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AMINO ACID-MEDIATED EPSPs AT PRIMARY AFFERENT SYNAPSES WITH SUBSTANTIA GELATINOSA NEURONES IN THE RAT SPINAL CORD

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SUMMARY

1. Fast excitatory postsynaptic potentials (EPSPs) evoked by stimulation of Aδ and C fibres were examined by intracellular recording from substantia gelatinosa (SG) neurones in a transverse slice preparation of adult rat spinal cord.

2. Single low-intensity stimuli applied to the dorsal root activated Aδ fibres and evoked monosynaptic EPSPs in 70% of SG neurones. In 5% of SG neurones, increasing the intensity and duration of stimulation evoked solely C fibre-mediated EPSPs. About 20% of neurones received both Aδ and C fibre input from primary afferents.

3. Low concentrations of tetrodotoxin (TTX, ~ 50 nM) blocked EPSPs evoked by stimulation of Aδ fibres without affecting those evoked by C fibre stimulation. Higher concentrations of TTX (500 nM) also blocked C fibre-evoked responses.

4. EPSPs evoked by Aδ and C fibre stimulation reversed in polarity at membrane potentials near 0 mV, similar to the reversal potential of spontaneous EPSPs and of the potential change evoked by exogenous glutamate.

5. Aδ and C fibre-evoked EPSPs were depressed by kynurenate and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX); C fibre-evoked EPSPs appeared to be less sensitive.

6. In the presence of TTX, only 50% of SG neurones were depolarized by L-glutamate. However, neurones which exhibited no direct response to L-glutamate received afferent-evoked EPSPs which were sensitive to CNQX. In sensitive neurones, the depolarization evoked by L-glutamate was depressed by only ~ 15% in the presence of CNQX, whereas afferent-evoked EPSPs recorded from the same neurone were almost completely suppressed. Combined application of DL-2-amino-5-phosphonovaleric acid (APV) and CNQX depressed the response to L-glutamate by only ~ 25%.

7. These findings suggest that Aδ and C fibres use L-glutamate or a related amino acid as a transmitter at synapses with substantia gelatinosa neurones. The

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postsynaptic actions of this transmitter are mediated predominantly by non *N*-methyl-D-aspartic acid (NMDA) receptors. The failure of CNQX and APV to completely block the L-glutamate-evoked depolarization of substantia gelatinosa neurones raises the possibility that exogenously applied L-glutamate activates a non-NMDA receptor distinct from that which mediates the actions of the synaptically released afferent transmitter.

INTRODUCTION

The transmission of cutaneous sensory information from the periphery to the central nervous system is mediated by primary sensory neurones that terminate in the dorsal horn of the spinal cord (Light & Perl, 1979; Brown, 1982). Many fine afferent fibres that conduct in the Aδ and C fibre range respond to noxious peripheral stimuli. These afferent fibres terminate predominantly on postsynaptic neurones in laminae I and II of the dorsal horn (Réthelyi, 1977; Light & Perl, 1979; Ribiero-da-Silva & Coimbra, 1982; Nagy & Hunt, 1983). Lamina I neurones include spinothalamic projection cells (Giesler, Menétrey & Basbaum, 1979; Granum, 1986; Lima & Coimbra, 1988) and neurones with segmental projections (Cervero, Iggo & Molony, 1979). Lamina II neurones form local interneuronal circuits which are thought to integrate afferent information from different fibre classes and to relay this information to projection neurones located in lamina I and in deeper regions of the dorsal horn (Kumazawa & Perl, 1978; Light, Trevino & Perl, 1979).

The mechanisms underlying chemical transmission at Aδ and C fibre synapses in the dorsal horn are still not resolved. Immunocytochemical and biochemical studies have provided evidence for the presence and release of amino acids (Roberts, 1974; Takeuchi, Onodera & Kawagoe, 1983) and peptides (Hökfelt, Elde, Johansson, Luft, Nilsson & Arimura, 1976; Tuchscherer & Seybold, 1985) from primary afferent fibres that terminate in the superficial dorsal horn. Neurones in the superficial dorsal horn are excited by L-glutamate and other acidic amino acids (Schneider & Perl, 1988), and also by ATP (Jahr & Jessell, 1983; Fyffe & Perl, 1984) and by several neuropeptides (Murase, Nedeljkov & Randić, 1982; Murase & Randić, 1984; Urban & Randić, 1984). However, direct analysis of the contribution of these agents to chemical transmission at Aδ and C fibre synapses in the superficial dorsal horn has not been possible because of the difficulty in obtaining stable intracellular recordings from identified postsynaptic neurones receiving fine fibre input.

The use of *in vitro* spinal cord slice preparations has begun to provide a more detailed analysis of primary afferent transmission in the dorsal horn. Intracellular recordings from spinal cord slices isolated from immature rats or hamsters has demonstrated that activation of primary afferent input evokes both fast and slow EPSPs in dorsal horn neurones (Urban & Randić, 1984; King, Thompson, Urban & Woolf, 1988; Schneider & Perl, 1988; Gerber & Randić, 1989). Some afferent-evoked fast EPSPs are blocked by excitatory amino acid antagonists (Schneider & Perl, 1988; Gerber & Randić, 1989), supporting the idea that L-glutamate or a related compound is a fast-acting afferent transmitter. However, it has also been reported that some afferent-evoked fast EPSPs recorded from dorsal horn neurones are insensitive to excitatory amino acid antagonists (Schneider & Perl, 1988), raising the

possibility that agents other than excitatory amino acids may play a role in fast synaptic transmission. The monosynaptic nature of the afferent input to dorsal horn neurones and the class of afferent fibres responsible for the postsynaptic excitation was, however, not addressed in these studies.

We have examined the monosynaptic input to substantia gelatinosa neurones from identified Aδ and C fibre afferents in an *in vitro* slice preparation of adult rat spinal cord (Yoshimura & Jessell, 1989*a*). Here, we describe the use of this preparation to evaluate the role of excitatory amino acids as transmitters at Aδ and C fibre synapses with substantia gelatinosa neurones.

METHODS

Preparation of the spinal cord slice

Male adult Sprague–Dawley rats (100–250 g) were anaesthetized with urethane (1·5 g/kg, I.P.), a lumbosacral laminectomy was performed and a 2 cm segment of the lumbar spinal cord with the ventral and dorsal roots attached was isolated and submerged in pre-oxygenated Krebs solution. After removal of the dura mater, all ventral and dorsal roots were cut near the root entry zone, with the exception of the L4 or L5 dorsal root on one side. The pia-arachnoid membrane was then removed, with the exception of the area around the site of entry of the preserved dorsal root. The spinal cord segment was fixed to a Plexiglas stage with cyanoacrylate glue. A transverse slice (500 μm thick) which retained the attached dorsal root was cut on a vibratome and placed on a nylon mesh in a recording chamber. The slice was completely submerged and perfused continuously with Krebs solution (14–16 ml/min) equilibrated with 95% O_2–5% CO_2 at 36$\pm$1 °C. The composition of the Krebs solution was (in mM): 117 NaCl, 3·6 KCl, 2·5 $CaCl_2$, 1·2 $MgCl_2$, 1·2 NaH_2PO_4, 25 $NaHCO_3$ and 11 glucose. Detailed methods for preparation of spinal cord slices used in this study are described elsewhere (Yoshimura & Jessell, 1989*a*).

Recording and dorsal root stimulation

Intracellular recordings were performed from neurones located in the substantia gelatinosa (lamina II of Rexed, 1952) with 4 M-potassium acetate-filled microelectrodes which had DC tip resistances of 120–200 MΩ. In experiments to determine the reversal potential of fast excitatory postsynaptic potentials and of glutamate-evoked responses, caesium chloride (CsCl) or caesium acetate (CsAc) electrodes were used. Signals were amplified with a high-input impedance bridge amplifier (Axoclamp 2A; Axon Instruments), stored and analysed on a PDP 11/73 computer. A DC pen recorder (Gould 2600S) was used to monitor membrane potential continuously.

The minimum stimulus intensities required to activate Aα/β, A δ and C fibres were determined by monitoring the compound action potential in an isolated sciatic nerve preparation using silver-wire hook electrodes. The minimum stimulus intensities and durations required to activate Aβ, Aδ and C fibres were 1·7 V, 0·1 ms; 1·9 V, 0·1 ms and 5·5 V, 0·4 ms, respectively. No C fibre-evoked responses were detected in sciatic nerve with stimulus intensities less than 5 V (0·4 ms). The same suction electrode was then used to activate dorsal root afferents which had a length of 6–10 mm in the *in vitro* slice preparation. With a given stimulating electrode, the minimum stimulus intensity was quite constant among slice preparations, ranging from 1·9 to 2·0 V, 0·1 ms duration for Aδ fibres and from 5·5 to 6 V, 0·4 ms duration stimulation for C fibres. Activation of dorsal root afferents at intensities less than 5 V, 0·4 ms did not produce long-latency EPSPs, presumed to be mediated by C fibres.

Identification of substantia gelatinosa neurones

Substantia gelatinosa neurones were identified by their location and morphological features. When viewed under a dissecting microscope with transmitted illumination, the substantia gelatinosa was discernible as a translucent band in the superficial dorsal horn; however, it was difficult to distinguish with certainty the border between laminae I and II. To avoid recording from lamina I neurones, recording electrodes were inserted under visual control into the middle of

substantia gelatinosa. The identity of these neurones was confirmed in selected instances by intracellular injection of 2·5% Lucifer Yellow CH in 0·1 M-LiCl or 5% carboxyfluorescein in 0·1 M-potassium acetate as described previously (Yoshimura & Jessell, 1989*a*).

Application of drugs

Drugs were applied by superfusing slices with solutions of known drug concentration or by pressure application from pipettes with tip diameters of 5–10 μm. The solution in the recording chamber (0·5 ml) was completely replaced by the perfusing solution within 20 s. Drugs used were tetrodotoxin (TTX; 10–500 nM), cobalt (Co; 2 mM), cadmium (Cd; 0·2 mM), L-glutamate (Glu; 0·1–10 mM), kynurenate (Kyn; 0·5–2 mM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 1–10 μM), L-aspartic acid β-hydroxamate (50–300 μM), bicuculline methiodide (10 μM), strychnine (1–10 μM), *N*-methyl-D-aspartic acid (NMDA; 50–100 μM), DL-2-amino-5-phosphonovaleric acid (APV; 50–100 μM), kainate (KA; 50–100 μM), quisqualate (QA; 10–40 μM), α-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA; 10–20 μM) and caesium chloride (1–2 mM).

RESULTS

Intracellular recordings were obtained from 279 substantia gelatinosa neurones that received fast EPSPs driven by Aδ and/or C afferent fibres. Stable recordings could be obtained from slices maintained *in vitro* for up to 24 h and recordings were made from single substantia gelantinosa neurones for up to 6 h. The resting membrane potential of substantia gelatinosa neurones examined was -68 ± 1 mV (mean ± S.E.M., $n = 69$), the input resistance was 242 ± 18 MΩ ($n = 52$) and the time constant was 21 ± 5 ms ($n = 5$). Neurones with resting membrane potentials more positive than -55 mV were not analysed further with the exception of neurones impaled with electrodes filled with CsAc or CsCl (see below). Neurones with input resistances of less than 50 MΩ were also discarded.

EPSPs evoked by primary afferent stimulation

A high proportion of afferent-evoked EPSPs recorded from substantia gelatinosa neurones appear to be monosynaptic (Yoshimura & Jessell, 1989*a*). The latency of primary afferent-evoked EPSPs is constant in response to repetitive (20 Hz) stimulation and failures are not observed, even though the amplitude of EPSPs is markedly attenuated by high-frequency stimulation. These EPSPs persist and maintain constant latency in the presence of medium containing 5 mM-Ca^{2+} and 5 mM-Mg^{2+}. This finding contrasts with primary afferent fibre-evoked fast inhibitory postsynaptic potentials (IPSPs) that are di- or polysynaptic in origin (Jahr & Yoshioka, 1986). These IPSPs have a variable latency and exhibit failures with high-frequency stimulation, and after perfusion with solutions containing high divalent cation concentrations. In this study we have confined our analysis to substantia gelatinosa neurones receiving monosynaptic primary afferent-evoked EPSPs, based on these criteria.

Comparison of the latency of evoked EPSPs with the conduction velocity and stimulus intensity required to activate primary afferent fibres indicates that short-latency EPSPs (Fig. 1*Aa*) are mediated by Aδ fibres and that the long-latency EPSPs (Fig. 1*Ab*) are mediated by C fibres. Short-latency EPSPs were detected in 70% of substantia gelatinosa neurones after stimulation of dorsal root fibres with single square pulses of 1·9–5·0 V and 0·1 ms duration. The conduction velocity of these afferent fibres varied between 3 and 9 m/s, which is within the range obtained

in vivo for Aδ fibres (Light & Perl, 1984). In about 5% of substantia gelatinosa neurones, low-intensity stimulation did not evoke synaptic responses, but an increase in the intensity and duration of stimulation (to > 5·5 V; 0·4 ms) evoked fast EPSPs of long and constant latency. The conduction velocity of afferent fibres

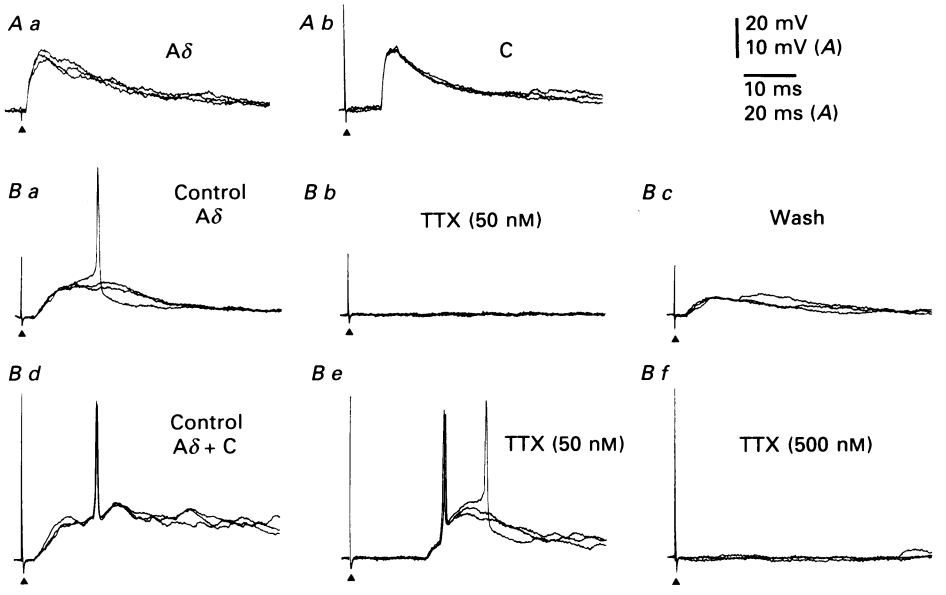

Fig. 1. Isolation of C afferent fibre-evoked input in the presence of low concentrations of TTX. Records displayed were obtained from two neurones (*A* and *B*). In this and subsequent figures, ▲ indicates the time of dorsal root stimulation. *A*, low-intensity stimulation of a dorsal root afferent produces Aδ fibre-evoked short-latency EPSPs (*A a*). During the wash-out phase following application of 500 nM-TTX, high-intensity stimulation evokes only C fibre-mediated, constant-latency EPSPs (*A b*). High-intensity stimulation in the complete absence of TTX evokes polysynaptic inputs which obscure any recognizable C fibre inputs. *B*, superfusion with TTX (50 nM) reversibly blocks Aδ fibre-evoked EPSPs (*Ba–Bc*) and the initial component of the mixed Aδ- and C fibre-evoked EPSPs (*Bd*, *Be*). TTX (500 nM) blocks both Aδ and C fibre-evoked responses (*Bf*). Note that action potentials were obtained in the presence of 50 nM-TTX (*Be*) and that the EPSPs recorded in the presence of 50 nM-TTX had long and constant latencies (*Be*). Three superimposed traces are shown.

mediating these EPSPs was 0·4–2·0 m/s, which is within the C fibre range (Light & Perl, 1984). In about 20% of substantia gelatinosa neurones, increasing the intensity and duration of stimulation evoked short- and long-latency EPSPs, suggesting that these neurones received both Aδ and C fibre input. Afferent fibres conducting in the Aα/β range (> 30 m/s) did not evoke monosynaptic EPSPs in substantia gelatinosa neurones. However, in about 20% of neurones low-intensity stimulation (< 1·7 V; 0·1 ms), which is insufficient to activate Aδ fibres, did evoke variable latency EPSPs with amplitudes of 1–7 mV. These EPSPs may reflect polysynaptic inputs driven by activation of Aα/β fibres.

Because most substantia gelatinosa neurones that exhibited monosynaptic C fibre-evoked responses also received an Aδ fibre-mediated monosynaptic input and often

polysynaptic EPSPs, it was usually possible to analyse C fibre-evoked input only in the subset of neurones that did not receive a preceding Aδ fibre input. To confirm the identity of C fibre-evoked EPSPs, we used low concentrations of TTX (~ 50 nM) which reversibly blocked Aδ afferent fibre-mediated EPSPs revealing more clearly, C fibre-evoked EPSPs (Fig. 1*B*). Higher concentrations of TTX (500 nM) also blocked C fibre-evoked EPSPs (Fig. 1*Bf*). C fibre-evoked EPSPs presented here were recorded in the absence of TTX but in many cases their identity was confirmed by their resistance to low concentrations of TTX. The ability to record action potentials in substantia gelatinosa neurones in the presence of 50 nM-TTX (Fig. 1*Be*) suggests that dorsal horn neurones also express TTX-insensitive Na^+ channels.

Reversal potential of Aδ and C fibre-mediated EPSPs and of potential change evoked by L-glutamate

To examine whether activation of Aδ and C fibres results in similar postsynaptic effects on substantia gelatinosa neurones, we compared the reversal potential of EPSPs evoked by these two classes of afferents. In most neurones, EPSPs evoked by Aδ and C fibre afferents increased in amplitude with membrane hyperpolarization. However, in about 20% of neurones, there was either no change or a decrease in the amplitude of EPSPs upon membrane hyperpolarization (not shown). This subset of neurones exhibited anomalous rectification, which was detected as a large increase in membrane conductance upon hyperpolarization from rest to −100 mV (Yoshimura & Jessell, 1989*b*). Perfusion with caesium blocked the anomalous rectification in these neurones and in these circumstances there was an increase in the amplitude of EPSPs with membrane hyperpolarization (not shown). These findings suggest that the reduction in EPSP amplitude upon hyperpolarization results primarily from anomalous rectification and not from a voltage-dependent blockade of NMDA receptors which occurs at hyperpolarized potentials (Mayer, Westbrook & Guthrie, 1984; Nowak, Bregestovski, Ascher, Herbert & Prochiantz, 1984). The fast EPSP, however, does have an NMDA receptor-mediated component (see below).

The reversal potential of afferent fibre-evoked EPSPs were examined with CsCl- or CsAc-filled electrodes to reduce or abolish outward currents. After impalement of substantia gelatinosa neurones, the resting membrane potential decreased to between −40 and 0 mV over a period of 20–60 min. This probably results from the gradual blockade of K^+ channels that contribute to the resting membrane potential. To avoid any contribution of glycine or GABA-mediated IPSPs to the synaptic potential, recordings were performed in the presence of strychnine (5 μM) and bicuculline (10 μM). Under these conditions, there was a linear relationship between membrane potential and EPSP amplitude from −50 to +30 mV for both Aδ and C fibres. The reversal potential of Aδ fibre-mediated fast EPSPs was -4 ± 2 mV ($n = 7$). C fibre-evoked EPSPs reversed in polarity at a membrane potential of -6 ± 2 mV ($n = 3$). Moreover, the reversal potentials of Aδ and C fibre-evoked EPSPs recorded from the same neurone were almost identical (Fig. 2*A*). Spontaneous EPSPs recorded from substantia gelatinosa neurones had a similar reversal potential ($-5{\cdot}0 \pm 2$ mV; $n = 7$) (Fig. 2*B*).

L-Glutamate is a candidate as the transmitter of Aδ and C fibre-evoked fast EPSPs at synapses with substantia gelatinosa neurones. We therefore determined the

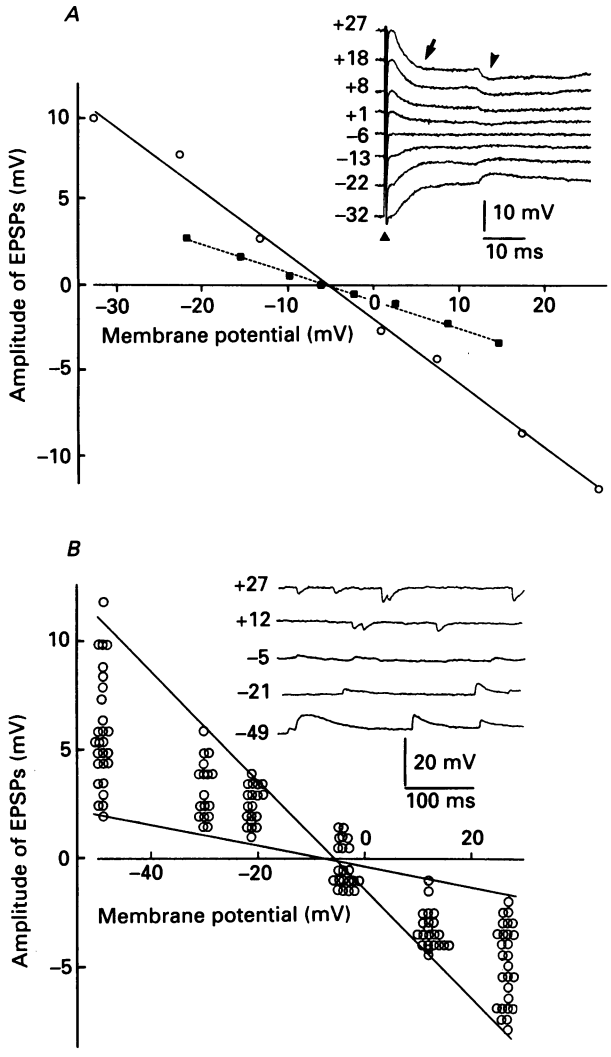

Fig. 2. Reversal potential of Aδ and C fibre-evoked EPSPs and spontaneous EPSPs. Traces show records obtained from two (*A* and *B*) neurones. *A*, plot of the amplitude of Aδ and C fibre-evoked EPSPs *vs.* membrane potentials obtained from records shown in the inset. Aδ (○) and C fibre (■)-evoked EPSPs reversed in polarity at a similar membrane potential. In the inset, the arrow shows Aδ and the arrow-head shows C fibre-evoked EPSPs. The EPSP amplitude was measured at the membrane potentials indicated on the left of each trace (mV). *B*, plot of the amplitude of spontaneous EPSPs *vs.* membrane potential obtained from records shown in the inset. The reversal potential of spontaneous EPSPs is about −7 mV. The lines indicate the limits of the variation in EPSP amplitude.

reversal potential of the potential change evoked by exogenous L-glutamate. Application of L-glutamate by superfusion (1 mM) or pressure ejection (100 mM) resulted in a membrane depolarization. The amplitude of the depolarization was linearly related to membrane potential, and reversed at a membrane potential of

0 ± 2 mV ($n = 4$) (Fig. 3). Synaptic activation and L-glutamate-evoked depolarization of substantia gelatinosa neurones may therefore be mediated by similar ionic mechanisms.

Effect of excitatory amino acid antagonists on Aδ and C fibre-evoked EPSPs

To test further the possibility that L-glutamate or a related amino acid is the fast transmitter at Aδ fibre afferent synapses, we examined the sensitivity of EPSPs to

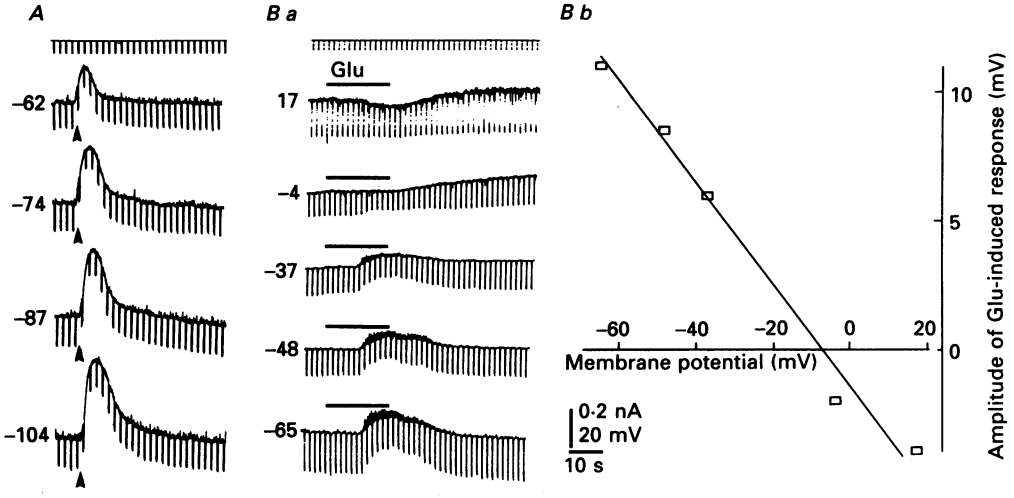

Fig. 3. Reversal potential of L-glutamate-induced depolarization. Records shown were obtained from two neurones (*A* and *B*). *A*, the depolarization recorded in response to pressure application of L-glutamate (Glu; arrow-head) increases in amplitude with membrane hyperpolarization. Downward deflections indicate hyperpolarizing potentials evoked by current injection (0·07 nA, 300 ms at 0·6 Hz). *B*, L-glutamate-induced responses are reversed in polarity by membrane depolarization. L-Glutamate was applied during the period indicated by bar (*Ba*). Plot of the amplitude of L-glutamate-evoked depolarization *vs.* membrane potential obtained from *Ba* gives a reversal potential of about -7 mV (*Bb*).

excitatory amino acid receptor antagonists. Kynurenate (0·5–2 mM), which inhibits both NMDA and non-NMDA classes of excitatory amino acid receptors (Evans, Evans, Pook & Sunter, 1987; Kemp, Grimwood & Foster, 1987), depressed Aδ fibre-evoked and spontaneous EPSPs in a dose-dependent manner (Fig. 4*A*). The reduction of EPSP amplitude in the presence of kynurenate (0·5 and 1 mM) was by $47{\cdot}9 \pm 4{\cdot}3\%$ ($n = 9$) and $55{\cdot}2 \pm 5{\cdot}1\%$ ($n = 9$) respectively. The depression of the EPSPs produced by kynurenate reached a maximum within 5–10 min and returned to control levels within 10–20 min. We also examined the sensitivity of afferent-evoked EPSPs to the amino acid antagonist 6-cyano-6-nitroquinoxaline-2,3-dione (CNQX) (Honoré, Davies, Drejer, Fletcher, Jacobsen, Lodge & Nielsen, 1988). In spinal cord slices, this compound acts as a selective antagonist of non-NMDA receptors. CNQX had no effect on resting membrane potential and input resistance of substantia gelatinosa neurones. The amplitude of Aδ fibre-evoked EPSPs was reduced by $77{\cdot}9 \pm 2{\cdot}0\%$ ($n = 12$) in the presence of 5 μM-CNQX (Fig. 4*B*; Table 1).

The suppression of EPSPs was maximal within 7 min and EPSPs returned to control amplitude within 10–15 min after wash-out of CNQX. Spontaneous EPSPs onto these neurones were also depressed.

To determine whether NMDA receptors contribute to Aδ fibre-evoked EPSPs, we used the NMDA antagonist DL-2-amino-5-phosphonovaleric acid (APV) (Watkins &

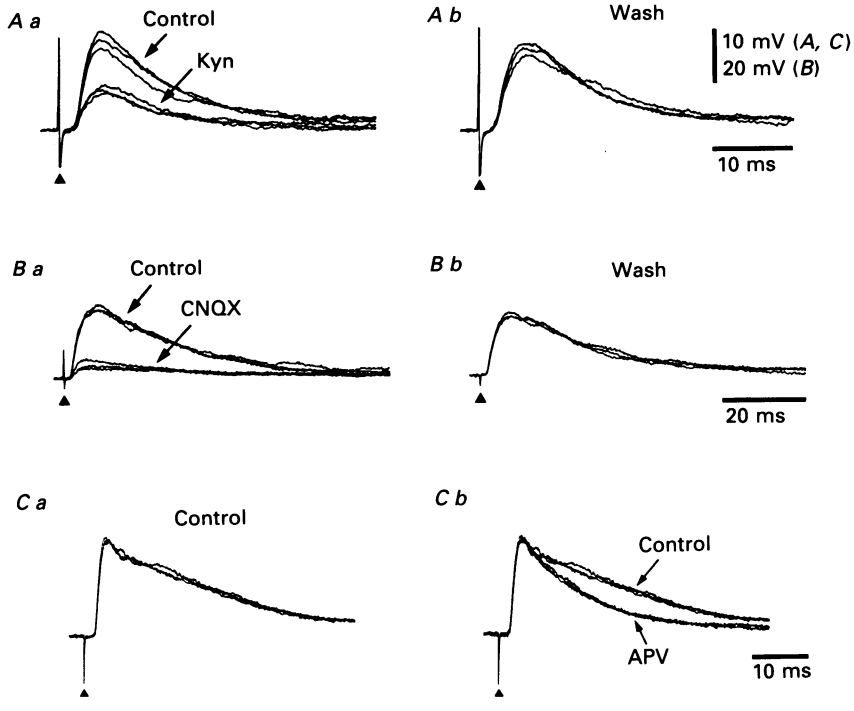

Fig. 4. Depression of Aδ fibre-evoked EPSPs by the amino acid receptor antagonists kynurenate, CNQX and APV. Records shown were obtained from three neurones (*A*, *B* and *C*). *A*, depression of EPSPs by kynurenate. Records obtained before (control) and during (Kyn) application of kynurenate (0·5 mM) are superimposed (*Aa*). The effect of kynurenate is reversible (*Ab*). *B*, depression of EPSPs by CNQX. Records obtained before (control) and during (CNQX) application of CNQX (5 M) are superimposed (*Ba*). The effect of CNQX is reversible (*Bb*). *C*, low-intensity dorsal root stimulation elicits Aδ fibre-evoked monosynaptic EPSPs (*Ca*). In this cell, APV (100 μM) did not affect the peak amplitude of the EPSP but depressed the falling phase of the EPSP (*Cb*).

Evans, 1981; Mayer & Westbrook, 1987). In about 50% of substantia gelatinosa neurones APV (100 μM) produced a small (< 20%) decrease in the rate of rise and in the amplitude of Aδ fibre-evoked EPSPs (not shown). Virtually all neurones exhibited a decrease in the half-decay time of the EPSP (28·1 ± 6·0%, $n = 8$) (Fig. 4*C*). This finding is consistent with other studies showing that NMDA receptors contribute primarily to later components of EPSPs (Dale & Roberts, 1985; Forsythe & Westbrook, 1988). In about 30% of neurones APV abolished polysynaptic afferent-evoked inputs without affecting fast, Aδ fibre-evoked EPSPs. However, in other neurones variable-latency polysynaptic inputs were resistant to APV (not

shown). The small effect of APV on fast EPSPs suggests that amino acid receptors of the non-NMDA type mediate the major component of the Aδ fibre-evoked monosynaptic EPSP.

We also examined the sensitivity of C fibre-mediated EPSPs to excitatory amino acid receptor antagonists. Kynurenate (1 mM) depressed C fibre-evoked EPSPs by

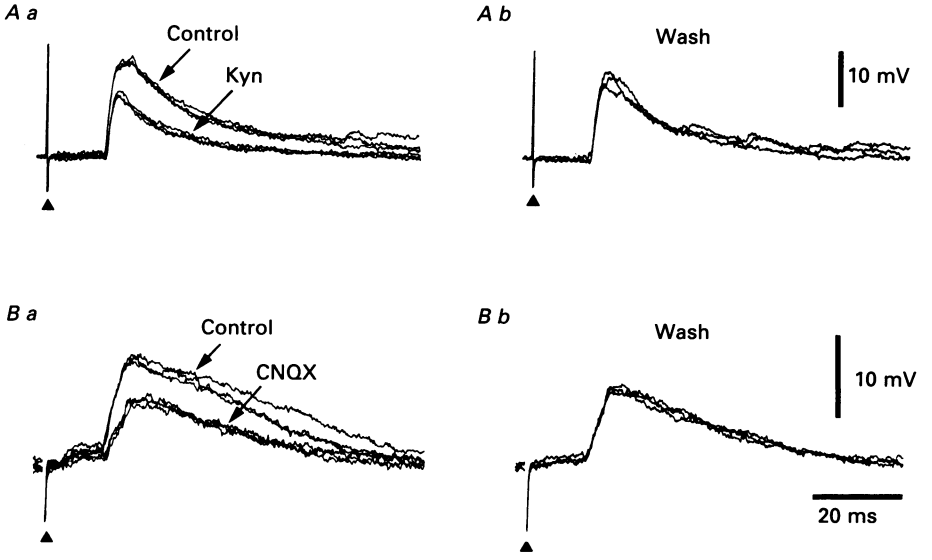

Fig. 5. Depression of C fibre-evoked EPSPs by kynurenate and CNQX. Records *A* and *B* were obtained from two different neurones. *A*, C fibre-evoked EPSPs recorded before (control) and during (Kyn) application of kynurenate (0·5 mM) are superimposed (*Aa*). The effect of kynurenate is reversible (*Ab*). *B*, C fibre-evoked EPSPs recorded before (control) and during (CNQX) application of CNQX (5 μM) are superimposed (*Ba*). The effect of CNQX is reversible (*Bb*).

24·0±7·8% ($n = 6$) and CNQX (5 μM) depressed the peak amplitude of C fibre-evoked EPSPs by 54·3±9·4% ($n = 4$) (Fig. 5, Table 1). Thus, C fibre-mediated EPSPs appear to be less sensitive to excitatory amino acid antagonists than Aδ fibre-evoked EPSPs (see Discussion)

Sensitivity of substantia gelatinosa neurones to exogenous excitatory amino acids

We next examined the chemosensitivity of substantia gelatinosa neurones that received monosynaptic Aδ and C fibre inputs. Application of L-glutamate (1 mM) produced a membrane depolarization in 90% of substantia gelatinosa neurones (Fig. 6*A*). The amplitude of the L-glutamate-evoked depolarization was 11·6±1·3 mV ($n = 83$). However, in the presence of TTX (500 nM), Co^{2+} (2 mM) or Cd^{2+} (0·2 mM), only about 50% of the neurones responded to L-glutamate (1–3 mM) suggesting that L-glutamate has a direct action on only a subset of substantia gelatinosa neurones (Fig. 6*B*). Neurones that did not respond to L-glutamate in the presence of TTX nevertheless exhibited large Aδ and C fibre-evoked EPSPs that were blocked by amino acid receptor antagonists (Fig. 6*Bc*). Thus, the lack of response to L-glutamate

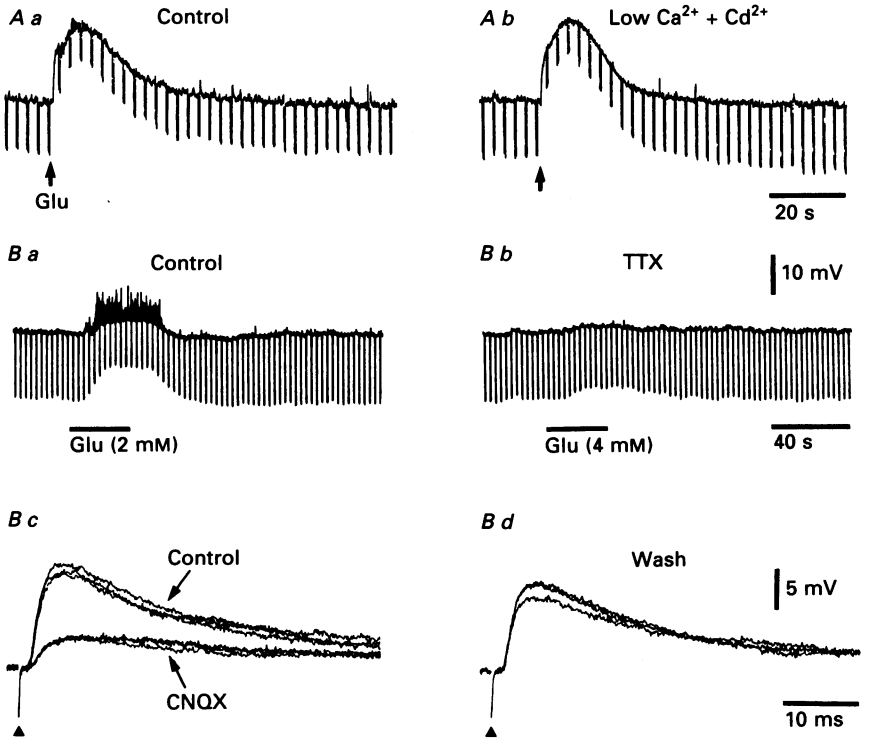

Fig. 6. Variation in L-glutamate sensitivity of substantia gelatinosa neurones. *A*, example of L-glutamate-sensitive gelatinosa neurone. Pressure application of L-glutamate (Glu; 100 mM) evokes a pronounced depolarization (*Aa*) which persists in the presence of low Ca^{2+} and Cd^{2+} (*Ab*). *B*, example of a substantia gelatinosa neurone that is not directly activated by L-glutamate. L-Glutamate (2 mM) evokes a small depolarization (*Ba*), but this is almost completely abolished in the presence of TTX (500 nM; *Bb*). However, the same neurone receives a monosynaptic Aδ fibre-evoked EPSP (*Bc*, control) that is reversibly blocked by CNQX (*Bc*, CNQX: *Bd*).

TABLE 1. Effect of amino acid antagonists on responses evoked by afferent nerve stimuli and by exogenous agonists

	Kynurenate (1 mM)	CNQX (5 μM)	APV (100 μM)
Aδ fibre EPSP	55·2±5·1 (9)	77·9±2·0 (12)	21·8±3·3 (8)
C fibre EPSP	24·0±7·8 (6)	54·3±9·4 (4)	ND
L-Glutamate (1 mM)	34·8±5·0 (16)	16·7±4·4 (18)	11·4±4·5 (5)
Kainate (100 μM)	ND	52·4±5·1 (14)	ND
AMPA (20 μM)	ND	48·5±10·2 (4)	ND

Numbers indicate percentage depression of responses in presence of antagonist, ±S.E.M. (*n*). ND = not determined. Kynurenate ($P = 0.006$) and CNQX ($P = 0.004$) produced a significantly greater suppression of Aδ fibre-evoked EPSPs than C fibre EPSPs. Statistical significance was determined with paired Student's *t* test.

does not result from the absence of excitatory amino acid receptors on these neurones; rather, exogenously applied L-glutamate appears not to gain access to the receptors.

One possible explanation for this is that L-glutamate is rapidly sequestered by an uptake mechanism that is widely distributed on glial or neuronal cells within the slice

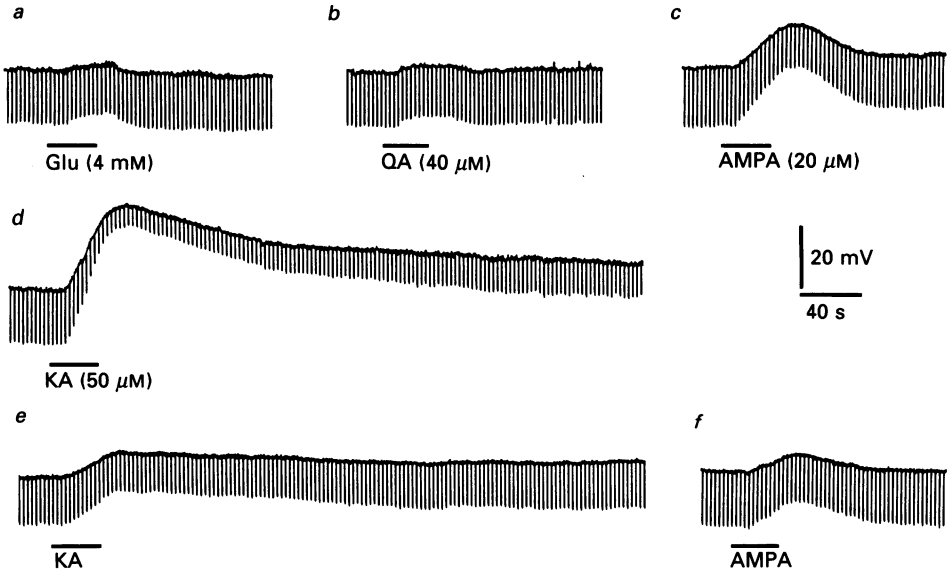

Fig. 7. Effects of CNQX on depolarization evoked by excitatory amino acids. All records were obtained from the same neurone. TTX (500 nM) was present throughout the experiment. Application of L-glutamate (Glu; 4 mM) and quisqualate (QA; 40 μM) produced only a small depolarization (*a*, *b*), whereas application of AMPA and kainate (KA) resulted in a marked depolarization (*c*, *d*). Superfusion with CNQX (5 μM) depressed both the kainate and AMPA-induced depolarization (*e*, *f*). L-Glutamate and agonists were applied by superfusion during the time indicated by the bars.

(Fagg & Lani, 1979; Garthwaite, 1985). If this is the case, it might be expected that the L-glutamate sensitivity of neurones deep within the slice would be lower than that of more superficial neurones. We examined the sensitivity of substantia gelatinosa neurones to L-glutamate as a function of their depth within the slice. Neurones near to the surface showed only a slightly greater sensitivity to applied L-glutamate than those at the centre of the slice (not shown). Many superficial neurones were unresponsive or responded with only a very small depolarization and several deeper neurones responded with a large depolarization. Thus the depth at which a neurone is located within the slice does not appear to account for the marked variation in L-glutamate sensitivity of individual neurones.

We next compared the sensitivity of substantia gelatinosa neurones to non-NMDA receptor agonists that differ in their abilities to be transported by the high-affinity L-glutamate uptake system. Quisqualate, which is a substrate for the high-affinity L-glutamate uptake system (Lodge, Curtis, Johnston & Bornstein, 1980), evoked little or no response in L-glutamate-insensitive neurones (Fig. 7*A b*). However, the selective quisqualate receptor agonist α-amino-3-hydroxy-5-methylisoxazole pro-

pionic acid (AMPA; 10–20 μM), which is not a substrate for the high-affinity L-glutamate uptake system (Bridges, Nieto-Sampedro, Kadri & Cotman, 1987), evoked a marked depolarization of L-glutamate-insensitive neurones (five of six neurones examined, Fig. 7*Ac*). Similarly, kainate (50–100 μM) which also is not a

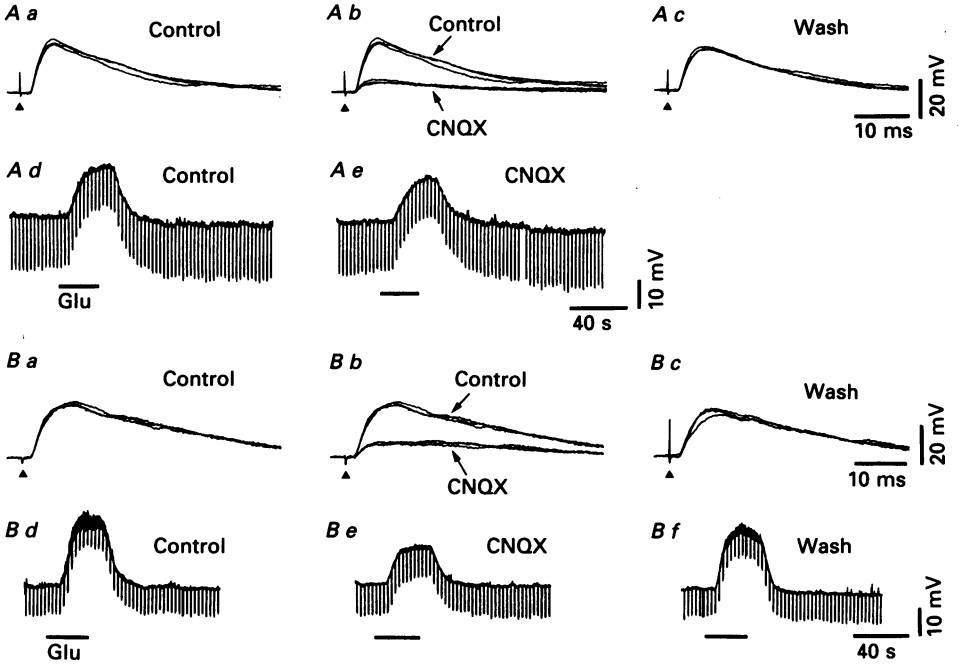

Fig. 8. Example of L-glutamate-sensitive neurones in which the effects of CNQX on Aδ fibre-evoked EPSPs and L-glutamate-evoked depolarization are compared. Records shown were obtained from two neurones (*A* and *B*). Aδ fibre-evoked EPSPs (*Aa–Ac*, *Ba–Bc*) and L-glutamate-induced responses (Glu; *Ad*, *Ae*, *Bd–Bf*) are shown before (left), during (middle) and after (right) application of CNQX (5 μM). CNQX reversibly depresses the Aδ fibre-evoked EPSPs (*Ab*, *Bb*). The L-glutamate-induced depolarization is not significantly affected by CNQX (*Ad*, *Ae*). In the neurone shown in *B*, CNQX markedly depresses Aδ fibre-evoked EPSPs and produces a smaller but significant decrease in the L-glutamate-induced depolarization. This neurone illustrates the maximum degree of depression of L-glutamate-evoked response in the presence of CNQX.

substrate for the uptake system (Stallcup, Bulloch & Baetge, 1979), evoked a large depolarization in all twenty-three neurones examined (Fig. 7*Ad*). Both the AMPA- and kainate-evoked depolarizations were markedly reduced by CNQX (Fig. 7*Ae* and *Af*; Table 1). Since L-glutamate is an agonist at both kainate and quisqualate receptors in the spinal cord (Watkins & Evans, 1981), these findings are consistent with the idea that the lack of L-glutamate sensitivity of some substantia gelatinosa neurones under our experimental conditions results, at least in part, from the rapid uptake of L-glutamate.

In an attempt to examine further the contribution of uptake to the L-glutamate sensitivity of substantia gelatinosa neurones we used the uptake inhibitor L-aspartic acid β-hydroxamate (L-AABH). There was a clear enhancement of the L-glutamate-

evoked depolarization when neurones that were sensitive to application of L-glutamate in the absence of uptake blockers were superfused with L-AABH (not shown). However, superfusion with L-AABH did not confer L-glutamate sensitivity to neurones that were previously insensitive. We also tested whether synaptically-

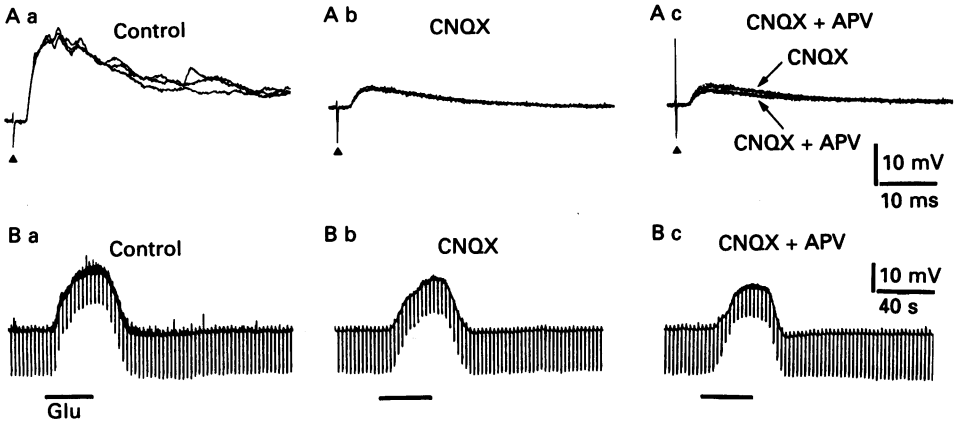

Fig. 9. Effects of CNQX and APV on Aδ fibre-evoked EPSPs and on L-glutamate-induced depolarization. Records were obtained from the same neurone. *A*, Aδ fibre-evoked EPSPs are depressed 5 min after the onset of application of CNQX (5 μM, *Ab*). EPSPs are also depressed by superfusion with a solution containing CNQX (5 μM) and APV (100 μM; *Ac*). The extent of depression in the presence of both antagonists is similar to that in the presence of CNQX alone. *B*, bath application of L-glutamate (Glu; 2 mM) produces a marked depolarization of this neurone (*Ba*). CNQX (5 μM) did not markedly affect the response to L-glutamate, although spontaneous EPSPs were blocked (*Bb*). Superfusion with both CNQX (5 μM) and APV (100 μM) reduces the response to L-glutamate by 25% (*Bc*).

evoked responses in these neurones were enhanced by blockade of L-glutamate uptake. The amplitude and duration of EPSPs activated by dorsal root stimulation were not affected by L-AABH. Available L-glutamate uptake blockers may therefore be ineffective in preventing the removal of L-glutamate from the extracellular space surrounding some neurones.

Evidence for a CNQX-insensitive, APV-insensitive L-glutamate receptor on a subset of substantia gelatinosa neurones

Amino acid receptor antagonists were not equally effective in blocking the depolarization of substantia gelatinosa neurones evoked by L-glutamate and by afferent synaptic input. There was only a small (16·7 ± 4·4%; $n = 18$) reduction in the L-glutamate-evoked depolarization of substantia gelatinosa neurones in the presence of CNQX (5 μM), whereas Aδ fibre-evoked EPSPs recorded from the same neurones were blocked by 77·9 ± 2·0% ($n = 12$) (Fig. 8*A*; Table 1). The L-glutamate-evoked depolarization was blocked by about 35% in the presence of kynurenate (1 mM). Kynurenate is therefore more effective than CNQX as an antagonist of L-glutamate-evoked depolarization, probably because it acts as antagonist at both NMDA and non-NMDA receptors, but less effective in blocking afferent fibre-evoked EPSPs. APV, a selective NMDA antagonist, reduced the L-glutamate-evoked

depolarization by about 11%. However, in the presence of both APV and CNQX, the response to L-glutamate was inhibited by only about 25% (Fig. 9), whereas Aδ fibre-evoked EPSPs were depressed by over 90%. Thus, NMDA receptors do not mediate the major component of the CNQX-resistant L-glutamate-evoked depolarization. A component of the L-glutamate-mediated depolarization of substantia gelatinosa neurones is therefore resistant to both non-NMDA and NMDA receptor antagonists.

DISCUSSION

Chemical transmission at synapses formed by Aδ and C fibres with substantia gelatinosa neurones in the dorsal horn of the spinal cord constitutes an early step in the central processing of somatosensory information. The neurotransmitters released by identified classes of cutaneous afferent fibres in the superficial dorsal horn have not been well characterized. In this study, we have used a transverse slice preparation of adult rat spinal cord to examine the identity of Aδ and C fibre transmitters and their interaction with postsynaptic receptors on substantia gelatinosa neurones. Our results suggest that both Aδ and C fibres release L-glutamate or a related substance as a transmitter. The postsynaptic excitatory actions of this transmitter appear to be mediated primarily *via* excitatory amino acid receptors of the non-NMDA class. Taken together with previous studies demonstrating that Ia afferent fibres release an L-glutamate-like transmitter (Jahr & Yoshioka, 1986), it appears that many and perhaps all classes of somatosensory afferent fibres that terminate in the spinal cord release the same fast excitatory transmitter.

Primary afferent input to the substantia gelatinosa

The *in vitro* slice preparation we have used permits direct analysis of monosynaptic connections between Aδ and C fibre afferents and postsynaptic neurones in the substantia gelatinosa. Anatomical studies of the mammalian dorsal horn have provided evidence that C fibres provide the major afferent input to the substantia gelatinosa, whereas Aδ fibres terminate predominantly in lamina I and have a less pronounced input to the substantia gelatinosa (Réthelyi, 1977; Light & Perl, 1979; Gobel, Falls & Humphrey, 1981; Sugiura, Lee & Perl, 1986; Sugiura, Terui & Hosoya, 1989). Our intracellular recordings suggest that there is a prominent monosynaptic input from Aδ afferents within the substantia gelatinosa. Over 70% of substantia gelatinosa neurones received monosynaptic input from afferent fibres conducting in the Aδ range. Intracellular recordings from neurones in the superficial dorsal horn in horizontal slices of hamster spinal cord have also revealed that a high proportion of superficial dorsal horn neurones receive A fibre input (Schneider & Perl, 1988). Our studies raise the possibility that there is a more significant Aδ fibre input to the rat substantia gelatinosa than previously appreciated. The difference between *in vitro* and *in vivo* studies could arise because substantia gelatinosa neurones in slice preparations lack descending or segmental inputs that normally suppress Aδ inputs *in vivo*.

We observed that only about 25% of substantia gelatinosa neurones received a detectable monosynaptic C fibre input. This is lower than might be predicted from

the density of C fibre terminals observed in the substantia gelatinosa (Réthelyi, 1977; Gobel *et al.* 1981; Sugiura *et al.* 1986; Sugiura *et al.* 1989). There are several possible reasons for an under-representation of C fibre inputs in the transverse slice preparation. Firstly, studies in which the central projection of single C fibres have been visualized in guinea-pig spinal cord by labelling with *Phaseolus* lectin have shown that the terminal arbor of somatic C fibres is located at a considerable distance from the point of entry of the afferent fibre into the spinal cord (Sugiura *et al.* 1989). In our experiments slices were obtained from lumbar levels which receive primarily somatic inputs. Thus it is possible that the transverse orientation of the slice severs the longitudinally directed branches of many C fibres before the collateral branch point, reducing the extent of C fibre input. Alternatively, it is possible that C fibres are simply more sensitive to the trauma involved in preparing the slice. The number of C fibre inputs may also have been underestimated because most substantia gelatinosa neurones received an input from Aδ fibres, masking the later C fibre input. Because of these problems, our analysis of the transmitter identity of afferent fibres is probably restricted to a subpopulation of C fibre inputs. There is no evidence, however, to suggest that the properties of synapses mediated by this subset of C fibres is atypical.

To confirm the identity of C fibre inputs we made use of an observation that mouse dorsal root ganglion neurones with unmyelinated fibres and the sensory endings of C fibres have action potentials that are relatively resistant to TTX (Yoshida, Matsuda & Samejima, 1978; Kirchhoff, Reeh & Waddell, 1989). Consistent with this, we found that action potential propagation by Aδ fibres within the dorsal root was blocked by low concentrations of TTX (50 nM), whereas propagation along C fibres was blocked only by a tenfold higher concentration. These findings provide a pharmacological method for segregating Aδ and C fibre inputs to the superficial dorsal horn, and may permit the analysis of C fibre-evoked EPSPs in the absence of contaminating Aδ input.

Identification of Aδ and C fibre transmitters

Our experiments provide evidence that fast EPSPs at synapses between Aδ and C fibres and substantia gelatinosa neurones are mediated by an excitatory amino acid transmitter, probably L-glutamate. Previous studies in newborn rat spinal cord have shown that Ia afferent fibres release an L-glutamate-like transmitter (Jahr & Yoshioka, 1986). Moreover, synaptic transmission between dorsal root ganglion and dorsal horn neurones in cell culture can be blocked by excitatory amino acid antagonists (Jahr & Jessell, 1985). The reversal potential of Aδ and C fibre-mediated EPSPs that we observed is in good agreement with the reversal potential of fast EPSPs recorded at sensory neurone–spinal cord synapses in culture (MacDonald, Pun, Neale & Nelson, 1983).

It has been reported that some afferent-evoked EPSPs in hamster spinal cord slices are insensitive to the excitatory amino acid antagonist kynurenate (Schneider & Perl, 1988). In contrast, we found that all Aδ and C fibre-evoked monosynaptic EPSPs were sensitive to kynurenate and also to the non-NMDA receptor antagonist CNQX. CNQX was a more potent antagonist than kynurenate at non-NMDA receptors and suppressed Aδ fibre-evoked EPSPs by about 80% and C fibre-evoked

EPSPs by about 55%. The difference between our results and those of Schneider & Perl could result from the use of a more effective antagonist of the non-NMDA subclass of receptors and the restriction of our analysis to monosynaptic afferent-evoked EPSPs recorded from substantia gelatinosa neurones. Although C fibre-evoked EPSPs were less sensitive than Aδ fibre inputs to CNQX, this may not necessarily reflect an inherent difference in the properties of receptors located postsynaptic to Aδ and C fibre terminals. Instead there may be a delayed access of antagonists to C fibre synapses. In support of this, the blockade of C fibre-evoked EPSPs during application of CNQX was gradual, whereas the onset of blockade of Aδ fibre-evoked EPSPs was much more rapid. The delayed access of antagonists to C fibre synapses may result from the positioning of many C fibre terminals as the central element of glomerular complexes (Maxwell & Réthelyi, 1987).

Comparison of the effect of NMDA and non-NMDA antagonists on Aδ and C fibre-evoked EPSPs indicates that the initial component of the EPSP is mediated by non-NMDA receptors. NMDA receptors appear to contribute to later phases of EPSP since the duration of EPSPs was decreased by APV. These findings are in agreement with previous studies on the contribution of non-NMDA and NMDA receptors of synapses on spinal neurones in culture (Forsythe & Westbrook, 1988). In contrast, in *Xenopus* tadpole spinal cord, some sensory neurone-evoked synaptic potentials appear to be mediated predominantly by NMDA receptors (Dale & Roberts, 1985).

L-Glutamate insensitive substantia gelatinosa neurones

Although all Aδ and C fibre-evoked monosynaptic EPSPs recorded from substantia gelatinosa neurones were sensitive to excitatory amino acid receptor antagonists, only about 50% of these neurones responded directly to exogenously applied L-glutamate. A high proportion of L-glutamate-insensitive dorsal horn neurones has also been reported in hamster spinal cord (Schneider & Perl, 1988). However, L-glutamate-insensitive substantia gelatinosa neurones received afferent-evoked EPSPs that were blocked by the amino acid receptor antagonists CNQX, kynurenate and APV indicating that they do express excitatory amino acid receptors. Exogenously applied L-glutamate therefore fails to activate excitatory amino acid receptors on this subset of neurones. There are two possible reasons for the lack of effect of L-glutamate.

Firstly, L-glutamate may be removed from the extracellular space by an effective uptake process. In support of this, excitatory amino acid agonists such as kainate and AMPA that are not substrates for high-affinity uptake mechanisms (Stallcup *et al.* 1979; Bridges *et al.* 1987) depolarize substantia gelatinosa neurones that fail to respond to L-glutamate. The high-affinity uptake of L-glutamate inhibitor L-AABH was effective in potentiating the L-glutamate-evoked depolarization in L-glutamate-sensitive neurones but did not confer sensitivity on neurones previously insensitive to L-glutamate. L-AABH may therefore be unable to overcome a highly effective uptake system in the microenvironment to some neurones. Alternately, there may be more than one class of L-glutamate uptake system, only one of which is insensitive to L-AABH.

Secondly, L-glutamate has been reported to induce a rapid desensitization of receptors which maintain a sustained response to kainate (Trussell, Thio, Zorumski

& Fischbach, 1988; Mayer & Vyklicky, 1989). Thus, it is possible that L-glutamate reaches the receptors which mediate the afferent-evoked EPSP but causes these receptors to desensitize before any depolarization can be detected.

Antagonist resistant L-glutamate responses

Our analysis of the pharmacological properties of substantia gelatinosa neurones raises the possibility that L-glutamate interacts with two distinct classes of non-NMDA receptors. Evidence for this comes from a comparison of the antagonist sensitivity of afferent-evoked EPSPs with L-glutamate-evoked depolarization in the same neurones. CNQX is a potent antagonist of the response of substantia gelatinosa neurones to Aδ and C fibre input, to kainate and to AMPA, whereas responses to L-glutamate are inhibited by only about 25%. Moreover, APV does not block the CNQX-resistant component of the L-glutamate-evoked depolarization in these neurones. One interpretation of these observations is that substantia gelatinosa neurones express two classes of non-NMDA receptors: (i) a CNQX-sensitive receptor activated by the afferent synaptic transmitter, by kainate and AMPA, and perhaps by L-glutamate if it were not removed by uptake; and (ii) a receptor which is activated by L-glutamate but which is not blocked by CNQX. Since L-glutamate appears not to gain access to the CNQX-sensitive synaptic receptors on many neurones, the CNQX-insensitive non-NMDA receptor may be located at non-synaptic sites. L-Glutamate-evoked responses which are insensitive to amino acid receptor antagonists have also been observed in spinal cord neurones *in vivo* (Honoré *et al.* 1988). Further studies are clearly required to characterize this CNQX- and APV-insensitive L-glutamate receptor and to determine whether this reflects the expression of a novel L-glutamate receptor by substantia gelatinosa neurones.

More generally, our results provide direct evidence that Aδ and C fibres release a transmitter(s) which acts primarily on a CNQX-sensitive population of postsynaptic excitatory amino acid receptors. L-Glutamate is likely to be the transmitter released by both classes of fibres, although we cannot exclude that Aδ and C fibres release distinct transmitters which interact with similar populations of postsynaptic receptors. Our studies also show that C fibres which are known to contain and release neuropeptides also release an L-glutamate-like fast transmitter. The interaction of L-glutamate with non-NMDA receptors is likely to be responsible for the initial signalling of cutaneous sensory input in the superficial dorsal horn. Activation of NMDA and neuropeptide receptors and the recruitment of local dorsal horn circuits may modulate the information transmitted *via* this initial synaptic event, which is likely to be common to all classes of somatosensory afferent fibres.

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