptors. CNQX has been found to cause a reduction in desensitization at non-NMDA receptors (Rossier *et al.*, 1991). Similar effects which cause a paradoxical enhancement of responses by antagonists have been observed at GABA_A (Simmonds, 1983) and strychnine-sensitive glycine (Long *et al.*, 1989) receptors. The latter phenomenon would be expected to result in an increase in the potency of CNQX following concanavalin A treatment.

The use of domoate and AMPA as agonists revealed a degree of selectivity of NBQX (approximately thirty fold) which was not apparent with kainate and quisqualate (Long *et al.*, 1990; Watkins *et al.*, 1990). A similar but smaller difference has been observed with CNQX (Watkins *et al.*, 1990; Ishida & Shinozaki, 1991) and it suggests that domoate and AMPA are more selective agonists for sub-types of non-NMDA receptors than either kainate or quisqualate and that NBQX discriminates better between these receptors than does CNQX (Sheardown *et al.*, 1990). In particular the 'kainate' receptor appears to be characterized by high agonist sensitivity to domoate (Agrawal & Evans, 1986) and the weak activity of AMPA at C fibres is likely to be due to a relatively low affinity and/or efficacy of this agonist for

kainate receptors in this tissue. L-Aspartate and NMDA would appear to be devoid of affinity and efficacy at C fibre kainate receptors.

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