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UNEVEN DISTRIBUTION OF EXCITATORY AMINO ACID RECEPTORS ON VENTRAL HORN NEURONES OF NEWBORN RAT SPINAL CORD

BY K. ONODERA AND A. TAKEUCHI

From the Department of Physiology, Juntendo University, School of Medicine, Hongo, Tokyo, Japan

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SUMMARY

1. The distribution of excitatory amino acid receptors on ventral horn neurones was investigated using slices of newborn rat spinal cord.

2. The neurone and the tip of the pipette used to inject amino acids were visualized using Lucifer Yellow under a fluorescent microscope. The pipette was precisely located on the soma and dendrite of the neurone under visual control, and L-glutamate (Glu), L-aspartate (Asp), *N*-methyl-D-aspartate (NMDA), kainate (KA) and quisqualate (Quis) were ionophoretically applied with a short pulse. The potential changes were intracellularly recorded from the soma.

3. Sensitivity to Glu as tested with short pulses (1–2 ms) was almost the same at the soma and along dendrites.

4. The amplitude of the responses to NMDA produced at the soma and the proximal part of the dendrite was about the same as that of Glu, but smaller than that of Glu at the distal part of the dendrite. Suppression of the Glu potential by an NMDA receptor antagonist, 2-amino-5-phosphonovaleric acid (APV), was greater at the soma than at the dendrite, suggesting that the contribution of NMDA receptors to the Glu potential was greater at the soma.

5. Sensitivity to Asp was about one-half that to Glu sensitivity on the soma and even less on the dendrite. Sensitivity to KA was high at the soma and low at the dendrite. However, Quis responses were produced throughout the neurone.

6. The Quis response induced by the application of a short pulse showed two phases: a fast response followed by a very slow depolarization that lasted more than 10 s.

7. The fast Quis response was easily desensitized and insensitive to APV. The time course of the fast Quis potential was shorter than that of Glu.

8. The slow Quis response was more pronounced at the dendrites than at the soma and was reduced by the intracellular injection of EGTA, suggesting the contribution of Ca^{2+} in the cell, possibly mediated by a second messenger system.

9. Experimental results suggest that the distribution of excitatory amino acid receptors differs between the soma and the dendrites of spinal neurones.

INTRODUCTION

L-Glutamate (Glu) has been proposed as a neurotransmitter in primary afferents in the spinal cord based on its higher concentration in dorsal roots and dorsal columns than in ventral roots (Duggan & Johnston, 1970; Roberts, Keen & Mitchell, 1973; Jones, Jordan, Morton, Stagg & Webster, 1974), its endogenous release by nerve stimulation (Fagg, Jordan & Webster, 1976; Kawagoe, Onodera & Takeuchi, 1985, 1986; Takeuchi, 1987) and the depolarization of spinal neurones induced by ionophoretic and pressure application of Glu, which has a reversal potential close to that of evoked excitatory postsynaptic potentials (EPSPs), *in vivo* and *in vitro* (Watkins & Evans, 1981; Finkel & Redman, 1983; Salt & Hill, 1983; Mayer & Westbrook, 1984; Nelson, Pun & Westbrook, 1986).

Studies with specific agonists suggest that there are at least three types of excitatory amino acid receptors in the spinal neurone: *N*-methyl-D-aspartate (NMDA), kainate (KA) and quisqualate (Quis) receptors. Glu is a mixed agonist of these receptors (Watkins & Evans, 1981; Mayer & Westbrook, 1987). When the selective NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV) was applied to the spinal cord, polysynaptic responses produced by dorsal root stimulation were almost completely suppressed, while monosynaptic responses remained relatively intact (Davies & Watkins, 1982, 1983; Onodera & Takeuchi, 1986). The fast monosynaptic EPSPs have been suggested to be mediated by non-NMDA receptors (Jahr & Jessell, 1985; Nelson *et al.* 1986). However, in cultured hippocampal and spinal neurones of mice with synaptic connections and in *Xenopus* embryo neurones, the falling phase of monosynaptic EPSP has fast and slow components (Dale & Roberts, 1985; Forsythe & Westbrook, 1988). The slow component of monosynaptic EPSP is reduced by APV in *Xenopus* embryos and spinal neurones of chick and rat embryos (Dale & Roberts, 1985; O'Brien & Fischbach, 1986*a*, *b*; Forsythe & Westbrook, 1988; Ziskind-Conhaim, 1990). These results suggest that NMDA receptors also contribute to monosynaptic transmission.

Glutamate receptors are not uniformly distributed over the surface of embryonic chick motoneurones. The sensitivity of isolated motoneurones to Glu was greatest at the soma and decreased with distance along the processes. However, in cultures containing interneurones, the highest sensitivity occurred along motoneurone processes (O'Brien & Fischbach, 1986*b*). Furthermore, in cultured chick neurones, sharply defined Glu hot spots were present on the neurite at sites corresponding to points of contact with neighbouring neurites (Trussell, Thio, Zorumski & Fischbach, 1988). By measuring miniature synaptic currents in cultured hippocampal neurones, it was found that each excitatory synapse on neurites had varying numbers of NMDA and non-NMDA receptors, though 20% of synapses contained only non-NMDA receptors (Bekkers & Stevens, 1989). Therefore, it is assumed that aggregations of receptors exist on dendrites with physiological connections and different distributions of receptor subtypes occur on the surface of dendrites depending on their synaptic connections. Characterization of the distribution of receptors and their relation to the synapse seems important for understanding synaptic transmission in spinal motoneurones.

The first step to characterize the receptors on soma and dendrites would be to

locate the site of amino acid receptors on them. Several attempts have been made to apply excitatory amino acids to spinal motoneurones (Takahashi, 1978; Zieglgänsberger & Champagnat, 1979; Flatman, Lambert & Engberg, 1985). However, motoneurones in the spinal cord have long, expanded dendrites, and it is difficult to apply amino acids precisely. Therefore, in the present experiments, spinal neurones were visualized by injection of Lucifer Yellow. With this method neurones were relatively easily stained up to the end of the dendrite and amino acids were applied to localized parts of soma and dendrites under visual control. It was found that the chemosensitivities of soma and dendrites to Glu, L-aspartate (Asp), Quis, KA and NMDA were different. Preliminary results of some of these experiments have been reported (Onodera & Takeuchi, 1988, 1989).

METHODS

The spinal cord was dissected from a 5- to 7-day-old newborn rat anaesthetized with ether. After being dissected, the spinal cord was quickly placed in artificial cerebrospinal fluid (CSF) and the pia mater was carefully removed. The spinal cord was then placed in CSF–agar (2% agar + 2% Agarose-L, Inashokuhin) with the agar temperature below 35 °C. An agar block containing the spinal cord was cut out, and a 300 μm thick slice was prepared using a vibratome (Cambridge). The slice was placed in a small bath and CSF saturated with 95% O_2 and 5% CO_2 was continuously superfused. The bath temperature was kept at 29 °C.

A conventional intracellular microelectrode was filled with 1·5 M-KCl solution that contained Lucifer Yellow (Sigma, Lucifer Yellow CH, 0·5%). The resistance of the microelectrode was 20–30 MΩ. After the microelectrode had been inserted into a ventral horn neurone, inward current pulses (0·2 nA and 100 ms in duration) were applied through a bridge circuit (CEZ 3100, Nihon Kohden). The cell body and dendrites were visible under fluorescent microscope (OPTIPHOT with FFD2, Nikon) within a few min. If the resting potential of the cell was below 55 mV the neurone was discarded. The most frequently inserted were large cells, whose soma exceeded 25 μm in diameter. These cells had high sensitivity to Glu. On some occasions, cells were encountered which had a high resting potential of over 80 mV and low resistance. These cells, which had small cell bodies and many fine processes, did not generate an action potential. These cells were probably glial cells, as reported by Takahashi & Tsuruhara (1987).

A micropipette was filled with Glu solution (0·5 M), the pH of which had been adjusted to about 8·0. To compare the effects of Glu to other amino acids, a double-barrelled micropipette was used, one barrel being filled with Glu and the other with either NMDA (0·5 M), KA (0·5 M), Quis (0·5 M) or Asp (0·5 M). Lucifer Yellow was dissolved in the amino acid solutions (0·3%). The tip of the pipette, which was visible under fluorescent microscope, was moved to various parts of the neurone under visual control. Amino acids were ionophoretically applied by passing currents through the pipette in series with a 1 GΩ resistor. The pH of amino acids was adjusted to about 8·0. A backing potential of about 1 V was usually used. Ionophoretic application of Glu close to the neurone produced membrane potential changes, which were recorded by a microelectrode inserted in the soma. All amino acids, inhibitors of amino acid receptors (kynurenate and APV) and EGTA were obtained from Sigma.

The composition of artificial CSF used was as follows (mM): NaCl, 130; KCl, 4·5; $CaCl_2$, 2·0; $MgCl_2$, 1·0; glucose, 20 and $NaHCO_3$, 10. The pH was adjusted to 7·2. After the neurone was visualized by the Lucifer Yellow injection, the normal solution was replaced with Mg^{2+}-free solution containing TTX (1 μM, Sankyo Co. Ltd) unless otherwise noted.

When concentrated Lucifer Yellow solution was applied to the cut end of the ventral root of dissected spinal cord, Lucifer Yellow was taken up into motoneurones. When the spinal cord was sliced into 300 μm sections the motoneurones stained by retrograde uptake of Lucifer Yellow were visible under a fluorescent microscope (Fig. 1*A* and *B*). The size and location of these motoneurones were the same as those of neurones that were intracellularly stained, as shown in Fig. 1*C* and *D* and Fig. 3*A* and *C*. The large neurones of the cell body were selected for experiments. It has been

reported that all neurones over 25 μm in diameter in the spinal cords of newborn rat are motoneurones (Takahashi, 1990).

When the preparation was illuminated with ultraviolet light for a long period, usually over 10 min, the neurone began to fire spontaneously. To avoid possible damage to the neurone, the period of ultraviolet illumination was kept as short as possible during the experiment, usually less

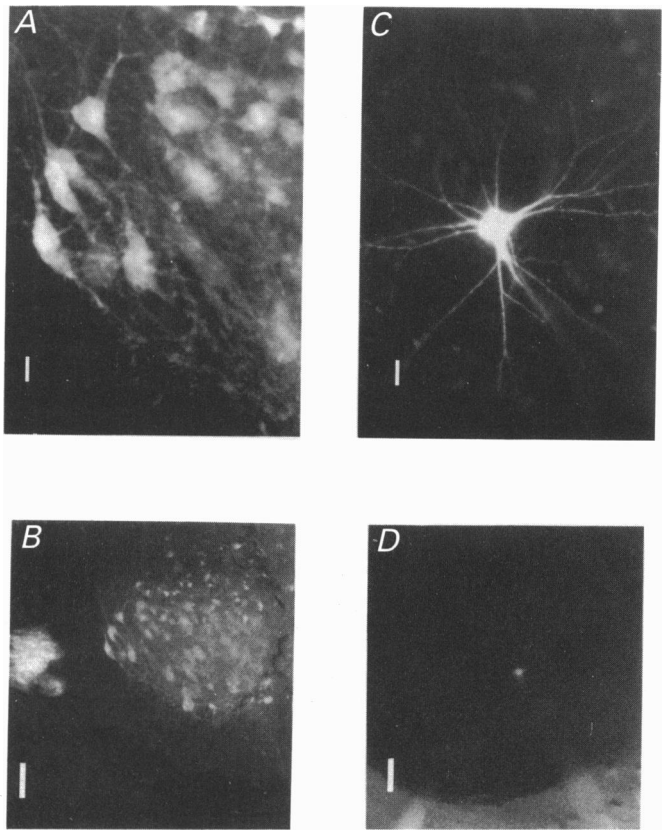

Fig. 1. Ventral horn neurones in the slice of a newborn rat spinal cord. Left (*A*, *B*), Lucifer Yellow was taken up from the ventral roots. *B* is the picture of the ventral horn with ventral root that is seen as the light part on the left side. *A* was enlarged from *B*. Right (*C*, *D*), Lucifer Yellow was injected from a recording microelectrode. *D* is at lower magnification. The neurone was photographed after the experiment. The bars in *A* and *C*, and *B* and *D*, are 20 μm and 100 μm, respectively.

than 1 or 2 min. Therefore, after the injection pipette was located on the neurone membrane under visual control, potential changes were recorded in the dark. At the end of each series of experiments, photographs were taken to record the position of the tip of the micropipette. A neutral density (ND) filter was used to reduce the amount of illumination during observations. Visible light was also filtered to remove ultraviolet light.

RESULTS

Ventral horn neurones in the spinal slices

A pair of silver electrodes 100 μm in diameter was placed on the dorsal horn of the spinal slice and a short stimulus current was applied (Fig. 2). When the stimulus

strength was weak, a synaptic stimulation with a short latency was produced (Fig. 2*Ab*). As the stimulus strength was increased to twice that of *Ab* in Fig. 2, the amplitude of EPSP was increased and showed a long summated response (Fig. 2*Aa*). Application of Glu to this neurone produced a Glu potential as shown in Fig. 2*Ac*.

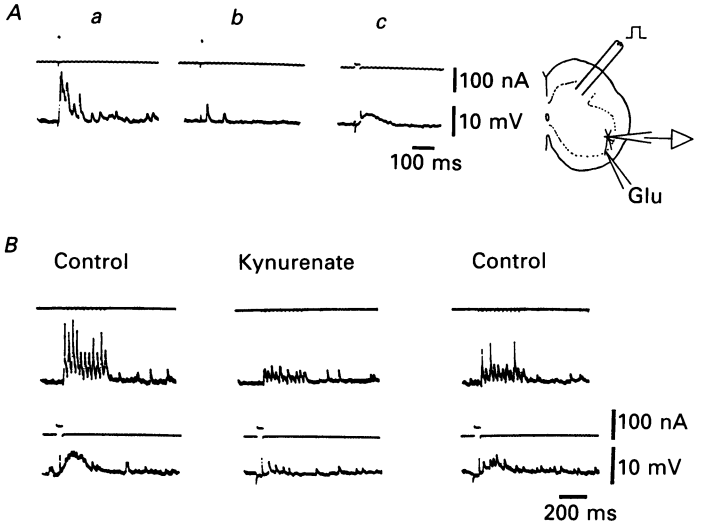

Fig. 2. Synaptic potentials and Glu potentials recorded from a motoneurone. A pair of silver electrodes (100 μm in diameter) was placed on the surface of the dorsal horn in the slice and a brief pulse was applied. Synaptic potentials were recorded intracellularly from the neurone in the ventral horn. The duration of stimulation at *Aa* was twice that at *Ab*. *Ac* is the Glu potential induced from the same neurone. *B*, the effect of kynurenate on synaptic potentials and Glu potentials. A train of stimulation was applied. By adding 1 mM of kynurenate to the perfusion solution, the synaptic potentials and Glu potentials were decreased. When kynurenate was washed out, the potentials recovered. The resting potentials of neurones *A* and *B* were 65 and 75 mV, respectively.

Figure 2*B* shows the effect of kynurenate, a non-specific inhibitor of amino acid receptors (Perkins & Stone, 1982; Ganong, Lanthorn & Cotman, 1983; Elmslie & Yoshikami, 1985; Jahr & Jessell, 1985), on EPSPs and the Glu potential. Application of 1 mM-kynurenate in perfusion solution suppressed EPSPs and Glu potential to the same extent (Fig. 2*B*).

Glutamate potential on the soma and dendrites

As shown in Fig. 3*A*, the Glu injection pipette was located on the edge of the soma, and as shown in Fig. 3*C*, the pipette was placed at the dendrite 80 μm from the edge of the soma, and Glu was applied. In this experiment one barrel of a double-barrelled micropipette was filled with Glu, the other with NMDA. Potential changes induced by ionophoretic application of amino acid are shown in Fig. 3*B* and *D*.

Glu was applied to the soma and dendrite of the same neurone by short pulses (1–5 ms), and responses induced by 2 ms pulses are shown in Fig. 4*a* (soma) and *b*

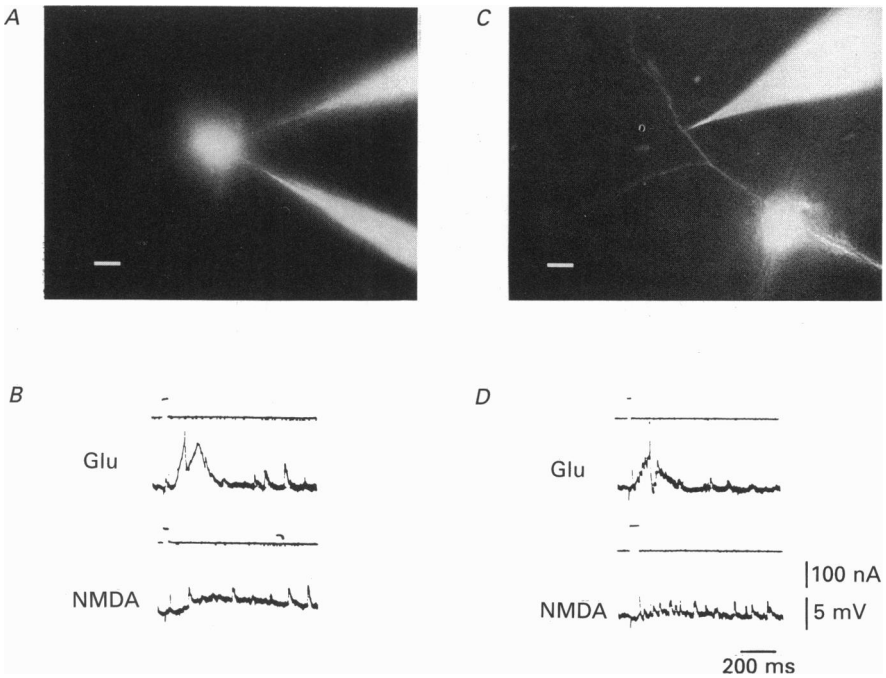

Fig. 3. The location of electrodes at the neurone (*A* and *C*) and electrical responses (*B* and *D*). The upper electrode in *A* or *C* is the double-barrelled pipette for the application of Glu and NMDA. The electrodes were located on the soma (*A*) and at the dendrite (*C*). The other electrode is a recording electrode inserted in the soma. *A* and *C* were photographed after the potential recordings (*B* and *D*) were made. The bars in *A* and *C* indicate 20 μm.

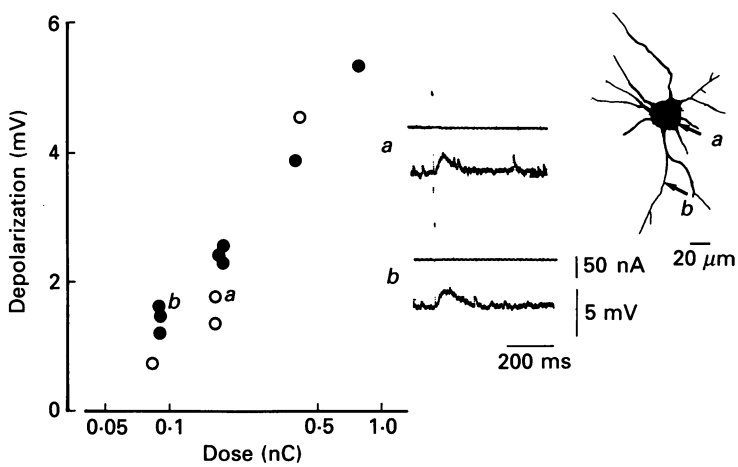

Fig. 4. The dose–depolarization curve of Glu potentials on the soma (○) and dendrite (●) of the same neurone. The positions of Glu application are shown in the illustrated neurone by *a* and *b*. Sample records of the Glu potential at *a* and *b* are shown in inset. The resting potential of the neurone was 55 mV.

(dendrite). Peak times at the soma and dendrite were 30 ms (*a*) and 31 ms (*b*), respectively. Figure 4 shows the dose–depolarization curves recorded on the soma (○) and on the dendrite (●). All points fall on approximately the same line. As discussed later, the falling phase of the Glu potential at the dendrite (*b*) was slightly

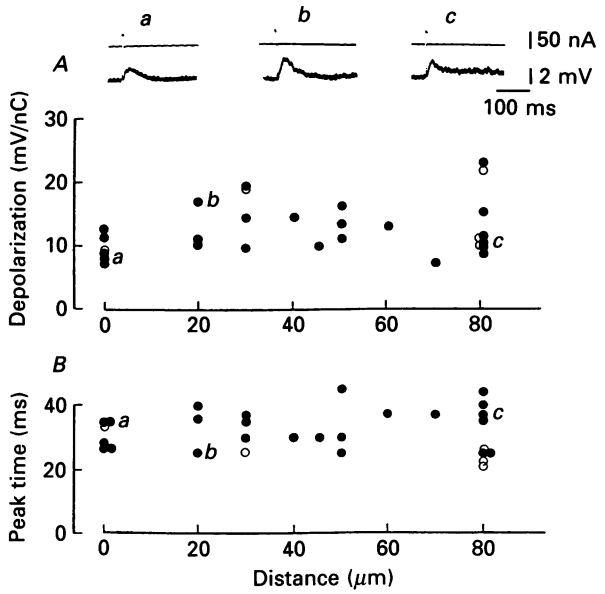

Fig. 5. Glu responses recorded along the dendrite. Each potential was induced by application for 1 (○) or 2 ms (●). *A*, peak amplitudes of Glu potentials. *B*, peak time of Glu potentials. Peak amplitudes are indicated as the values induced by unit charge (nC) for the generation of responses. The values at distance 0 were estimated from the data at the somata. Distance was measured between the edge of the soma and the tip of the pipette along the dendrite from photographs that were taken after the potential recording. Records at the top of the figure are sample records at three points on the dendrite induced by 2 ms pulses.

longer than at the soma (*a*), although the rising phase of the Glu potential was almost the same at both sites. The Glu potential generated on the dendrite usually had a long tail potential.

The Glu potential was produced by 1 or 2 ms pulses at various points on the soma and dendrites (Fig. 5, inset), and the peak time and amplitude of the Glu potential per unit dose (nC) are plotted against distance from the soma in Fig. 5. Peak time and depolarization are relatively constant along the dendrites of different neurones. The mean values for peak time and depolarization were $33{\cdot}1 \pm 1{\cdot}18$ ms (mean $\pm$ S.E.M. $n = 25$) and $12{\cdot}2 \pm 0{\cdot}76$ mV/nC ($n = 25$), respectively.

Sensitivity to NMDA

Sensitivities to NMDA and Glu were tested on the soma and dendrites by using a double-barrelled pipette. Application of NMDA and Glu to the soma at the same dose (1·4 nC) produced depolarization of 5·8 and 4·3 mV, respectively (Fig. 6*a*). The rise

and fall of the depolarization induced by NMDA were 3–5 times longer than those of Glu potentials. The dose–depolarization curves for Glu and NMDA on the soma were approximately the same as illustrated in Fig. 6*a*. The injection pipette was then moved to the dendrite, about 70 μm from the soma, as indicated in the inset by arrow

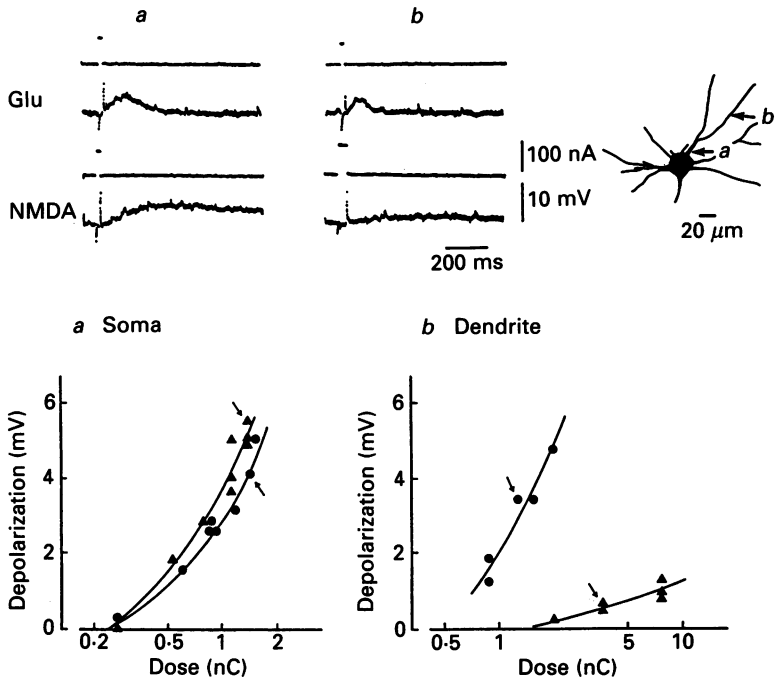

Fig. 6. Responses of NMDA and Glu at soma and dendrite of the same neurone. The double-barrelled pipette, filled with NMDA and Glu, was located at the soma (*a*) and dendrite (*b*) as shown in the illustration by arrows. NMDA and Glu were applied ionophoretically with a short pulse that is monitored on the upper record. The two graphs are the dose–depolarization curves of NMDA (▲) and Glu (●) at the soma (*a*) and dendrite (*b*).

b. The application of 1·2 nC Glu produced a depolarization of 3·6 mV, whereas a larger dose of NMDA (5 nC) applied to the same point produced only a 0·6 mV depolarization. Sensitivity to Glu at the dendrite as estimated from the dose–depolarization curves in Fig. 6*b* was 2·0 mV/nC. This value was approximately the same as that obtained in the soma of the same neurone (2·8 mV/nC), but the sensitivity to NMDA at the dendrite (0·12 mV/nC) was much less than that at the soma (3·5 mV/nC).

The distribution of sensitivity to NMDA along the dendrite is shown in Fig. 7. Sensitivities varied from neurone to neurone, probably due to the distance between the pipette and the membrane. Therefore, the relative sensitivities to NMDA were evaluated from the ratio of NMDA sensitivity to Glu sensitivity at the same point. The mean relative sensitivity to NMDA at the soma and at the dendrite near the

soma (0–10 μm) was $0{\cdot}6 \pm 0{\cdot}10$ ($n = 14$); this value decreased as the distance from the soma increased (Fig. 7). The mean sensitivity of NMDA was $5{\cdot}0 \pm 0{\cdot}96$ mV/nC ($n = 14$) and that of Glu was $9{\cdot}9 \pm 2{\cdot}05$ mV/nC ($n = 14$) at the soma.

The above observations suggest that the density of NMDA receptors is lower in dendrites than in soma. If this is true, it is expected that the NMDA response

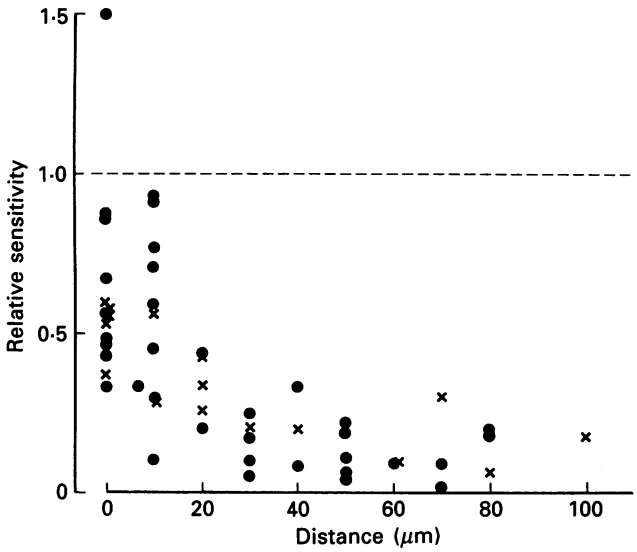

Fig. 7. Relative sensitivities of NMDA and Asp along the dendrite. The sensitivities of Glu and other amino acids (mV/nC) were estimated from the dose–depolarization curves, and the ratio of amino acid sensitivity to Glu sensitivity was plotted along the distance from the soma. ●, NMDA; ×, Asp.

contributes greatly to the Glu potential produced at the soma. This possibility was tested by applying 50 μM APV in the bath solution. It was found that the Glu potential at the soma was decreased to 19% of the control level by APV (Fig. 8*b*), whereas that of the dendrite was decreased to 55% of the control (Fig. 8*a*). The NMDA response was almost abolished by APV. Therefore a large part of the Glu potential produced at the soma may be mediated by the activation of NMDA receptors, and the contribution of non-NMDA receptors to Glu responses may be large at the dendrite.

Sensitivity to aspartate

Aspartate was applied at the soma and dendrite of the same neurone. The amplitude of the depolarization produced by Asp at the soma was about half that of Glu responses induced by the same dose at the same point (Fig. 9*a*). At the dendrite 30 μm apart from the soma, Asp responses were about one-fifth of Glu responses (Fig. 9*b*). The time course of the Asp potential was slightly longer than that of the Glu potential. When the relative sensitivity of Asp to that of Glu at the same point was

measured, it was found to be decreased as the distance from the soma increased, as shown in Fig. 7 (×).

Sensitivity to kainate

Glutamate and KA were applied to the soma and dendrite of the same neurone (Fig. 10*A*). At the soma (*a*), the same depolarization (0·8 mV) was induced by 70 pC of Glu

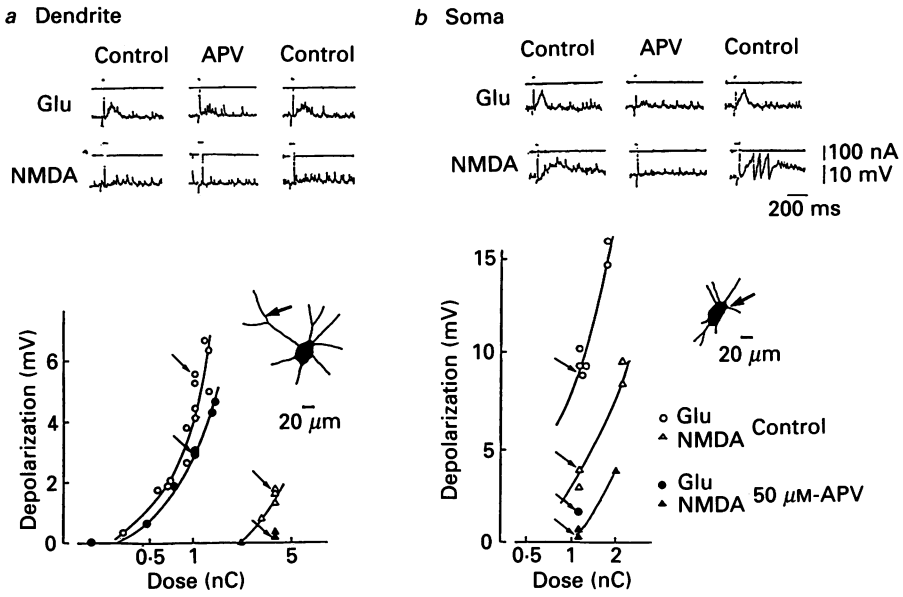

Fig. 8. Effect of APV on Glu responses on soma and dendrite. Glu and NMDA were applied from a double-barrelled pipette. The positions of application are shown by arrows in the illustration. The injection currents are shown in the upper traces. The duration of current was 20 ms in all experiments except for that of NMDA (50 ms) at the dendrite. NMDA produced large responses on the soma and very small responses on the dendrite. APV (50 μM) added to the bath solution diminished the responses of NMDA on soma and dendrite. APV reduced the Glu potential to 19% of the control at the soma, but 55% of the Glu potential remained at the dendrite. After APV solution was replaced with normal solution these responses recovered completely.

and 130 pC of KA. However, at the dendrite about 50 μm from the soma, KA (3 nC) produced only a small depolarization (*b*), though a considerable depolarization (3·5 mV) was induced by the application of Glu (2 nC) at the same spot. The rise and fall of KA potentials was slow compared with those of Glu potentials. The low sensitivity of KA at the dendrite is shown in another cell in Fig. 10*B*. In this case the respective barrels of an injection pipette were filled with Quis and KA. The amplitude of the Quis responses was almost the same on dendrite and soma, whereas very small depolarization was generated by KA on the dendrite. The relative value of KA sensitivity to Glu sensitivity was measured and plotted against the distance along the dendrite. The relative KA sensitivity on the soma was about 0·5, but this decreased to about 0·25 on the dendrite (Fig. 10*C*).

Sensitivity to quisqualate

The application of Quis induced membrane depolarizations at all points on the neurone where Glu potentials were produced. Figure 11 shows an example recorded on the dendrite 50 μm from the soma. Quis and Glu were applied with a current pulse

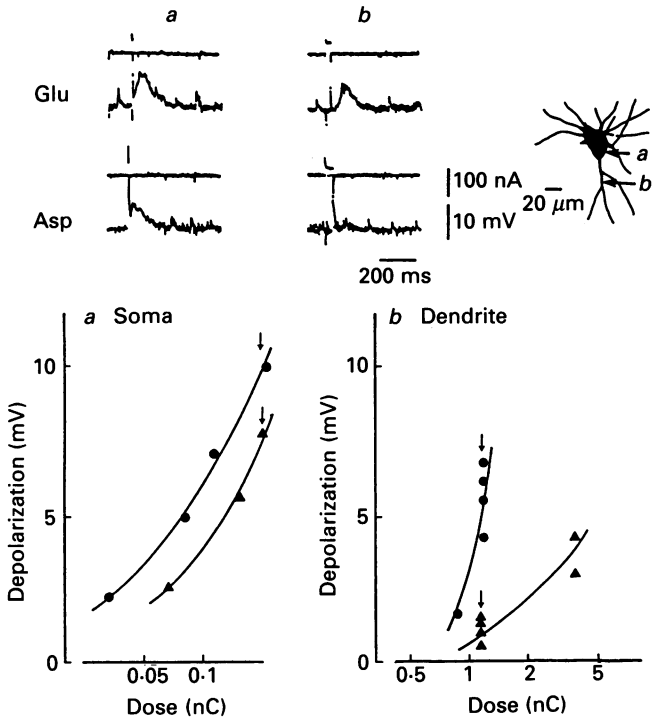

Fig. 9. Responses induced by Asp on the soma and dendrite of the same neurone. Glu and Asp were applied with a short current by a double-barrelled pipette at the same points at the soma (*a*) and dendrite (*b*). The positions of the tip of pipette are shown by arrows in the picture of neurone. The injection currents are shown in the upper traces. The graphs in the figure represent the dose-depolarization curves of Glu (●) and Asp (▲), on the soma (*a*) and dendrite (*b*).

of 5 ms duration. The Quis response had two phases: a fast response ($Quis_{fast}$) followed by a very slow depolarization ($Quis_{slow}$) that lasted for over 10 s. The fast responses could be observed only when the injection pipette was located close to the neurone membrane. The time course of $Quis_{fast}$ was shorter than that of the Glu response, the peak time and the half-time of the falling phase of $Quis_{fast}$ being $69 \pm 9\%$ ($n = 10$) and $46 \pm 11\%$ ($n = 10$) of those of the Glu potential induced at the same spot, respectively. Figure 11 also shows that the Glu response had a slow tail potential (Glu_{slow}). The dose–depolarization curves of the fast and slow responses of Glu and Quis are presented on the right side of Fig. 11.

The amplitude of $Quis_{fast}$ reached a maximal value as the dose of Quis was increased. The peak amplitude of $Quis_{fast}$ was approximately the same on the soma

and dendrites of different neurones (Fig. 12*B*), the mean being $0{\cdot}9 \pm 0{\cdot}07$ mV ($n = 25$). Although the sensitivities (mV/nC) of responses estimated from the dose–depolarization curves varied, the highest sensitivities to Glu_{fast}, $Quis_{fast}$ and $Quis_{slow}$ were 27·3, 10·6 and 20·8 mV/nC, respectively. The ratio of $Quis_{slow}$ to $Quis_{fast}$ is

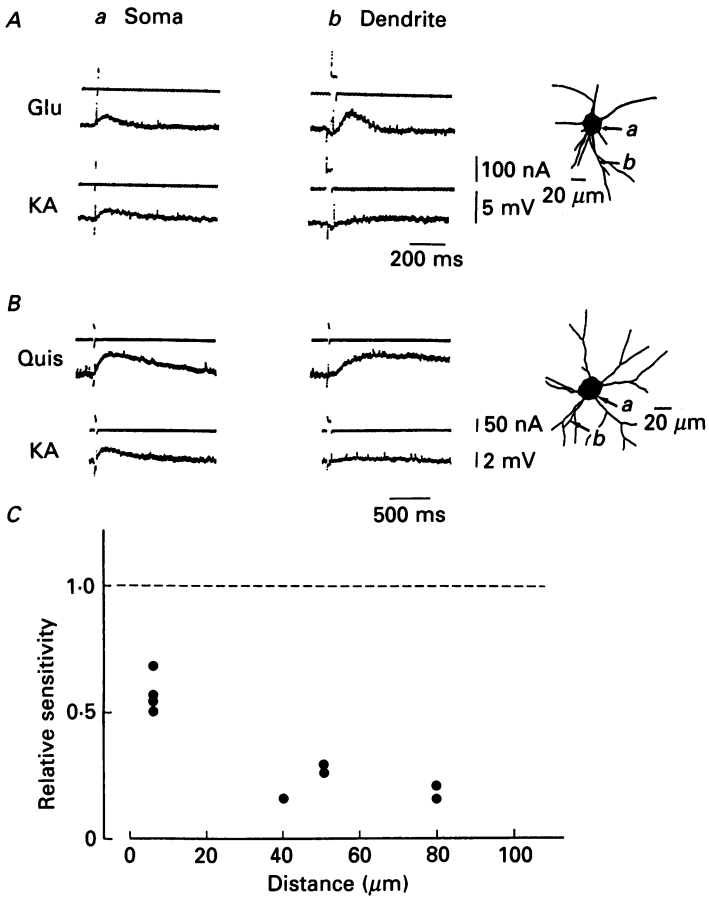

Fig. 10. KA responses on the soma and the dendrite of the same neurone induced by local application with a short current. KA responses were compared with the responses of Glu (*A*) or those of Quis (*B*) using the double-barrelled pipette. KA responses on the dendrite (*b*) were small compared with those on the soma (*a*). Durations of injection current in *A*: 1 ms (Glu) and 2 ms (KA) at soma, 30 ms (Glu) and 50 ms (KA) at dendrite. In *B*, durations of injection were 20 ms for both Quis and KA at the soma, and 20 ms for Quis and 50 ms for KA at the dendrite. *C*, relative sensitivity of KA to Glu sensitivity. The sensitivities were estimated from the dose–depolarization curves.

shown in Fig. 12*A*. $Quis_{slow}$ was relatively small on the soma and tended to be large on remote points of the dendrite.

Another difference between $Quis_{slow}$ at soma and dendrite was that of time course. When Quis was applied to the soma, the time course of $Quis_{slow}$ was relatively short compared with that produced at the dendrite and lacked a large tail potential (Fig.

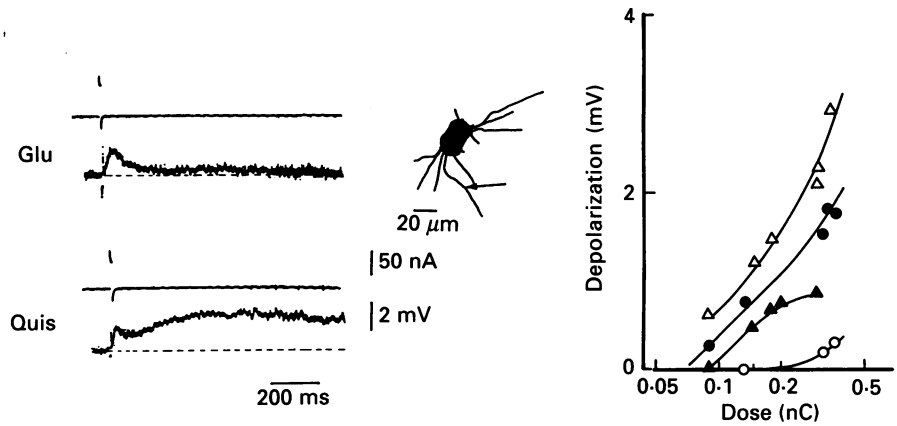

Fig. 11. Quis and Glu responses on the dendrite. A double-barrelled pipette was located close to the dendrite (indicated by an arrow in the inset). Slow and fast phases of response were observed as a result of the application of Glu and Quis with a pulse of 5 ms. The injection currents are shown in upper traces. Right, the dose–response curves of Glu (○, ●) and Quis (△, ▲). Each has fast (●, ▲) and slow (○, △) responses.

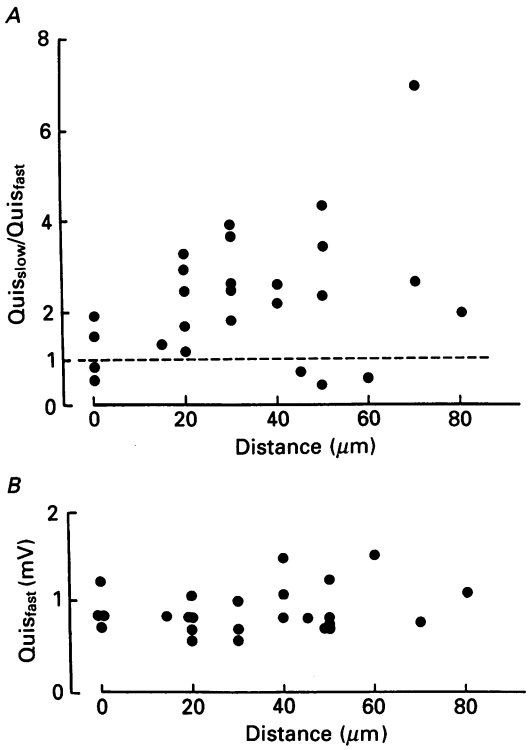

Fig. 12. Quis responses induced by an application of 2–5 ms duration at various points along the dendrite. The Quis response had fast and slow phases of depolarization (Fig. 11). The amplitudes of maximum response of $Quis_{fast}$ obtained at various points along the dendrite are plotted in *B*. *A*, relative amplitude of $Quis_{slow}$ compared with $Quis_{fast}$. The points at distance 0 were obtained on the soma.

10*Ba*). Application of 50 μM-APV had no effect on $Quis_{fast}$. $Quis_{slow}$ produced at the dendrite was not reduced by APV. However, the $Quis_{slow}$ produced at the soma was slightly reduced (by 25%) by 50 μM-APV. Quis may have partly activated NMDA receptors on the soma membrane. Foster & Fagg (1987) reported that Quis has an

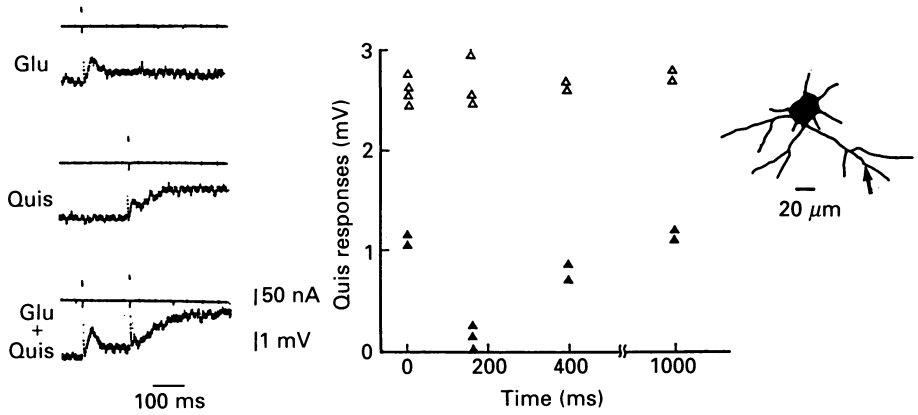

Fig. 13. Desensitization of the Quis response. The fast Quis response was almost completely abolished by a 150 ms prior application of Glu at the same point on the neurone (indicated by an arrow in the inset). Right, the interval between Glu and Quis applications is shown by the horizontal scale. The ordinate indicates the peak amplitudes of fast Quis (▲) and slow Quis (△) responses.

affinity for NMDA binding sites, although the affinity to NMDA receptors was higher than that for Quis receptor sites.

$Quis_{fast}$ was easily desensitized by repetitive applications of Quis or prior application of Glu to the same spot. Figure 13 shows an example where Glu and Quis were applied to a dendrite (indicated by the arrow in the inset). The top and middle traces in Fig. 13 are responses produced by single applications of Glu and Quis, respectively. As shown in the bottom trace, the application of Glu about 180 ms before Quis injection almost completely abolished $Quis_{fast}$ but produced little change in $Quis_{slow}$. The amplitudes of $Quis_{fast}$ and $Quis_{slow}$ are plotted against the interval between Glu and Quis injection on the right of Fig. 13. These results suggest that $Quis_{slow}$ and $Quis_{fast}$ are mediated by different receptors and that the density of the receptors responsible for $Quis_{slow}$ is higher at the dendrite than at the soma.

Removal of Ca^{2+} from the perfusion solution did not change the fast and slow Quis responses. But when a recording electrode was filled with EGTA (1·75 mM) and injected into the neurone, $Quis_{slow}$ tended to be depressed, while $Quis_{fast}$ remained unchanged. An example is shown in Fig. 14. Quis and Glu were applied to the dendrite about 80 μm from the soma and their responses 5 min after EGTA injection are shown at the top of the figure. $Quis_{slow}$ and Glu_{slow} were relatively small as compared with the ordinary responses, and they decreased further as time passed. At 13 min after EGTA injection the slow phases were almost completely abolished, while Glu_{fast} and $Quis_{fast}$ were unchanged (Fig. 14, top right). The peak amplitudes of Quis and Glu potentials are plotted against time after EGTA injection in the lower part of Fig. 14.

DISCUSSION

When the soma and dendrites of neurones in spinal slices were visualized by the injection of Lucifer Yellow, the tip of the Glu pipette containing Lucifer Yellow could be brought to localized parts of the neurone under visual control. Close application

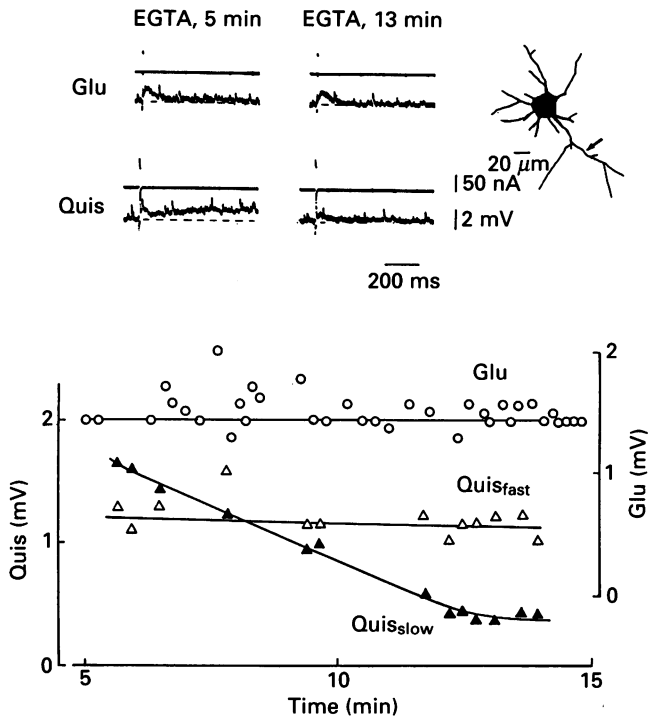

Fig. 14. Effect of intracellular injection of EGTA. The recording microelectrode was filled with EGTA (1·7 mM) and injected into the neurone. Quis and Glu were applied at the dendrite (shown in the inset by an arrow). The upper traces are sample records of 5 and 13 min after EGTA injection. The peak amplitudes (in mV) of fast responses of Quis and Glu and that of the slow Quis response are plotted in the lower part. Horizontal scale, time after EGTA injection.

of Glu with short pulses of 2 ms duration produced a Glu potential that reached its peak in $33{\cdot}1 \pm 1{\cdot}18$ ms (mean $\pm$ S.D., $n = 25$). If the concentration of Glu matched the time course of the Glu response, the distance between the tip of the pipette and the surface of the neurone could be calculated from the peak time using the diffusion equation (Takeuchi & Takeuchi, 1964). Assuming that the diffusion constant would be that of glutamine ($7{\cdot}62 \times 10^{-6}$ cm^2/s at 25 °C) and that Glu was injected instantaneously in a homogeneous infinite media, the distance between the tip of the pipette and the surface of the membrane would be about 10 μm assuming 30 ms of peak time. However, this value may be rather overestimated, because a distance of 10 μm could be relatively easily detected under the fluorescent microscope and it was possible to bring the pipette closer to the neurone membrane. Therefore, the diffusion

constant of glutamine used for the calculation would be large for the diffusion of Glu in the spinal cord. Since the neurone is surrounded by glia, the pathway for the diffusion of Glu may be limited, resulting in an increase in the apparent diffusion constant.

Since potential changes were recorded from the soma while amino acids were applied to dendrites, the passive cable properties of motoneurones must be taken into consideration (Rall, 1977). However, since the present experiments were carried out on short dendrites within 100 μm of the soma, the decrement in potential would be small. It has been shown that the equivalent distance from soma as an electrical function is longer than 1 mm in cat spinal motoneurones (Fleshman, Segev & Burke, 1988; Clements & Redman, 1989).

High sensitivity to Glu was observed throughout the neurone membrane. The sensitivity of the soma membrane to NMDA and KA was similar to that to Glu, whereas the dendrite showed relatively low sensitivity to NMDA and KA. These results suggest that the densities of NMDA and KA receptors in soma and dendrites are different. Sensitivity to Asp was approximately half that to Glu on soma, and it was further decreased on dendrite. The distribution of sensitivities to Asp was similar to that of NMDA.

From studies using cultured neurones (Kiskin, Krishtal & Tsyndrenko, 1986; Zorumski & Yang, 1988) and oocytes injected with brain mRNA (Rassendren, Lory, Pin, Bockaert & Nargeot, 1989; Lerma, Kushner, Zukin & Bennett, 1989), it has been suggested that Quis and KA activate the same excitatory receptor or the same receptor–channel complex. However, different properties of Quis and KA have been reported in cultured brain neurones of the rat. The whole-cell currents induced by Quis differed from those induced by KA in their dose–response characteristics, desensitization patterns and selective blockade by kynurenate (Perouansky & Grantyn, 1989). In the present experiment it was found that the application of Quis to the dendrite membrane produced depolarization, while KA applied to the same spot produced no appreciable change in potential (see Fig. 10*B*). Quis and KA applied to the soma membrane produced the same degree of depolarization. Therefore, different receptors may be responsible for Quis and KA responses, and the distribution of Quis and KA receptors may be different between soma and dendrite membranes.

It has been found that NMDA, Quis and KA receptor sites show a transient increase in the immature brain (Nicoletti, Indarola, Wroblewski & Costa, 1986; Silverstein, Chen & Johnston, 1986; Hamon & Heinemann, 1988; Tremblay, Roisin, Represa, Charriaut-Marlangue & Ben-Ari, 1988; Erdö & Wolff, 1989; Insel, Miller & Gelhard, 1990; Luhmann & Prince, 1990). Further experiments will be necessary to study whether the distribution of receptors on the neurone membrane in neonatal rats, as observed in the present experiments, is the same as that in adult animals.

The application of Quis produced two kinds of responses on the neurones: fast and slow depolarizations. The time course of the fast Quis response was shorter than the Glu response produced at the same spot and was easily desensitized by the prior application of Glu. These observations correspond to the fact that in patch clamp experiments the open time of a single channel activated by Quis is shorter than one activated by Glu (Ascher, Bregestovski & Nowak, 1988; Ascher & Nowak, 1988;

Cull-Candy, Howe & Ogden, 1988; Cull-Candy & Usowicz, 1989*a*, *b*). In cultured neurones Quis produced a fast depolarization followed by a slow response (Jessell, Yoshioka & Jahr, 1986; Trussell *et al.* 1988; Joels, Yool & Gruol, 1989), and the fast depolarization was easily desensitized (Kiskin *et al.* 1986; Trussell *et al.* 1988; Tang, Dichter & Morad, 1989; Mayer & Vyklický, 1989). These observations are similar to those of the present experiment. However, the slow depolarization in cultured neurones was abolished by APV (Trussell *et al.* 1988), whereas in the present experiment the amplitude of $Quis_{slow}$ produced at the dendrite was little affected by APV. Furthermore, the slow response observed in the present experiment lasted for more than 10 s after a close ionophoretic application with 2–5 ms pulses. Although the possibility of slow diffusion of Quis cannot be excluded (Johnston, Lodge, Bornstein, Curtis, 1980; Lodge, Curtis, Johnston, Bornstein, 1980; Sawada, Higashi & Yamamoto, 1985; Horne & Simmonds, 1989), the application of other agonists like KA and NMDA to the same spot produced responses that lasted only several hundred milliseconds. It has been reported that the short application of Quis to cultured Purkinje neurones produced prolonged components that might be mediated by intracellular messenger systems (Joels *et al.* 1989). The extremely slow time course and the reduction of response induced by EGTA injection in the present experiment might be attributed to a second messenger system that regulates the mobilization of intracellular calcium (Nicoletti *et al.* 1986; Sugiyama, Ito & Hirono, 1987; Murphy & Miller, 1988; Sladeczek, Récasens & Bockaert, 1988; Furuya, Ohmori, Shigemoto & Sugiyama, 1989; Weiss, 1989). Slow depolarization was also observed after Glu was applied at the dendrite where $Quis_{slow}$ was dominant, although the potential was smaller than $Quis_{slow}$ (Fig. 11, Glu). The results suggest that the receptor for $Quis_{slow}$ is also activated by Glu.

Since Quis receptors are widely distributed over the motoneurone membrane, this receptor may make an important contribution to synaptic transmission. It has been proposed that monosynaptic transmission of motoneurone may be mediated through Quis receptors, probably activated by Glu (Jessell *et al.* 1986). Similarities between the time course of EPSPs and the Quis response support the above supposition (Forsythe & Westbrook, 1988; Tang *et al.* 1989). Further experiments are certainly needed to clarify the physiological significance of different types of amino acid receptors, but the receptors responsible for slow responses, as observed in the present experiment, are also widely distributed on dendrites and may play some physiological role in neural transmission.

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REFERENCES

ASCHER, P., BREGESTOVSKI, P. & NOWAK, L. (1988). *N*-Methyl-D-aspartate-activated channels of mouse central neurones in magnesium-free solutions. *Journal of Physiology* **399**, 207–226.

ASCHER, P. & NOWAK, L. (1988). Quisqualate- and kainate-activated channels in mouse central neurones in culture. *Journal of Physiology* **399**, 227–245.

BEKKERS, J. M. & STEVENS, C. F. (1989). NMDA and non-NMDA receptors are co-localized at individual excitatory synapses in cultured rat hippocampus. *Nature* **341**, 230–233.

CLEMENTS, J. D. & REDMAN, S. J. (1989). Cable properties of cat spinal motoneurones measured by combining voltage clamp, current clamp and intracellular staining. *Journal of Physiology* **409**, 63–87.

CULL-CANDY, S. G., HOWE, J. R. & OGDEN, D. C. (1988). Noise and single channels activated by excitatory amino acids in rat cerebellar granule neurones. *Journal of Physiology* **400**, 189–222.

CULL-CANDY, S. G. & USOWICZ, M. M. (1989*a*). Whole-cell current noise produced by excitatory and inhibitory amino acids in large cerebellar neurones of the rat. *Journal of Physiology* **415**, 533–553.

CULL-CANDY, S. G. & USOWICZ, M. M. (1989*b*). On the multiple-conductance single channels activated by excitatory amino acids in large cerebellar neurones of the rat. *Journal of Physiology* **415**, 555–582.

DALE, N. & ROBERTS, A. (1985). Dual component amino-acid-mediated synaptic potentials: excitatory drive for swimming in *Xenopus* embryos. *Journal of Physiology* **363**, 35–59.

DAVIES, J. & WATKINS, J. C. (1982). Actions of D and L forms of 2-amino-5-phosphonovalerate and 2-amino-4-phosphonobutyrate in the cat spinal cord. *Brain Research* **235**, 378–386.

DAVIES, J. & WATKINS, J. C. (1983). Role of excitatory amino acids receptors in mono- and polysynaptic excitation in the cat spinal cord. *Experimental Brain Research* **49**, 280–290.

DUGGAN, A. W. & JOHNSTON, G. A. R. (1970). Glutamate and related amino acids in cat spinal roots, dorsal root ganglia and peripheral nerves. *Journal of Neurochemistry* **17**, 1205–1208.

ELMSLIE, K. S. & YOSHIKAMI, D. (1985). Effects of kynurenate on root potentials evoked by synaptic activity and amino acids in the frog spinal cord. *Brain Research* **330**, 265–275.

ERDÖ, S. L. & WOLFF, J. R. (1989). Transient increase in ligand binding to quisqualate and kainate sites in cerebral cortex of immature rats. *Neuroscience Letters* **104**, 161–166.

FAGG, G. E., JORDAN, C. C. & WEBSTER, R. A. (1976). The release of endogenous amino acids from the cat spinal cord *in vivo*. *British Journal of Pharmacology* **58**, 440–441P.

FINKEL, A. S. & REDMAN, S. J. (1983). The synaptic current evoked in cat spinal motoneurones by impulses in single group 1a axons. *Journal of Physiology* **342**, 615–632.

FLATMAN, J. A., LAMBERT, J. D. C. & ENGBERG, I. (1985). The variation in action of excitatory amino acids in relation to distance of iontophoretic application to spinal motoneurones. *Acta Physiologica Scandinavica* **124**, 421–427.

FLESHMAN, J. W., SEGEV, I. & BURKE, R. E. (1988). Electrotonic architecture of type-identified α-motoneurons in the cat spinal cord. *Journal of Neurophysiology* **60**, 60–85.

FORSYTHE, I. D. & WESTBROOK, G. L. (1988). Slow excitatory postsynaptic currents mediated by *N*-methyl-D-aspartate receptors on cultured mouse central neurones. *Journal of Physiology* **396**, 515–533.

FOSTER, A. C. & FAGG, G. E. (1987). Comparison of L-[^{3}H]glutamate, D-[^{3}H]aspartate, DL-[^{3}H]AP5 and [^{3}H]NMDA as ligands for NMDA receptors in crude postsynaptic densities from rat brain. *European Journal of Pharmacology* **133**, 291–300.

FURUYA, S., OHMORI, H., SHIGEMOTO, T. & SUGIYAMA, H. (1989). Intracellular calcium mobilization triggered by a glutamate receptor in rat cultured hippocampal cells. *Journal of Physiology* **414**, 539–548.

GANONG, A. H., LANTHORN, T. H. & COTMAN, C. W. (1983). Kynurenic acid inhibits synaptic and acidic amino acid-induced responses in the rat hippocampus and spinal cord. *Brain Research* **273**, 170–174.

HAMON, B. & HEINEMANN, U. (1988). Developmental changes in neuronal sensitivity to excitatory amino acids in area CA1 of the rat hippocampus. *Developmental Brain Research* **38**, 286–290.

HORNE, A. L. & SIMMONDS, M. A. (1989). The pharmacology of quisqualate and AMPA in the cerebral cortex of the rat *in vitro*. *Neuropharmacology* **28**, 1113–1118.

INSEL, T. R., MILLER, L. P. & GELHARD, R. E. (1990). The ontogeny of excitatory amino acid receptors in rat forebrain. I. *N*-Methyl-D-aspartate and quisqualate receptors. *Neuroscience* **35**, 31–43.

JAHR, C. E. & JESSELL, T. M. (1985). Synaptic transmission between dorsal root ganglion and dorsal horn neurons in culture: antagonism of monosynaptic excitatory postsynaptic potentials and glutamate excitation by kynurenate. *Journal of Neuroscience* **5**, 2281–2289.

JESSELL, T. M., YOSHIOKA, K. & JAHR, C. E. (1986). Amino acid receptor-mediated transmission at primary afferent synapses in rat spinal cord. *Journal of Experimental Biology* **124**, 239–258.

JOELS, M., YOOL, A. J. & GRUOL, D. L. (1989). Unique properties of non-*N*-methyl-D-aspartate excitatory responses in cultured Purkinje neurons. *Proceedings of the National Academy of Sciences of the USA* **86**, 3404–3408.

JOHNSTON, G. A. R., LODGE, D., BORNSTEIN, J. C. & CURTIS, D. R. (1980). Potentiation of L-glutamate and L-aspartate excitation of cat spinal neurones by the stereoisomers of threo-3-hydroxyaspartate. *Journal of Neurochemistry* **34**, 241–243.

JONES, I. M., JORDAN, C. C., MORTON, I. K. M., STAGG, C. J. & WEBSTER, R. A. (1974). The effect of chronic dorsal root section on the concentration of free amino acids in the rabbit spinal cord. *Journal of Neurochemistry* **23**, 1239–1244.

KAWAGOE, R., ONODERA, K. & TAKEUCHI, A. (1985). Release of endogenous glutamate from the frog spinal cord following dorsal root stimulation. *Biomedical Research* **6**, 239–245.

KAWAGOE, R., ONODERA, K. & TAKEUCHI, A. (1986). The release of endogenous glutamate from the newborn rat spinal cord induced by dorsal root stimulation and substance P. *Biomedical Research* **7**, 253–259.

KISKIN, N. I., KRISHTAL, O. A. & TSYNDRENKO, A. Y. (1986). Excitatory amino acid receptors in hippocampal neurons: kainate fails to desensitize them. *Neuroscience Letters* **63**, 225–230.

LERMA, J., KUSHNER, L., ZUKIN, R. S. & BENNETT, M. V. L. (1989). *N*-Methyl-D-aspartate activates different channels than do kainate and quisqualate. *Proceedings of the National Academy of Sciences of the USA* **86**, 2083–2087.

LODGE, D., CURTIS, D. R., JOHNSTON, G. A. R. & BORNSTEIN, J. C. (1980). In vivo inactivation of quisqualate: studies in the cat spinal cord. *Brain Research* **182**, 491–495.

LUHMANN, H. J. & PRINCE, D. A. (1990). Transient expression of polysynaptic NMDA receptor-mediated activity during neocortical development. *Neuroscience Letters* **111**, 109–115.

MAYER, M. L. & VYKLICKÝ, L. JR (1989). Concanavalin A selectively reduces desensitization of mammalian neuronal quisqualate receptors. *Proceedings of the National Academy of Sciences of the USA* **86**, 1411–1415.

MAYER, M. L. & WESTBROOK, G. L. (1984). Mixed-agonist action of excitatory amino acids on mouse spinal cord neurones under voltage clamp. *Journal of Physiology* **354**, 29–53.

MAYER, M. L. & WESTBROOK, G. L. (1987). The physiology of excitatory amino acids in the vertebrate central nervous system. *Progress in Neurobiology* **28**, 197–276.

MURPHY, S. N. & MILLER, R. J. (1988). A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. *Proceedings of the National Academy of Sciences of the USA* **85**, 8737–8741.

NELSON, P. G., PUN, R. Y. K. & WESTBROOK, G. L. (1986). Synaptic excitation in cultures of mouse spinal cord neurones: receptor pharmacology and behaviour of synaptic currents. *Journal of Physiology* **372**, 169–190.

NICOLETTI, F., IADAROLA, M. J., WROBLEWSKI, J. T. & COSTA, E. (1986). Excitatory amino acid recognition sites coupled with inositol phospholipid metabolism: Developmental changes and interaction with α_1-adrenoceptors. *Proceedings of the National Academy of Sciences of the USA* **83**, 1931–1935.

O'BRIEN, R. J. & FISCHBACH, G. D. (1986*a*). Excitatory synaptic transmission between interneurons and motoneurons in chick spinal cord cell cultures. *Journal of Neuroscience* **6**, 3284–3289.

O'BRIEN, R. J. & FISCHBACH, G. D. (1986*b*). Modulation of embryonic chick motoneuron glutamate sensitivity by interneurons and agonists. *Journal of Neuroscience* **6**, 3290–3296.

ONODERA, K. & TAKEUCHI, A. (1986). Dual actions of substance P on the motoneurons of the newborn rat spinal cord *in vitro*. *Biomedical Research* **7**, 245–252.

ONODERA, K. & TAKEUCHI, A. (1988). Excitatory amino acid receptors in ventral horn neurons of the rat. *Biomedical Research* **9**, suppl. 2, 63–66.

ONODERA, K. & TAKEUCHI, A. (1989). Distribution of amino acid receptors on ventral horn neurons of spinal cord. *Proceedings of the International Union of Physiological Sciences* **17**, 407.

PERKINS, M. N. & STONE, T. W. (1982). An iontophoretic investigation of the actions of convulsant kynurenines and their interaction with the endogenous excitant quinolinic acid. *Brain Research* **247**, 184–187.

PEROUANSKY, M. & GRANTYN, R. (1989). Separation of quisqualate- and kainate-selective glutamate receptors in cultured neurons from the rat superior colliculus. *Journal of Neuroscience* **9**, 70–80.

RALL, W. (1977). Core conductor theory and cable properties of neurons. In *The Nervous System. Cellular Biology of Neurons*, ed. KANDEL, E. R., pp. 39–97. American Physiological Society, Bethesda, MD, USA.

RASSENDREN, F. A., LORY, P., PIN, J. P., BOCKAERT, J. & NARGEOT, J. (1989). A specific quisqualate agonist inhibits kainate responses induced in *Xenopus* oocytes injected with rat brain RNA. *Neuroscience Letters* **99**, 333–339.

ROBERTS, P. J., KEEN, P. & MITCHELL, J. F. (1973). The distribution and axonal transport of free amino acids and related compounds in the dorsal sensory neuron of the rat, as determined by the dansyl reaction. *Journal of Neurochemistry* **21**, 199–209.

SALT, T. E. & HILL, R. G. (1983). Neurotransmitter candidates of somatosensory primary afferent fibres. *Neuroscience* **10**, 1083–1103.

SAWADA, S., HIGASHI, M. & YAMAMOTO, C. (1985). Inhibitors of high-affinity uptake augment depolarizations of hippocampal neurons induced by glutamate, kainate and related compounds. *Experimental Brain Research* **60**, 323–329.

SILVERSTEIN, F. S., CHEN, R. & JOHNSTON, M. V. (1986). The glutamate analogue quisqualic acid is neurotoxic in striatum and hippocampus of immature rat brain. *Neuroscience Letters* **71**, 13–18.

SLADECZEK, F., RÉCASENS, M. & BOCKAERT, J. (1988). A new mechanism for glutamate receptor action: phosphoinositide hydrolysis. *Trends in Neurosciences* **11**, 545–549.

SUGIYAMA, H., ITO, I. & HIRONO, C. (1987). A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature* **325**, 531–533.

TAKAHASHI, T. (1978). Intracellular recording from visually identified motoneurons in rat spinal cord slices. *Proceedings of the Royal Society of London* B **202**, 417–421.

TAKAHASHI, T. (1990). Membrane currents in visually identified motoneurones of neonatal rat spinal cord. *Journal of Physiology* **423**, 27–46.

TAKAHASHI, T. & TSURUHARA, H. (1987). Slow depolarizing potentials recorded from glial cells in the rat superficial dorsal horn. *Journal of Physiology* **388**, 597–610.

TAKEUCHI, A. (1987). The transmitter role of glutamate in nervous systems. *Japanese Journal of Physiology* **37**, 559–572.

TAKEUCHI, A. & TAKEUCHI, N. (1964). The effect on crayfish muscle of iontophoretically applied glutamate. *Journal of Physiology* **170**, 296–317.

TANG, C., DICHTER, M. & MORAD, M. (1989). Quisqualate activates a rapidly inactivating high conductance ionic channel in hippocampal neurons. *Science* **243**, 1474–1477.

TREMBLAY, E., ROISIN, M. P., REPRESA, A., CHARRIAUT-MARLANGUE, C. & BEN-ARI, Y. (1988). Transient increased density of NMDA biding sites in the developing rat hippocampus. *Brain Research* **461**, 393–396.

TRUSSELL, L. O., THIO, L. L., ZORUMSKI, C. F. & FISCHBACH, G. D. (1988). Rapid desensitization of glutamate receptors in vertebrate central neurons. *Proceedings of the National Academy of Sciences of the USA* **85**, 4562–4566.

WATKINS, J. C. & EVANS, R. H. (1981). Excitatory amino acid transmitters. *Annual Review of Pharmacology and Toxicology* **21**, 165–204.

WEISS, S. (1989). Two distinct quisqualate receptor systems are present on striatal neurons. *Brain Research* **491**, 189–193.

ZIEGLGÄNSBERGER, W. & CHAMPAGNAT, J. (1979). Cat spinal motoneurones exhibit topographic sensitivity to glutamate and glycine. *Brain Research* **160**, 95–104.

ZISKIND-CONHAIM, L. (1990). NMDA receptors mediate poly- and monosynaptic potentials in motoneurons of rat embryos. *Journal of Neuroscience* **10**, 125–135.

ZORUMSKI, C. F. & YANG, J. (1988). AMPA, kainate, and quisqualate activate a common receptor–channel complex on embryonic chick motoneurons. *Journal of Neuroscience* **8**, 4277–4286.