Comparison of Primary Afferent and Glutamate Excitation of Neurons in the Mammalian Spinal Dorsal Horn

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The actions of L-glutamate and agonists, agents blocking their membrane receptors and dorsal root afferent volleys, were compared on intracellularly recorded neuronal activity in an *in vitro* horizontal slice preparation of the hamster spinal dorsal horn. Bath-applied L-glutamate or L-aspartate (≤ 1 mM) rapidly depolarized and excited less than a third of the dorsal horn neurons sampled. Bathing solutions containing low Ca^{2+} eliminated synaptic transmission in the slices but failed to block the excitatory effects of L-glutamate for the majority of the neurons tested. *N*-Acetylaspartylglutamate had no effect on dorsal horn neurons at concentrations up to 1 mM.

Neurons excited by L-glutamate were most commonly located in the superficial dorsal horn (laminae I and II). Neurons insensitive to L-glutamate were more broadly distributed, with a number being located in laminae III–V.

Kynurenic acid, 2-amino-4-phosphonobutyric acid, and 2,3-piperidine dicarboxylic acid selectively antagonized rapid, short-lasting synaptic components of the dorsal cord potentials. Kynurenic acid reversibly antagonized intracellularly recorded L-glutamate-induced excitation, spontaneous synaptic potentials, and fast synaptic potentials evoked by dorsal root volleys. Compounds with strong antagonist actions at the NMDA receptor, 2-amino-5-phosphonovaleric acid and D- α -amino adipic acid, were much less effective in suppressing the effects of L-glutamate or in blocking synaptic potentials.

We conclude that a subset of spinal neurons directly excited by dorsal root fibers have excitatory membrane receptors activated by L-glutamate. This conclusion is consistent with the concept that L-glutamate or a substance binding to the receptors it activates is released from the central terminals of some primary afferent fibers and mediates fast synaptic transmission from them to certain spinal neurons in the dorsal horn.

The distribution, uptake, and release of L-glutamate in regions of the spinal cord known to receive terminations from afferent fibers (Graham et al., 1967; Johnson and Aprison, 1970; Balcar and Johnston, 1972; Roberts, 1974) have made it a candidate for chemical transmission at synapses of the central terminals

of primary sensory neurons (see reviews by Curtis and Johnston, 1974; Puil, 1981). With few exceptions, previous reports have suggested an absence of selectivity in the excitatory action of L-glutamate on spinal and medullary neurons (Curtis et al., 1960; Zieglgansberger and Herz, 1971; Zieglgansberger and Puil, 1973; Galindo et al., 1977; Hill and Salt, 1982), supporting the contention that L-glutamate is a nonspecific, universal excitant (Curtis et al., 1960). However, there is evidence for a selective concentration of glutamate-related compounds in smaller dorsal root ganglion (DRG) neurons. For example, Cangro et al. (1985a) reported that phosphate-dependent glutaminase, an enzyme of possible importance for the synthesis of releasable pools of L-glutamate (Kvamme, 1983), is elevated only in a subpopulation of small DRG cells. Also, small DRG cells accumulate 6 times more glutamine, a precursor in the synthesis of L-glutamate, than larger DRG neurons (Duce and Keen, 1983).

The mammalian superficial dorsal horn is known to receive terminations principally from fine-diameter, primary afferent axons (Light and Perl, 1979). Our earlier observations on extracellularly recorded activity in an *in vitro* preparation indicated that L-glutamate and L-aspartate preferentially excited neurons located in this region compared to deeper layers (Schneider and Perl, 1985a). We report here a comparison of the effects of blocking compounds and other manipulations on the primary afferent and L-glutamate-related excitation of dorsal horn neurons in intracellular recordings made in the same kind of preparation. Preliminary results have been presented elsewhere (Schneider and Perl, 1985b).

Materials and Methods

Preparation of tissue. Slices of lumbosacral spinal cord were obtained from 3- to 4-week-old, free-ranging, male Syrian hamsters weighing 35–55 gm by a modification of the procedure described by Bagust et al. (1982). Under anesthesia with urethane (1.5 mg/gm), the animals were cooled to 23–25°C by partial immersion in ice water. The vertebral column from midthorax to sacrum was removed and placed in chilled (3–8°C) artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.5 mM MgSO_4 , 1.25 mM NaH_2PO_4 , 24 mM NaHCO_3 , and 10 mM glucose at pH 7.4 equilibrated with 95% O_2 /5% CO_2 . The spinal cord was exposed by cutting away the vertebrae on each side, freed from bone and muscle, and a single, horizontal knife cut was made by hand with a fragment of razor blade parallel to and midway between the dorsal and ventral surfaces. In less than 3 min these procedures produced a slice of dorsal spinal cord approximately 400–600 μm thick with dorsal roots and sensory ganglia attached.

The slice was placed together with dorsal rootlets and sensory ganglia onto a stiff, nylon grid suspended over a layer of Sylgard (Dow Corning) in the recording chamber (volume, 1.5 ml), and all tissue was superfused with oxygenated ACSF (12–16 ml/min) at room temperature (23–27°C). A single, lumbar dorsal rootlet was isolated, placed onto platinum wire electrodes in an adjacent well connected to the recording chamber (Fig. 1A) and covered with a mixture of petroleum jelly and mineral oil.

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Figure 1. Experimental arrangement for *in vitro* dorsal horn slice. *A*, Recording chamber; *B*, positions of recording (*Rec*) and stimulating (*St*) electrodes on dorsal root relative to tissue slice.

Synaptic activation of neurons in the preparation remained unchanged for 8–10 hr.

Electrical stimulation and intracellular recording. A pressor foot constructed from a specimen grid for electron microscopes was gently lowered onto the dorsal surface to improve mechanical stability. Microelectrode penetrations were made through the grid perpendicular to the pial surface at loci where field potentials evoked by dorsal root volleys were maximal (Fig. 1*B*). Intracellular recordings from single dorsal horn neurons and primary afferent fibers were obtained with fine-tipped, glass pipette electrodes (Brown and Flaming, 1977) filled with 3 M potassium acetate or 2 M potassium methylsulfate (DC resistance, 80–120 M Ω). Signals were amplified by an electrometer system with bridge circuitry and stored on magnetic tape using an FM recording system. The microelectrode was advanced by a piezoelectric driver (a generous gift of Dr. A. R. Martin) attached to a stepping, hydraulic microdrive. Negative constant current pulses 20–50 msec in duration and 0.5 nA or less were injected through the recording pipette to measure neuronal input resistance.

Neurons were identified according to the measured distance from the point of contact with the dorsal surface of the spinal cord. We have shown by intracellular marking that, in these slice preparations of adolescent hamster spinal cords, this procedure localizes neurons within 50 μ m of loci marked by iontophoresis from recording electrodes (Schneider and Perl, 1985a). On this basis, recording locations were specified as the superficial dorsal horn (laminae I and II) or nucleus proprius (laminae III to V). This segregation was aided by recording of field potentials and the nature of excitatory drive from the dorsal root stimuli since the input to the superficial dorsal horn is dominated by excitation from thin primary afferent fibers, and there is a notable change in the field potentials and source of excitation between lamina II and the dorsal limits of the nucleus proprius (Christensen and Perl, 1970; Kumazawa and Perl, 1978; Light and Perl, 1979). The present observations were dominated by recordings from the superficial dorsal horn because of a specific emphasis on this region. Anatomical localization of recording sites with respect to individual spinal laminae was not attempted.

Volleys in dorsal root afferents were initiated by electrodes located about 8 mm from the spinal cord and were monitored by electrodes near the spinal cord junction (Fig. 1*B*). Components of the electrical signs of volleys reflecting synchronized activity in myelinated (A α β , A δ) and unmyelinated (C) fibers could be identified by waveform and by conduction velocity (calculated from latency and measured conduction distance to the monitoring electrode). Volleys in A α β fibers conducted between 7 and 25 m/sec, whereas those in A δ fibers conducted in the range of 2–5 m/sec. The velocity of C-fiber volleys was less than 1 m/sec. The conduction velocities of afferent fibers in this preparation were relatively low compared with *in vivo* observations in cat and primate (Burgess and Perl, 1973), reflecting the below-normal bath temperatures and species-related differences in axonal diameters and myelination. These conduction velocity values are comparable to those

observed in young rats and guinea pigs (E. R. Perl, unpublished observations). Judgment about the relationship between the source of excitation to a neuron and the conduction velocity of afferent fibers was based upon a consistent correlation between a volley component and an intracellularly recorded synaptic potential.

Intracellular recordings from primary afferent fibers could be easily differentiated from postsynaptic discharges by a one impulse response to one stimulation of the dorsal roots at frequencies over 100 Hz with a near-constant latency (variation <0.2 msec) and by the absence of evoked or background synaptic potentials (see Fig. 4, D1–D3).

Drugs and solutions. Solutions were changed by switching between gravity-fed supply sources connected in parallel to the bath inflow tube. Synaptic transmission was blocked by changing to a modified ACSF with low Ca²⁺ (0.25 mM) and high Mg²⁺ (5–10 mM) at pH 7.4 equilibrated with 95% O₂/5% CO₂, or to a Tris-buffered medium containing Cd²⁺ (0.1–1 mM) or Mn²⁺ (1–2.4 mM) at pH 7.4 equilibrated with 100% O₂ (Llinás and Sugimori, 1980). Application of excitatory amino acids via the bathing medium has been found to produce effects identical to those obtained with iontophoretic administration of the same agents (Schneider and Perl, 1985a). Solutions containing *N*-acetylasparylglutamate (Bachem, Torrance, CA; Lot no. 948B), D,L-homocysteic acid (Sigma), kynurenic acid (Sigma), D- α -aminoadipate (Sigma), D(-)-2-amino-4-phosphonobutyric acid (Sigma), D(-)-2-amino-5-phosphonovaleric acid (Sigma), *trans*-free ($\pm$)-cis-2,3-piperidine dicarboxylic acid (Cambridge Research Biochemicals), and γ -D-glutamylglycine (Cambridge Research Biochemicals) were prepared in ACSF immediately before use.

Results

General characteristics of dorsal horn neurons in the sample

Intracellular recordings for 30 min to over 3 hr were obtained from 198 dorsal horn neurons. The mean resting membrane potential (V_m) of the 147 neurons for which stable permanent records were available was 65.2 ± 0.5 (SE) mV. The mean amplitude and duration of action potentials ($n = 155$) was 63.8 ± 0.6 mV and 1.9 ± 0.1 msec, respectively; the values for neurons located superficially (<200 μ m from the dorsal surface; 65.1 ± 0.6 mV, 2.0 ± 0.1 msec) were essentially the same as for deeply located cells ($\geq 200 \mu$ m; 65.2 ± 1.0 mV, 1.5 ± 0.1 msec). Thus, there was no evidence for a differential viability throughout the slice. Neuronal input resistance (R_N) was quite variable, ranging in 132 cells from 1.5 to 208.6 M Ω (mean, 50.2 ± 3.3) suggesting a heterogeneous population in size and geometry. These values overlap somewhat but are generally lower than those reported by Murase and Randic (1983), also apparently derived from a

Figure 2. Intracellular recordings of dorsal horn neurons of the *in vitro* slice. Each display in *A–C* is composed of 3 oscilloscope sweeps. Onsets of synaptic potentials activated by volleys in A- and C-fibers are indicated at *a* and *c*, respectively. *A* and *B*, Two neurons responding only to myelinated (A) afferent volleys. Neuron of *A* was located in nucleus proprius ($V_M = -52$ mV). Neuron of *B* was located in the superficial dorsal horn ($V_M = -63$ mV). *C* and *D*, Two neurons responding to volleys in both A- and unmyelinated C-fibers. Neuron of *C* was located in the superficial laminae ($V_M = -70$ mV). Arrows in *B* and *C* mark 2 consecutive oscilloscope sweeps following afferent stimulation just suprathreshold for volleys in A δ - (*B*) and C- (*C*) fibers. *D*, Ten superimposed responses of a nucleus proprius neuron ($V_M = -62$ mV) to volleys in both A- and C-fibers. Hyperpolarization was present in the absence of spike discharge. Current (1 nA) pulses (downward) injected through the recording electrode, one pulse for each sweep at delays separated by duration of the pulse, indicate changes in R_N . Action potentials are truncated. Downward arrowheads mark stimulus artifact. Calibrations for *A* and *B* are shown in *C*.

mixed population of dorsal horn neurons from a horizontal slice preparation. R_N of neurons (56.8 ± 4.0 M Ω , $n = 102$) in the superficial dorsal horn (<200 μ m) was higher than those (27.9 ± 3.8 M Ω , $n = 30$) in the nucleus proprius (≥ 200 μ m). Values for R_N reported by North and Yoshimura (1984) for lamina II neurons in a transverse slice preparation are much higher than ours and Murase and Randic's (1983) from horizontal slices.

We have no evidence to account for this difference of R_N from the observations on horizontal and on transverse slices; however, it may be significant that in all mammalian species the principal orientation of dendrites of superficial dorsal horn neurons is longitudinal, and these processes extend over distances often exceeding 500 μ m. Some such processes surely would be transected in transverse slices 500 μ m thick used by North and Yoshimura (1984). Given the number of our recordings from depths and field potential zones corresponding to laminae I and II and the scarcity of large neurons in these levels, it appears unlikely that our sample was unduly biased toward large neurons.

Single dorsal root volleys initiated postsynaptic potentials (PSPs) in all neurons of the sample (Fig. 2). In most of these cells, volleys confined to myelinated (A) afferent fibers activated depolarizing excitatory postsynaptic potentials (EPSPs) with relatively invariant initial latencies (± 0.2 msec). EPSPs were usually multiphasic with later components; the latter's usual relationship to stronger dorsal root stimulation suggested that the excitation involved more slowly conducting primary fibers and/or more complex circuits. Since the earliest synaptic potentials, particularly in the superficial dorsal horn, were regularly associated in fibers conducting at less than the maximal in the stimulated dorsal root, proof of the number of synaptic links in the activation was impossible. This was particularly true for the later components of complex EPSPs. Nonetheless, it appeared likely on the basis of comparison with other systems (Kostyuk, 1961; Perl, 1962) that those dorsal root-initiated EPSPs with essentially invariant latencies were the result of simple, i.e., monosynaptic, linkages. The duration of EPSPs activated by A-fibers often increased with increasing stimulus intensity, depending on the composition of the volley, over a range of 7–100 msec. Volleys in A-fibers also activated hyperpolarizing, inhibitory postsynaptic potentials (IPSPs) in about 40% (71/181) of the sample (Fig. 2, *B*, *D*).

In some neurons, it was possible to distinguish between PSPs activated by rapidly (A $\alpha\beta$) and slowly (A δ) conducting A-fibers. PSPs initiated by A $\alpha\beta$ fibers (Fig. 2, *A*, *D*) occurred at a short latency (2–4 msec) upon weak dorsal root stimulation conjointly with the A $\alpha\beta$ wave in the dorsal root volley (not shown). PSPs in other neurons (Fig. 2*B*) had a longer latency (5–10 msec), typically required higher stimulus voltages than those with short latency, and were coupled to the appearance of the A δ deflection. Many short-latency and a number of longer-latency PSPs showed the stable latencies associated in other systems to be associated with monosynaptic activation.

C-fiber components were conjoint with EPSPs in about 40% (77/196) of the population. Such EPSPs were defined as those appearing in parallel only with volleys containing activity in afferent fibers conducting at <1 m/sec and appearing at latencies consistent with the arrival of C-fiber input (15–50 msec). Examples are shown in Figure 2, *C* and *D*. The C-fiber EPSPs had durations comparable to those initiated by A-fiber volleys (15–90 msec).

The PSPs evoked by single volleys in A- and/or C-fibers were associated with a decrease in R_N . (Fig. 2*D*, cf negative pulses early and late in sweep.) The decrease in R_N contrasts with the increased R_N of the much longer lasting potentials reported by Urban and Randic (1984) to be initiated in some dorsal horn neurons by repeatedly stimulating dorsal roots.

Transmembrane recordings from neurons excited by dorsal root volleys were obtained from 20 to 360 μ m ventral to the

Figure 3. Location of neurons relative to the dorsal spinal surface related to excitatory components of the dorsal root volley (A) and sensitivity to bath application of 1 mM L-glutamate (B). Approximate boundaries of dorsal horn laminae are indicated at right. I, II, III–V, Rexed's laminae; WM, white matter.

dorsal surface (laminae I–V). As shown in Figure 3A, neurons activated by A- and by C-fiber components of the volleys were differentially distributed within the dorsal horn; this differential distribution was consistent with the termination pattern of thick and thin afferent fibers established in other species (Light and Perl, 1979). Neurons with EPSPs associated solely with the A-fiber component ("A only" in Fig. 3A) were located throughout the dorsal horn, whereas those exhibiting PSPs related to the C-fiber component ("C only," "A and C" in Fig. 3A) were most common 60–180 μm beneath the dorsal surface.

Effects of L-glutamate, L-aspartate and N-acetylaspartylglutamate (NAAG)

L-glutamate, at bath concentrations of 1 mM or less, depolarized and initiated discharges in about 30% (22/77) of the dorsal horn neurons on which it was tested (Figs. 4A1, 5B, 6A and B, 8A). Seven neurons were hyperpolarized by L-glutamate. Two neurons were strongly excited by bath concentrations of L-glutamate of 50–100 μM , but most excited cells required concentrations between 0.5 and 1 mM for marked depolarization and vigorous excitation. In the typical L-glutamate-sensitive neuron, increasing the concentration from threshold levels to 1 mM caused graded increases in the magnitude of the depolarization and the

frequency of discharges, and sometimes induced a depolarization block of activity. The depolarization often occurred independently of an increase in spontaneous PSPs. Six neurons demonstrated strong excitation only at concentrations of 3 mM (Figs. 8B, 9). L-Aspartate was tested in 10 neurons and showed effects identical to L-glutamate at the same concentrations (not shown). The depolarization produced by the highest concentration of L-glutamate tested upon 8 neurons averaged 17 ± 3 mV (4–29 mV) and was associated with a substantial reduction in R_N ($52 \pm 7.5\%$).

Fifty-five of the 77 dorsal horn neurons were judged insensitive to L-glutamate and/or L-aspartate because concentrations of 1 mM or greater did not alter V_M , R_N , or frequency of discharge (Figs. 4B, 11A). Concentrations of glutamate 1 mM in about $\frac{1}{5}$ (8/43) of these L-glutamate-insensitive neurons induced a small, subthreshold depolarization (4.0 ± 0.9 mV) accompanied by a small reduction in R_N ($11 \pm 3\%$, Figs. 4C, 5C, 11B); increasing L-glutamate concentrations to 3 mM or more did not provoke responses in the insensitive group (Fig. 4B). A reduction in R_N was never noted in the absence of changes in V_M . Neurons insensitive to L-glutamate were routinely recorded in the same preparation both superficially and more deeply than those excited by L-glutamate and had similar resting V_M 's. Furthermore,

Figure 4. Effects of L-glutamate or like agents in superfusate on dorsal horn elements. Horizontal bars mark time of application of tested agent. A1, L-Glutamate; A2, N-acetylaspartylglutamate (NAAG) action on a superficial neuron; B and C, two other superficial neurons. Calibration in C for A–C. Action potentials are truncated by chart recorder. Downward deflections in the baseline represent intracellular current injection to measure R_N . D, Myelinated primary afferent fiber conducting at 11.4 m/sec; D1, L-glutamate; D2, response to a single dorsal root volley; D3, repetitive (100 Hz) volleys. Downward arrowhead marks stimulus artifact.

the lack of sensitivity to L-glutamate appeared to be unrelated to the general condition of the preparation. In each of 4 instances, extracellularly recorded neurons that were unresponsive to L-glutamate were found to be strongly excited by bath application of 1 mM D,L-homocysteate. These observations indicate that insensitivity to L-glutamate is not the result of a general decrease in electrical signs of responsiveness.

At concentrations causing robust excitation in spinal neurons, L-glutamate neither excited myelinated primary afferent fibers nor altered their action potentials. This makes it unlikely that the excitatory effects observed in spinal neurons resulted from presynaptic actions on primary afferent terminals (see Curtis et al., 1984). High concentrations of L-glutamate (≥ 3 mM), however, occasionally caused a small (<4 mV) depolarization in primary afferent fibers (Fig. 4D1), suggesting that at such high glutamate concentrations, some type of presynaptic effect is possible.

NAAG, an acetylated dipeptide of aspartate and glutamate (Zaczek et al., 1983) has been reported to be present in DRG cells (Cangro et al., 1985b). The firing frequency, V_M , or R_N (Fig. 4A2) of 3 neurons strongly excited by L-glutamate and/or L-aspartate were not altered by synthetic NAAG at 1 mM concentration. Three other neurons that did not respond to 1 mM L-glutamate were also unresponsive to similar concentrations of NAAG.

Characteristics of L-glutamate-sensitive neurons: laminar position, afferent input, and R_N

The sensitivity of neurons to L-glutamate was related to their dorsal–ventral location and to their R_N . Twenty-one neurons excited by L-glutamate at concentrations of 1 mM or less were concentrated in a zone between 70 and 170 μ m below the dorsal surface of the cord (Fig. 3B). This distribution is coextensive with that of neurons activated by volleys in C-fibers (Fig. 3A) and is contained in the superficial laminae of the spinal level from which the slice was prepared (Schneider and Perl, 1985a). Fifty-five neurons insensitive to L-glutamate were more uniformly dispersed throughout a zone that encompasses the bulk of the dorsal horn in the hamster (40–341 μ m below the dorsal

surface). Since in some cases, a deeper-lying neuron was found to be responsive to L-glutamate while superficial ones were not, skewing of the distribution of L-glutamate-insensitive neurons toward deeper locations in the slice is not simply due to differential drug diffusion. Moreover, arguments can be mounted that the cut deep surface of our type of slice may offer less of a barrier to diffusion than the pia-covered dorsal surfaces. The distribution of neurons excited by L-glutamate and those not excited is consistent with results from extracellular recordings (Schneider and Perl, 1985a), even though our present sample, biased by a deliberate effort to study neurons located superficially, resulted in a relative paucity of neurons more than 200 μ m from the dorsal surface (Fig. 3).

The relationship of the dorsal root volley components to EPSPs and R_N to L-glutamate and/or L-aspartate responsiveness is summarized in Table 1. No difference in the excitatory effectiveness of primary A- and C-fibers relative to L-glutamate responsiveness was apparent, possibly related to a sample bias toward neurons receiving excitatory input from A-fibers compared with our earlier survey of extracellularly recorded units (Schneider and Perl, 1985a). There was no difference in the proportion of neurons excited (9/22) and not excited (19/55) by L-glutamate which had EPSPs associated with C-fibers (“C only” and “A and C” in Table 1). On the other hand, the sensitivity of neurons to L-glutamate was correlated with their R_N (Table 1); those excited by L-glutamate had a significantly ($p < 0.001$; t test, 2-tailed) greater R_N (81.1 ± 12.7 M Ω , $n = 15$) than those unaffected by it (47.5 ± 4.8 M Ω , $n = 43$).

Effects of L-glutamate on spontaneous PSPs

Subthreshold, transitory fluctuations in V_M were evident in 63% (55/88) of those neurons for which adequate records were available (Figs. 5A, 10). These events were similar in duration to EPSPs evoked by weak stimulation of the dorsal roots, varied in amplitude from 2 to 4 mV, and could be synchronized by volleys in the dorsal roots (not shown). Six neurons exhibited spontaneous hyperpolarizing PSPs, suggestive of spontaneous IPSPs, that could be synchronized by dorsal root volleys and reversed by injecting hyperpolarizing current through the re-

Figure 5. Action of L-glutamate on spontaneous EPSPs of 3 superficial dorsal horn neurons. *a1* and *a2* of *A* are expanded oscilloscope sweeps showing EPSPs before and during presentation of L-glutamate, respectively. Calibration in *C* applies to *A–C*.

cording pipette (not shown). This was taken as indicative of a reversal potential more negative than the recorded V_M .

Bath-applied L-glutamate (0.5–1 mM) increased the frequency of EPSP-like fluctuations for some L-glutamate-sensitive neurons (13/30) coincident with the decreases in V_M and R_N and increase in discharge frequency (Fig. 5, *A* and *B*). L-glutamate also increased the frequency of background EPSPs in a fraction of neurons (16/57) that were otherwise unaffected by this compound (not shown). Elevating L-glutamate concentrations from 1 to 3 mM did not enhance this effect.

Direct versus indirect effects of L-glutamate action

An amino acid added to the superfusion fluid could act upon neurons synaptically linked to a cell under study, a possibility consistent with the observation that L-glutamate often increased the frequency of background EPSPs. Superfusion with media containing low Ca^{2+} and high Mg^{2+} or with Mn^{2+} substituted for Ca^{2+} reversibly blocked the synaptic responses of neurons to dorsal root volleys (Fig. 6*A*, inset). Low- Ca^{2+} media also blocked L-glutamate-induced depolarization, background EPSPs, and the enhancement of L-glutamate upon background EPSP frequency in 52% (13/25) of tested neurons (Fig. 6*B*). The actions of low Ca^{2+} were manifested in 5–30 min and reversed within 10–45 min after return to the normal Ca^{2+} solution. Exposure to media with altered concentrations of divalent cations also reversibly reduced R_N in some neurons (Fig. 6); this effect did not appear to be correlated with responsiveness to L-glutamate.

Superfusion with low Ca^{2+} /high Mg^{2+} ACSF sufficient to completely block synaptic responses to dorsal root volleys either did not affect or only reduced the depolarization and excitation from

Table 1. Relationship of sensitivity to 1 mM L-glutamate to afferent volley composition and R_N

	Excitation from dorsal root			R_N
	A only	C only	A and C	
Excited by L-glutamate ($n = 22$)	13	4	5	81.1 ± 12.7
Not excited ($n = 55$)	36	1	18	47.5 ± 4.8

Excitatory components of dorsal root volleys and R_N for 77 dorsal horn neurons classified according to their sensitivity to L-glutamate. A only: neurons activated by dorsal root volleys containing only the A (myelinated) fiber component. C only: units activated only by volleys in C (unmyelinated) fibers. A and C: neurons activated by volleys in both A and C fibers. R_N : neuronal input resistance (mean $\pm$ SEM).

L-glutamate (Fig. 6*A*) in about 50% (12/25) of L-glutamate-sensitive neurons. These observations led us to conclude that some of the dorsal horn neurons were excited by L-glutamate binding postsynaptically, while others were activated indirectly via action on presynaptic elements.

Four neurons that were unresponsive to L-glutamate (1 mM) in normal ACSF were also unresponsive in low- Ca^{2+} media (not shown). Cells were never excited by superfusion of low Ca^{2+} /high Mg^{2+} ACSF alone; therefore, the absence of excitatory response to L-glutamate appeared unlikely to have been the result of a masking by tonically released inhibitory transmitter action.

Action of excitatory amino acid antagonists

Excitatory amino acid receptor antagonists—kynurenic acid (Kyn), D- α -amino adipic acid (DAA), D(-)-2-amino-4-phosphonobutyric acid (APB), D(-)-2-amino-5-phosphonovaleric acid (APV), γ -D-glutamylglycine (DGG), and ($\pm$)-cis-2,3-piperidine dicarboxylic acid (PDA)—differ in their antagonist properties. Kyn, APB, PDA, and DGG are believed to act at quisqualate, kainate, and N-methyl-D-aspartate (NMDA) subtypes of excitatory amino acid receptors, although their affinities for each subtype differ (Watkins and Evans, 1981; Perkins and Stone, 1982). DAA has its greatest affinity for the NMDA receptor but may also have actions at kainate receptors (Watkins and Evans, 1981). APV is the most selective of all the compounds; its actions are reported to be specific for the NMDA subtype (Watkins and Evans, 1981).

Dorsal root volleys evoke a complex that includes 3 negative waves (N_1 , N_2 , and N_C) in our *in vitro* preparation; these appeared comparable to those described *in vivo* in other species (Christensen and Perl, 1970; Beall et al., 1977; Kumazawa and Perl, 1978) (Fig. 7*A*). The first wave, N_1 , with a peak at about 4 msec, was initiated by volleys limited to A $\alpha\beta$ fibers (Fig. 7*A1*) and was prominent throughout the thickness of the slice. The second, smaller wave, N_2 (in monkey, N_3 ; Beall et al., 1977), with a peak at 9 msec, coincided with volleys in A δ fibers (Fig. 7*A2*) and appeared largest when the tip of the recording electrode was between 50 and 150 μm below the dorsal surface. This potential has been attributed to activity in neurons of the outermost superficial dorsal horn (Christensen and Perl, 1970). The third wave, N_C , with a peak at about 20 msec, was associated with volleys in C-fibers (Fig. 7*A3*) and was concurrent with EPSPs in single neurons evoked by C-fiber volleys. N_C was most conspicuous when the tip of the recording electrode was located between 50 and 200 μm below the dorsal surface.

Figure 6. Effect of low Ca^{2+} on 2 superficial dorsal horn neurons. *A* (same neuron as Fig. 4*A*), and *B*. *DR*, Response of each neuron to graded volleys in dorsal roots. *a* and *c*, Synaptic response to volleys in A- and C-fibers, respectively. Bottom three rows: responses to L-glutamate in normal ACSF (*control*), in medium containing 1.3 mM Mg^{2+} and either 1 mM (*A*) or 2.4 mM (*B*) Mn^{2+} (*Ca-free*) and after return to normal ACSF (*wash*). *Insets* in *A* show effect of *Ca-free* superfusate on response evoked by dorsal root stimulation at C-fiber intensities (*downward arrowheads*). Effect of *Ca-free* medium on response of neuron in *B* to dorsal root volleys same as in *A* (not shown). Calibration for *A* shown in *B*.

Kyn, APB, and PDA (0.5, 1 mM) produced differential suppression of these 3 waves (Fig. 7*B*) in 5–10 min. Kyn (1 mM) had only a small depressive action on the N_1 wave; in 9 experiments, this component was diminished by an average of only 12%. In contrast, Kyn suppressed N_2 and N_c by more than 40%. The effects of APB and PDA on the dorsal cord potentials were similar to those of Kyn. APV, DAA, and DGG had no effect at equivalent concentrations even when applied for 30 min (not shown). None of the compounds appeared to affect the amplitude, latency, or time course of the primary afferent volley.

Intracellularly recorded EPSPs initiated by dorsal root volleys were reversibly decreased in many (21/35) of the neurons tested with Kyn (0.5, 1 mM) within 1–2 min after the beginning of superfusion, with the maximum effect occurring in 5–7 min. The number of action potentials evoked by a volley decreased, and discharges were sometimes abolished without changes in V_M or R_N (Table 2, Fig. 8). Longer exposure (up to 30 min) to Kyn usually produced no further block. In 6 neurons, Kyn had little effect on IPSPs evoked by dorsal root volleys other than 2 instances in which the slope of the late phase of the afferent-evoked IPSP was increased (see Fig. 8*A*). At concentrations up to 3 mM Kyn had no effect on resting V_M , spike duration, or conduction velocity of myelinated primary afferent fibers (not shown).

Kyn antagonized the responses of certain dorsal horn neurons to afferent input from both A- and C-fibers (see Table 2). Thirteen of 21 neurons whose dorsal root EPSPs were depressed by Kyn were synaptically activated only by volleys in A-fibers. Figure 8*A* illustrates the effects of Kyn on a neuron in lamina

I or outer lamina II driven by volleys in $A\delta$ fibers. After superfusion for 4 min with 1 mM Kyn, the EPSP and action potential, but not the IPSP, were reversibly blocked. Eight of the 21 neurons received demonstrable excitatory input from C-fibers. Kyn selectively antagonized responses to volleys in the C-fibers but not the A-fibers in 3 neurons excited by both A- and C-fibers. Figure 8*B* shows an example of the effect of Kyn on a presumed lamina II or III neuron; superfusion of 1 mM Kyn for 1 min decreased the magnitude of the depolarization and reduced the number of discharges evoked by volleys in the C-fibers, while the earlier response to $A\alpha\beta$ volleys was changed little. The blocking effects of Kyn were not related to a particular preparation. Neurons whose responses were partially or completely blocked were observed in preparations in which other neurons studied either before or afterward were unaffected.

These observations taken together indicated that the blockade was related to synaptic actions by a subset of myelinated or unmyelinated primary afferent fibers. The proportion of shorter latency responses evoked by dorsal root volleys in those dorsal horn neurons excited by L-glutamate differs from our earlier observations made on extracellularly recorded unitary potentials (Schneider and Perl, 1985a). One possible explanation for this discrepancy is a sampling bias in the selection of neurons for the present intracellular recordings, a selection that likely was biased to larger elements. However, even in the intracellular sample, the amino acid receptor blockade frequently selectively reduced EPSPs associated with thinner afferent fibers ($A\delta$ and C).

Nearly all neurons (19/21) whose synaptic activation from

dorsal root stimulation was antagonized by Kyn were excited by bath application of L-glutamate (Figs. 8, 9). Excitation of most of these neurons (12/14) by L-glutamate was reversibly antagonized in a concentration-dependent manner by pretreatment with Kyn media (Fig. 9, left). As shown in Figure 9 (right), the effect of Kyn on responses to L-glutamate paralleled the effect on the excitatory responses to dorsal root volleys.

In all of the 6 neurons that were excited by L-glutamate and for which appropriate observations were available, background EPSPs and synaptic excitation were blocked by Kyn at concentrations similar to that for L-glutamate excitation. For example, in Figure 10A, 0.5 mM Kyn reversibly blocked spontaneous EPSPs in a superficial laminae neuron 4 min after addition to the bathing medium. The suppression of background EPSPs (Fig. 10B) and the response to dorsal root stimulation (not shown) in the same neuron by APV were much less than that by Kyn.

In contrast to the marked effects described above, the responses of 14 of 35 neurons to dorsal root volleys were unaltered by 1 mM Kyn even when added to the superfusate for up to 30 min. In a few neurons, the only effect of Kyn was an increased variability in the latency to afferent stimulation. Neurons that were resistant to Kyn also tended to be unresponsive to L-glutamate; only 2 of 14 neurons were excited by L-glutamate at concentrations up to 3 mM (Table 2). Background EPSPs persisted in 1 mM Kyn in 4 neurons that were resistant to Kyn and insensitive to L-glutamate, although in 2 cases their amplitude and frequency were diminished.

Figure 11 illustrates the failure of Kyn to block the synaptic activation of 2 neurons receiving input from A-fibers. As shown in Figure 11A, Kyn had no effect on an EPSP and action potential evoked by volleys in A $\alpha\beta$ fibers in a nucleus proprius neuron but may have reversibly attenuated a small, later depolarization (arrows). The example in Figure 11B also supports a differential action of Kyn on afferent fiber groups: Kyn had little effect on the synaptic response to volleys in A δ fibers of a neuron recorded in the superficial dorsal horn but reversibly diminished a small, later depolarization (arrows) related to C-fibers of the volley.

Discussion

A reevaluation of widely held views about the part played by L-glutamate-like agents and their membrane receptors for neurons in the spinal dorsal horn seems to be in order. First, our observations are inconsistent with the idea that L-glutamate is excitatory to all CNS neurons. In both the present and a previous sample (Schneider and Perl, 1985a), we found that marked somadendritic responsiveness to L-glutamate is limited to a subset (about a third) of dorsal horn neurons excited by primary af-

Figure 7. Action of excitatory amino acid antagonists on extracellular dorsal horn potentials. A1–A3, Single oscilloscope traces from recordings of activity in superficial dorsal horn evoked by dorsal root stimulation at 4.5, 20, and 75 V, respectively. See text for significance of N₁, N₂, and N_c. B, Three superimposed oscilloscope sweeps of response to volleys containing C-fiber activity before (*control*), during (*KYN*), and after (*wash*) the addition of 0.5 mM kynurenic acid. Bars in B indicate amplitude of potentials prior to KYN. Calibration for B same as A.

ferent input, particularly those located in the superficial dorsal horn. These observations are consistent with the concept that for rapid synaptic transmission some, but by no means all, primary afferent fibers activate a postsynaptic membrane receptor responsive to L-glutamate agonists.

Given the similarity of recording sites, latency of effects and the sequential observation of responsive and unresponsive neurons in a given preparation, the large number of neurons unresponsive to L-glutamate in our preparations cannot readily be

Table 2. Relationship of kynurenic acid-induced antagonism of response to dorsal root stimulation to afferent volley composition and sensitivity to 1 mM L-glutamate

	Excitation from dorsal root			Excitation by L-glutamate	Antagonism of L-glutamate excitation by kynurenic acid
	A only	C only	A and C		
Antagonized by kynurenic acid* (n = 21)	13	5	3#	19/21	12/14
Resistant to kynurenic acid** (n = 14)	11	1	2	2/14	1/1

* Response to dorsal root stimulation antagonized by 1 mM kynurenic acid.

** Response to dorsal root stimulation resistant to 1 mM kynurenic acid.

In the three neurons, kynurenic acid antagonized synaptic excitation by C-fibers.

Figure 8. Effect of kynurenic acid (KYN) on intracellular responses to dorsal root volleys in L-glutamate-excited neurons. *A*, Superficial laminae neuron (same cell as Fig. 1*B*). *B*, Nucleus proprius neuron. Records at *left* show effects of L-glutamate application; remaining panels show 3 consecutive, superimposed oscilloscope traces of the responses to dorsal root stimulation volleys (downward arrowheads) before (*control*), during (*KYN*), and after (*wash*) the addition of 1 mM kynurenic acid to the bathing medium. *a* and *c*, Same as in Figure 7. Calibrations in *B* apply to *A*.

attributed to diffusion barriers to the ligands or by an unexplained inactivation of certain L-glutamate receptors. Glial uptake of L-glutamate (Shank and Aprison, 1981) also does not appear to be a reasonable explanation of these observations since L-glutamate-sensitive neurons were commonly located in the superficial dorsal horn where the processes of ependymal glial cells are in closest apposition with the somata of neurons (Scheibel and Scheibel, 1968).

The observed differences in responsiveness cannot be accounted for on the basis of either moderate differences in L-glutamate receptor numbers or differences in membrane resistance alone. Broad-spectrum amino acid antagonists like Kyn blocked

or effectively interfered with synaptic activation evoked by dorsal root volleys in L-glutamate-sensitive neurons. Neither Kyn nor specific NMDA receptor blocking agents interfered with dorsal root excitation of neurons insensitive to L-glutamate. This commonality in receptor properties for the synaptic and agonist-induced excitation is inconsistent with a purely quantitative difference in receptor or membrane properties. Nonetheless, on the average, neurons unresponsive to L-glutamate did have a lower R_N than those excited by it. While differences in R_N could reflect variations in neuronal size, in the number and branching of processes, or in specific membrane properties, measurements made in different cells can reflect varying combinations of these factors. Additional data are necessary to evaluate these possibilities; although given the high proportion of lamina I and II recording sites in the sample, it seems likely that the L-glutamate-responsive neurons were relatively smaller than the unresponsive ones. A parsimonious explanation for the selectivity of excitation is that only some dorsal horn neurons have depolarizing membrane receptors activated by L-glutamate.

Mechanism of L-glutamate actions in vitro

The L-glutamate effects on many neurons were probably generated by a direct interaction by the amino acid with a specific membrane receptor that opens ion channels and causes a decrease in R_N and V_M (reviewed by Jessell et al., 1986). Our principal evidence for such direct action is the continued excitation by L-glutamate after block of synaptic transmission in low Ca^{2+} and the antagonism of L-glutamate-induced excitation by Kyn, a compound that interferes with all known subtypes of excitatory amino acid receptors. Excitation of other neurons by L-glutamate was Ca^{2+} dependent and thus may have been synaptically mediated, an interpretation consistent with the increase in frequency but not the amplitude of spontaneous EPSPs. However, this is equivocal evidence since amino acids can activate postsynaptic Ca^{2+} conductances that exist in dorsal horn

Figure 9. Responses of a superficial dorsal horn neuron to 3 mM L-glutamate (*left*) and to volleys in dorsal roots (*right*) before (*control*), during (*KYN*), and after (*wash*) bath application of 0.5 mM kynurenic acid. Stimulus artifact marked by downward arrowheads. Full amplitude of spikes not shown.

Figure 10. Spontaneous EPSPs in a neuron (same as for Fig. 9) before (control), during, and after (wash) exposure to 0.5 mM kynurenic acid (KYN, A) or 0.5 mM 2-amino-5-phosphonovaleric acid (APV, B).

neurons (Dingledine, 1983; Murase and Randic, 1983; MacDermott et al., 1986).

Since it was not possible to establish the monosynaptic nature of afferent-evoked EPSPs, the sites of action of agonist or antagonist compounds are uncertain. It is likely that some neurons were excited through interneurons by the actions of afferent volleys and L-glutamate. It also seems probable from the stability of the EPSP latency that some of the neurons both responsive and unresponsive to L-glutamate and Kyn were monosynaptically activated by primary afferent fibers. In any case, the observation that the afferent-evoked EPSPs of nearly all neurons insensitive to L-glutamate were resistant to Kyn sug-

gests that some spinal afferent pathways do not employ the excitatory amino acids at either the first- or any higher-order synapses to the neurons we studied. Furthermore, the lack of striking effect upon afferent evoked-IPSPs by Kyn suggests that many inhibitory interneurons are driven by synaptic mechanisms other than those involving L-glutamate or its agonists.

Our studies do not make it clear which subtype(s) of excitatory amino acid receptors mediated the actions of L-glutamate in the superficial dorsal horn. All these experiments were conducted in media with physiological concentrations of Mg^{2+} (1.5 mM), lower concentrations of which have been shown to selectively block ion channels activated by the NMDA receptor (Ault et al., 1980; Mayer et al., 1984). It is reasonable to assume that under such conditions this receptor was effectively blocked, yet synaptic activation by primary afferent input was vigorous. The ineffectiveness of APV, DAA, and DGG, strong antagonists for the NMDA receptor, in blocking spontaneous EPSPs, responses to dorsal root volleys, and excitation by L-glutamate also suggest a lack of contribution by NMDA receptors to excitation observed in our preparation. Therefore, we support the proposition that excitatory L-glutamate actions stem from the kainate and/or quisqualate types of receptors (Hill and Salt, 1982; Jessell et al., 1986).

Transmission from primary afferent fibers

The dipeptide NAAG has been described in somata of DRG cells (Cangro et al., 1985b) and reported to excite CNS neurons selectively (Bernstein et al., 1985; French-Mullen et al., 1985). However, in our studies, NAAG had no apparent effect on V_M , R_N , or firing frequency of dorsal horn neurons. Therefore, NAAG does not appear to activate the excitatory receptors in this group of neurons.

The neurons excited by L-glutamate in the present study were concentrated in the superficial dorsal horn, corroborating our

Figure 11. Effect of kynurenic acid (KYN) on responses to dorsal root stimulation for neurons unresponsive to L-glutamate. A, Nucleus proprius neuron; B, superficial dorsal horn neuron. Arrows indicate components of synaptic response reversibly antagonized by KYN. Other details and abbreviations same as in Figure 9.

observations on extracellularly recorded units (Schneider and Perl, 1985a). The superficial dorsal horn contains 3 to 4 times the number of receptors for ^3H -L-glutamate exist in the ventral horn (Greenamyre et al., 1984). It has been reported that laminae I and II accumulate high levels of ^3H -D-aspartate (Rustioni and Cuenod, 1982; Hunt, 1983), a compound that has been postulated to be taken up by neurons using glutamate or aspartate as a neurotransmitter (Cuenod et al., 1981). Nerve terminals and some small neurons located in laminae I and II demonstrate a glutamate-like immunoreactivity (Van Eyck et al., 1986).

However, the superficial dorsal horn is not the only region of the CNS that receives input from primary afferent fibers and is associated with glutamatergic action. Jahr and Yoshioka (1986) recently showed that Kyn blocks the monosynaptic activation of motor neurons by Ia afferent fibers *in vitro*, supporting a long-held contention that large-diameter sensory axons are glutamatergic. Excitatory amino acids have also been implicated in transmitting impulses from low-threshold, cutaneous mechanoreceptors in the rat trigeminal nucleus (Hill and Salt, 1982).

The parallel action of Kyn on L-glutamate and dorsal root evoked excitation supports the hypothesis that an L-glutamate-like compound is responsible for the central synaptic excitation by a substantial number of primary afferent neurons with thin as well as thick afferent fibers (see also Perkins and Dray, 1986). In fact, in the dorsal horn, the greatest proportion of primary fibers exhibiting glutamatergic transmission appears in the smaller diameter population. On the other hand, our present as well as our past observations suggest that more than one-half of the fast EPSP generation by primary afferents in the dorsal horn involves other mediators and receptors.

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