Glutamate receptor-mediated synaptic excitation in axons of the lamprey

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1. Spontaneous and evoked synaptic inputs were recorded *in vitro* in the axons of lamprey reticulospinal neurones. After isolation of the axon from its somata, synaptic inputs were recorded using microelectrode and whole-cell patch clamp recording techniques.

2. Single stimuli applied to the spinal cord elicited Ca^{2+}-dependent synaptic potentials with short latencies in reticulospinal axons. These synaptic inputs are capable of summation and generate sufficient depolarization to raise the membrane potential beyond threshold to initiate action potentials. Action potential initiation in the absence of the cell body indicates that these axons show synaptic integration.

3. Both evoked and spontaneous responses comprise at least two components of synaptic drive: a slow component (rise time of 9·6 ± 2·1 ms) with a reversal potential of −53 ± 19 mV and a fast component (rise time as fast as 0·85 ms) with a reversal potential of 0·3 ± 9·1 mV. The responses are Ca^{2+} dependent, and are blocked by the substitution of Ba^{2+} for Ca^{2+} in the saline solution.

4. The slow component of synaptic input was blocked by the γ-aminobutyric $acid_A$ ($GABA_A$) receptor antagonist bicuculline (5 μM). The fast component was blocked by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6-cyano-7-nitroqinoxaline-2,3-dione (CNQX; 10 μM) in Ringer solution containing physiological concentrations of Mg^{2+}. Following removal of Mg^{2+} from the superfusate a further excitatory component was identified that was blocked by application of the *N*-methyl-D-aspartate (NMDA) receptor antagonist 2-amino-5-phosphonopentanoate (AP5; 100 μM).

5. Comparison of the kinetic properties and the voltage sensitivity of the isolated components of evoked and spontaneous synaptic activity indicate that these responses are mediated by similar synaptic inputs.

6. These results suggest that axons and presynaptic terminals receive excitatory and inhibitory ionotropic receptor-mediated inputs. Summation of these inputs is possible indicating that the axons act as sites of synaptic integration similar to the role previously attributed only to neuronal dendrites and somata.

The classical view of neuronal signal processing has been that inputs to dendrites of neurones are passively transmitted to the soma where action potential initiation occurs. Active transmission to the terminal regions of the axons then completes a unidirectional flow of information (Ramon Y Cajal, 1909). This view of information flow has become less defined as studies have shown that dendrites are capable of spike generation (Llinás & Sugimori, 1979; Sugimori & Llinás, 1990), and more recently, that there is active propagation of spikes from soma to dendrites (Spruston, Schiller, Stuart & Sakmann, 1995). Even so, the view of axons as unidirectional transmission lines has remained unchallenged. Thus axon terminals function as output devices, though subject to modulation by axo-axonic synapses (Eccles, Schmidt & Willis, 1963; Alford, Christenson & Grillner, 1991) or autoreceptors (Starke, 1972; Baskys & Malenka, 1991) which have been considered inhibitory in nature. Evidence that presynaptic terminals or axons receive direct excitatory input remains circumstantial. Synaptosomal preparations isolated from mammalian brain contain ionotropic glutamate receptors (Kovalev & Hetey, 1987; Shimizu, Duan, Hori & Oomura, 1990; Barnes, Dev & Henley, 1994), while axons of dorsal roots are depolarized by the application of kainate. The latter effect, however, appears to be accompanied by an inhibition of action potentials in the axons (Agrawal & Evans, 1986). In

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paralysed cat, the activation of fictive locomotion has been shown to lead to antidromic spiking of spinal primary afferent fibres in the absence of sensory inflow (Dubuc, Rossignol & Lamarre, 1986; Dubuc, Cabelguen & Rossignol, 1988; Gossard, Cabelguen & Rossignol, 1989). Stimulation of locus coeruleus and raphe nuclei causes primary afferent depolarization, although the transmitter system involved is not known (Riddel, Jankowska & Eide, 1993). Stimulation-evoked depolarizations have been recorded in lamprey giant axons (Matthews & Wickelgren, 1979). This depolarization was slow and the properties of the recording suggested that the effect was mediated by current flow through synaptic gap junctions (Matthews & Wickelgren, 1978).

In the lamprey spinal cord rhythmic depolarizations of small axons of the motor system have been recorded during locomotor activity (Alford *et al.* 1991). These depolarizations are thought to result from the activation of both $GABA_A$ and $GABA_B$ receptors (Christenson, Bongianni, Grillner & Hökfelt, 1991; Alford & Grillner, 1991). Thus axo-axonic synapses are thought to be present in this preparation. Although no $GABA_B$ receptor-mediated depression has been recorded in the reticulospinal axons in the lamprey (Alford & Grillner, 1991), the presence of $GABA_A$ receptors or excitatory receptors on these axons has not been previously investigated; neither has the synaptic activation of such responses been investigated in any detail. Indeed, in vertebrate CNS systems no unambiguously identifiable synaptic response onto presynaptic terminals has been recorded, whether spontaneous or evoked, excitatory or inhibitory.

In this study we demonstrate both ionotropic glutamate and GABA receptor-mediated inputs to axons and presynaptic terminals of the lamprey spinal cord. This work has been published in abstract form (Holt & Alford, 1995).

METHODS

Lamprey ammoceotes (*Petromyzon marinus*) were anaesthetized with tricaine methyl sulphonic acid (MS222; 100 mg l^{-1}) and sections of the spinal cord, with or without the brainstem attached, were removed. The animal was then killed under anaesthesia. After removal, the tissue was immersed in Ringer solution (composition (mM): NaCl, 86; KCl, 2·1; $CaCl_2$, 2·6; $MgCl_2$, 1·8; glucose, 4; $NaHCO_3$, 26 (adapted from Wickelgren, 1977); bubbled with 95% O_2–5% CO_2 at a pH of 7·4. For those experiments in which Ba^{2+} was substituted for Ca^{2+} a Hepes-buffered solution was used as follows (mM): NaCl, 112; KCl, 2·1; $CaCl_2$, 2·6 (or $BaCl_2$); $MgCl_2$, 1·8; glucose, 4; Hepes, 2; bubbled with 100% O_2. During experiments, the tissue was positioned with staples in a Sylgard-lined recording chamber and maintained at 10 °C. Large reticulospinal axons run in the ventromedial tracts of the spinal cord while their cell bodies are located on the dorsal surface of the brainstem. For certain experiments it was necessary to stimulate the region surrounding the cell body while recording from an axon; therefore the preparation was twisted as illustrated in Fig. 2*A*. In experiments performed on the isolated spinal cord, it was removed in lengths of approximately twenty segments and pinned to the Sylgard-lined chamber. In all cases the protective meninx primitiva was removed from the tissue to facilitate electrode access. Whole-cell patch clamp recordings were achieved using the blind technique (Blanton, Lo Turco & Kriegstein, 1989) using pipettes pulled to a tip resistance of 5–10 MΩ and filled with patch solution containing (mM): potassium methane sulphonate, 102·5; NaCl, 1; $MgCl_2$, 1; EGTA, 5; Hepes, 5; and 0·05% w/v lysine fixable dextranamine conjugated fluorescein (Molecular Probes Inc.; pH 7·2). In a number of experiments caesium was substituted for potassium as the principal cation. Series resistances were in the range of 10–20 MΩ. For sharp electrode intracellular recordings, microelectrodes were pulled to a tip resistance of 20–30 MΩ when filled with 3 M potassium methyl sulphate.

For paired cell recordings between a presynaptic axon and postsynaptic soma, recordings were made from the postsynaptic cell with a patch pipette containing caesium as the principal cation and recordings from the presynaptic axon were with a microelectrode. The presynaptic recording was made from within 200 μm of the postsynaptic electrode to ensure adequate electrical access between the recording site and the synapse. The axons were stimulated by application of depolarizing current pulses sufficient to evoke single axonal spikes.

The tissue was stimulated with glass-coated tungsten microelectrodes with tip resistances of 1 MΩ (1–15 μA, 0·5 ms). Care was taken with the positioning of the stimulating electrode to avoid direct electrical stimulation of the recorded axon except when this was required (see Fig. 1). Rise times and decay times were measured between 10 and 90% of peak amplitude. Drugs were administered either by bath application (CNQX, AP5, NMDA: Tocris Cookson; bicuculline and tetrodotoxin (TTX): Sigma) or by pressure ejection (AMPA, kainate and NMDA: Tocris Cookson). Pressure ejection involved positioning a patch pipette containing a high agonist concentration immediately over the spinal cord and releasing a short pulse by 200 ms of pressure applied to the pipette interior. Drug release into the superfusate was monitored by inclusion of Fast Green (1%, w/v) in the pipette solution. Confirmation that the recorded cells were axons was obtained histologically. Before visualization, the tissue was fixed in paraformaldehyde, dehydrated in alcohol, cleared in methyl salycilate and mounted in a well slide under a coverslip. The tissue was imaged on a confocal microscope (Biorad MRC600) and was scanned at *z*-plane intervals of 1 μm. Cells were reconstructed on a Silicon Graphics workstation using VoxelView software (Vital Images).

RESULTS

Stimulus-evoked action potentials in axons

Recordings were made from axons of the ventromedial tracts of the isolated lamprey spinal cord. Microelectrode recordings of these axons reveals stable resting membrane potentials between −80 and −90 mV with little spontaneous activity discernible above the recording noise. Stimulus electrodes were placed adjacent to the recorded axon either ipsilaterally or contralaterally on the spinal cord. Single stimuli (1–30 μA) elicited transient depolarizing responses. Latencies from stimulation to initiation of the depolarizing responses were short and not highly variable (3·48 ± 0·79 ms: $n = 4$; Fig. 1*A* and *B*). Rise times (6·55 ± 1·26 ms) were similar to those recorded in lamprey spinal somata with variable decay

rates (47·6 ± 37·42 ms). Increasing the stimulus intensity caused a graded increase in response amplitude. In 8 of 10 neurones tested this depolarization led to spike initiation (Fig. 1*A*). This action potential did not result from a direct electrical stimulation of the axon. The evoked action potential showed no latency to onset (Fig. 1*C*) when the stimulating electrode was moved to a location immediately over the axon tracts the same distance from the recording site. The rise time of the subthreshold direct stimulation response (0·29 ± 0·05 ms; Fig. 1*D*) was also much faster than the rise time of the synaptic response. The slower rise time and delay to onset in Fig. 1*A* and *B* are indicative of a synaptic response as opposed to a response to direct stimulation. The responses were markedly attenuated by the application of CNQX (10 μM). In the presence of CNQX it was not then possible to evoke a spike unless the stimulus intensity was increased to the point at which direct stimulation of the axon occurred.

Axons are of reticulospinal neurones

The input impedances, spiking properties, time constants and dye fills of the recorded units were consistent with their being reticulospinal axons. (Input impedance ranged from 50 to 150 MΩ. Single exponentials fitted to the decay from −10 mV steps ranged from 2 to 9 ms. The axons will only spike once to depolarizing stimuli in current clamp regardless of depolarization.) However, should these recordings have been made from propriospinal axons with somata in the length of spinal cord used for recording, it would not be clear whether the depolarization induced in the above experiments was due to direct synaptic stimulation of synapses in which the axon represented the postsynaptic element, or whether stimulation induced a depolarization secondary to the excitation of the soma and dendrites. It was important to demonstrate that these recordings were solely from axons and that no component of the synaptic response could have originated from soma or dendrite. To demonstrate that

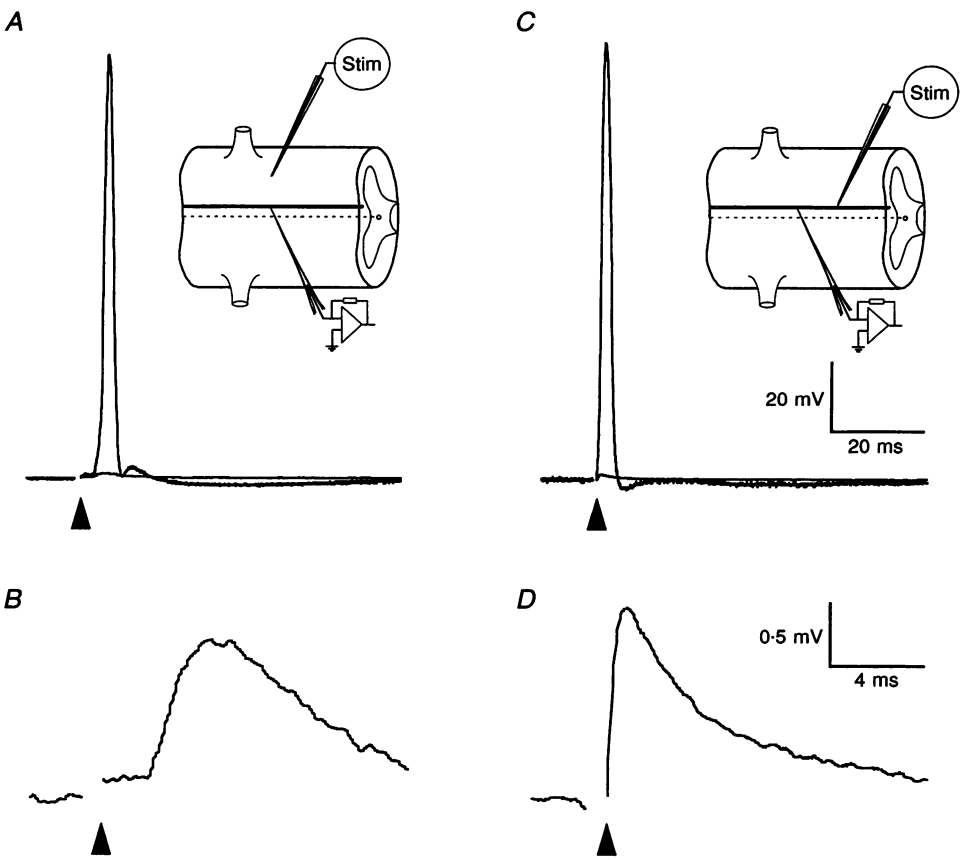

Figure 1. Synaptic inputs to reticulospinal axons may evoke Na^+ spikes

A, microelectrode recordings from a reticulospinal axon in the isolated spinal cord. Stimulation (Stim) of the spinal cord caused a synaptic response in the axon which was capable of generating an action potential. Sub- and superthreshold responses are shown. The subthreshold EPSP is shown enlarged below (*B*) to indicate the delay and rise time. Arrowheads indicate the stimulus artifact (removed for clarity). *C*, microelectrode recordings from the same axon as in *A* demonstrating direct stimulation of the axon. Sub- and superthreshold responses are shown. The subthreshold capacitative charging of the membrane is shown enlarged below (*D*). Note there is no delay and the rise time is fast. This is shown for comparison with the synaptic response (*A* and *B*) to indicate that the spike initiated by the synaptic response was not evoked following contamination with a direct electrotonic stimulation of the recorded axon.

axons could be isolated from the somata the entire CNS was utilized for recording (Fig. 2*A*; $n = 3$). Axons were recorded in whole-cell mode with patch pipettes in the spinal cord and stimuli were applied to either the spinal cord or the brainstem. Under these circumstances stimulation invariably led to the activation of an inward conductance at holding potentials of −70 mV (Fig. 2*B*). The addition of fluorescent dye to the patch pipette enabled the identification of these axons as reticulospinal (Fig. 2*A*). After recording, the tissue was maintained in the recording chamber overnight. The tissue was then fixed, cleared and imaged to reveal the axon filled in the spinal cord and brainstem and the soma located in the brainstem. These somata were identified as either Müller or Mauthner cells in different experiments. Clearly the soma and dendrites could be prevented from contributing to currents recorded in the axon by isolating the spinal cord from the brainstem.

Evoked responses are mediated by synapses on the axons

To understand the mechanism of inward current activation and the location of synaptic inputs to these neurones, a reduced preparation was employed comprising a short section of spinal cord. This preparation ensured that only the axon was present in the recordings. Axons were verified by the inclusion of fluorescent dye in the recording pipette. Thirty-four verified axons were recorded (diameters, 3·5–20 μm). In no case was a recording made from a neurone whose soma was located in the piece of spinal cord used; only large axons that extended to both ends of the tissue were filled with dye. At a holding potential of −70 mV,

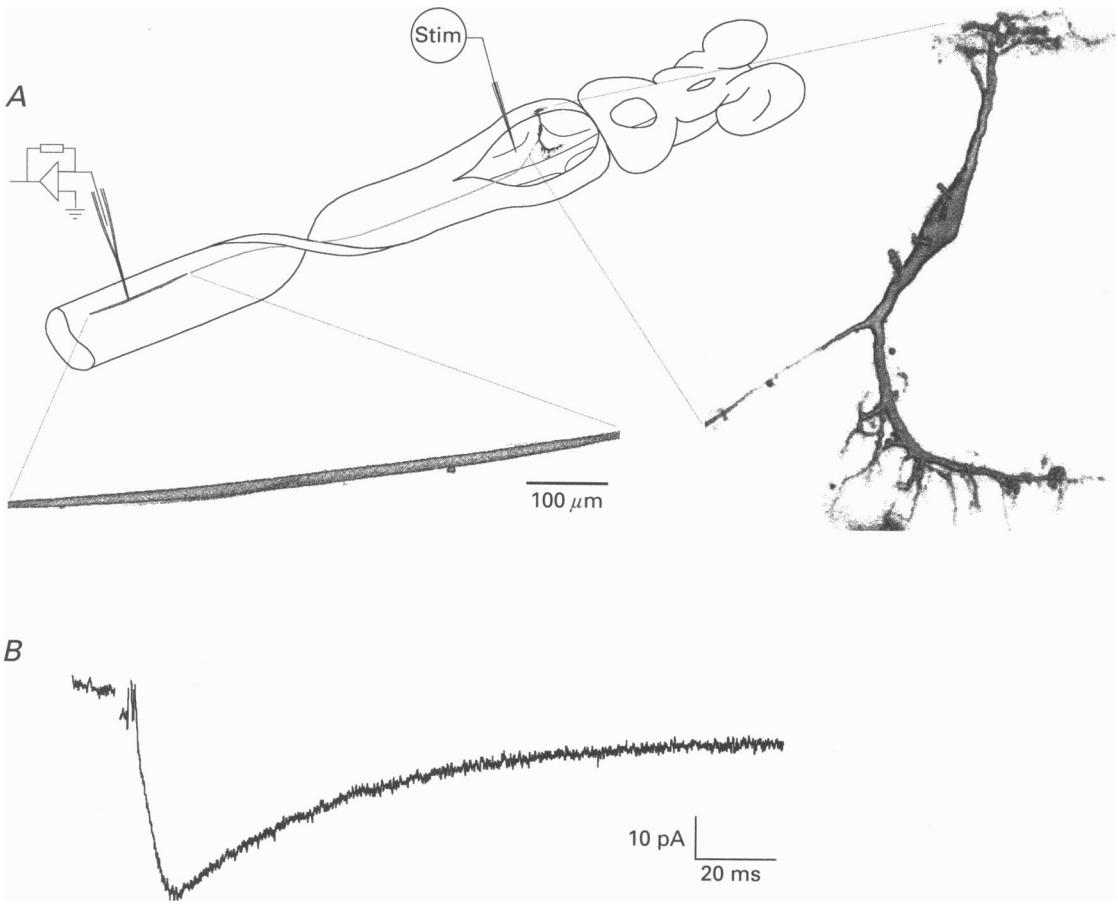

Figure 2. Identified reticulospinal axons receive excitatory inputs

A, a schematic illustration of the lamprey brain and spinal cord as situated during experiments. The recording patch electrode is shown attached to the axon in the spinal cord. The stimulating electrode was positioned in the brainstem ipsilateral to the cell body, but lateral to the course of the axon in the brainstem. The neurone was filled with fluorescent dye through the patch pipette. The tissue was subsequently maintained *in vitro* for approximately 12 h following the experiment which enabled the dye to be transported throughout the recorded neurone. Subsequent imaging of the tissue revealed the labelled axon and cell body, portions of which are illustrated. This axon was thus positively identified as from a Mauthner cell. Other identified axons were Müller neurones also with somata in the brainstem. *B*, voltage clamp recording of the response to stimulation (same cell as in *A*). Stimuli were applied to the brainstem to evoke a response in the axon recorded in the spinal cord. Stimuli applied to brainstem or to spinal cord invariably evoked an inward current at a holding potential of −70 mV (10 to 90% rise time ranged from 6·7 to 17·9 ms; mean, 10·58 ± 3·66 ms; $n = 3$).

stimulation evoked inward currents (rise time range, 0·85–27·8 ms; mean, 9·01 ± 0·96 ms; $n = 51$ axons of which 34 were identified histologically; no soma was ever loaded with dye in these experiments). The latency of onset of the recorded current (range, 2·1–7·4 ms; mean, 4·29 ± 0·39 ms) was much less variable than the rise time of the response.

The evoked responses may have been initiated by synaptic inputs to the axons or by a depolarization secondary to that of the surrounding soma and dendrites. Two possible sources of the latter depolarization are as follows: (1) depolarization of many surrounding dendrites and current passage through gap junctions between these axons and the dendrites; or (2) potassium liberated into the extracellular space and subsequent axonal depolarization. The following experiments were performed to confirm the evoked input was synaptic in origin.

Stimulation of the spinal cord adjacent to axons recorded in whole-cell configuration led to the activation of a mixed synaptic current. Current–voltage plots of this synaptic current indicate that both the early and late phases of the evoked response showed a reversal potential. This reversal was at positive holding potentials for the earliest phase of this response (25·6 mV) and at negative potentials for the later phase (−22·3 mV; Fig. 3*A* and *B*). In many recordings it was difficult to control membrane potential at depolarized levels because of the electrotonic properties of the axons under investigation. Consequently, in a further seventeen experiments the reversal potentials of the earliest component

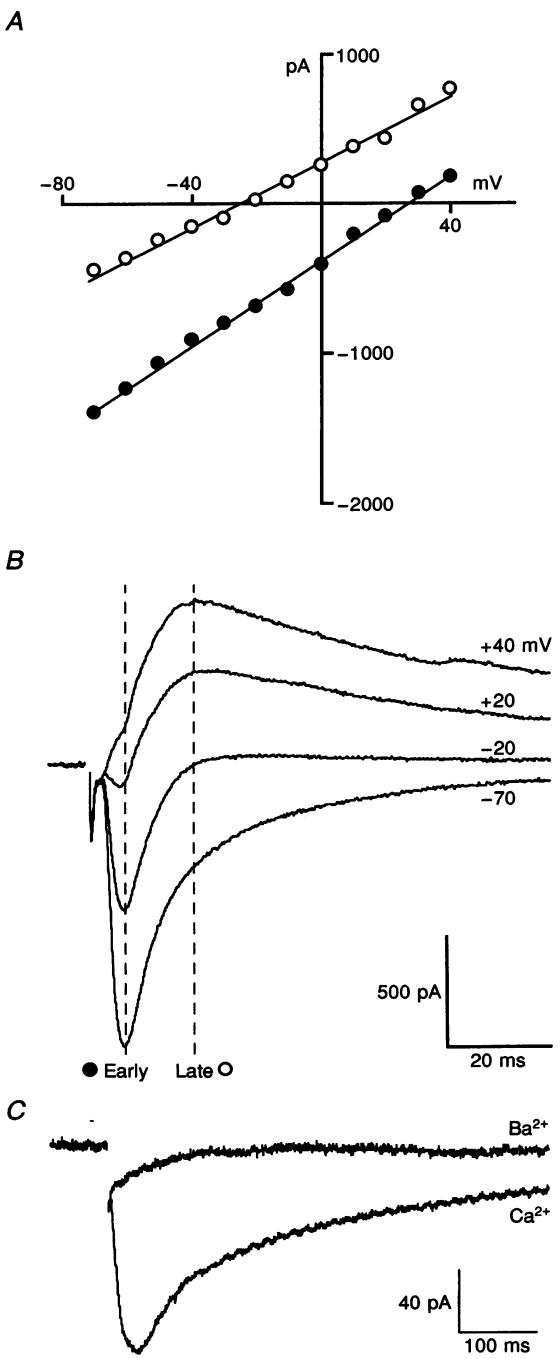

Figure 3. Properties of synaptically evoked currents in reticulospinal axons

A, the current–voltage relationship for the evoked response recorded from a reticulospinal axon in the isolated spinal cord is illustrated. The peak of the early component (●, measured 7·5 ms after the stimulus artifact onset as depicted by the first dashed line in *B*) reverses at +25·6 mV and the later component (○, measured 12 ms after the stimulus artifact onset as depicted by the second dashed line in *B*) reverses at −22·3 mV. (This later component is mediated by GABAergic inputs to the axons.) The reversal of the early evoked response was obtained in 7 of 18 axons tested. *B*, synaptic responses shown graphically in *A*. The response of the axon to stimulation at holding potentials of +40, +20, −20 and −70 mV are shown. The vertical dashed lines indicate the time points in *A* (early at 7·5 ms poststimulus, late at 12 ms poststimulus). *C*, synaptic responses are calcium dependent. When calcium in the Ringer solution is replaced with barium at the same concentration, the response of the axon to stimulation is eliminated. Traces recorded in the presence of barium or in the presence of calcium are shown.

were extrapolated. The peak current was measured at three holding potentials within 40 mV of the resting membrane potential then extrapolated to an estimated reversal potential for the earlier component (+25·1 ± 11·9 mV). This positive reversal potential may be due to either lack of an adequate space clamp of the axons under investigation or contamination of the response by a small non-reversing electrical component (see Fig. 9).

To test if the evoked responses in the axons required Ca^{2+}-dependent release of transmitter, Ca^{2+} was replaced with Ba^{2+} in the extracellular medium. Responses were evoked and recorded as described above. Following wash-in of Ba^{2+}-containing saline the responses were entirely abolished. As Ba^{2+} will not substitute for Ca^{2+} in evoking transmitter release, it is most likely that the evoked response in the axons required Ca^{2+}-dependent release of transmitter.

Pharmacological isolation of components of the response

The later component, which was most visible following depolarization of the membrane potential, was eliminated by the application of bicuculline (10 μM; $n = 6$; Fig. 4*A* and *B*). At −70 mV the evoked response was increased in amplitude and showed a faster decay (not significantly). At −20 mV the late outward component of the synaptic current was blocked by bicuculline application. Wash-out of bicuculline allowed recovery of this outward component. The subsequent addition of CNQX (10 μM) to the superfusate eliminated the early component to reveal an isolated slow outward component at −20 mV and an inward synaptic component at −80 mV. This response had a mean reversal potential of −53 ± 19 mV ($n = 3$; rise time, 9·6 ± 2·1 ms; decay time, 33·1 ± 8·9 ms; $n = 17$; see Fig. 4*C* and *D*).

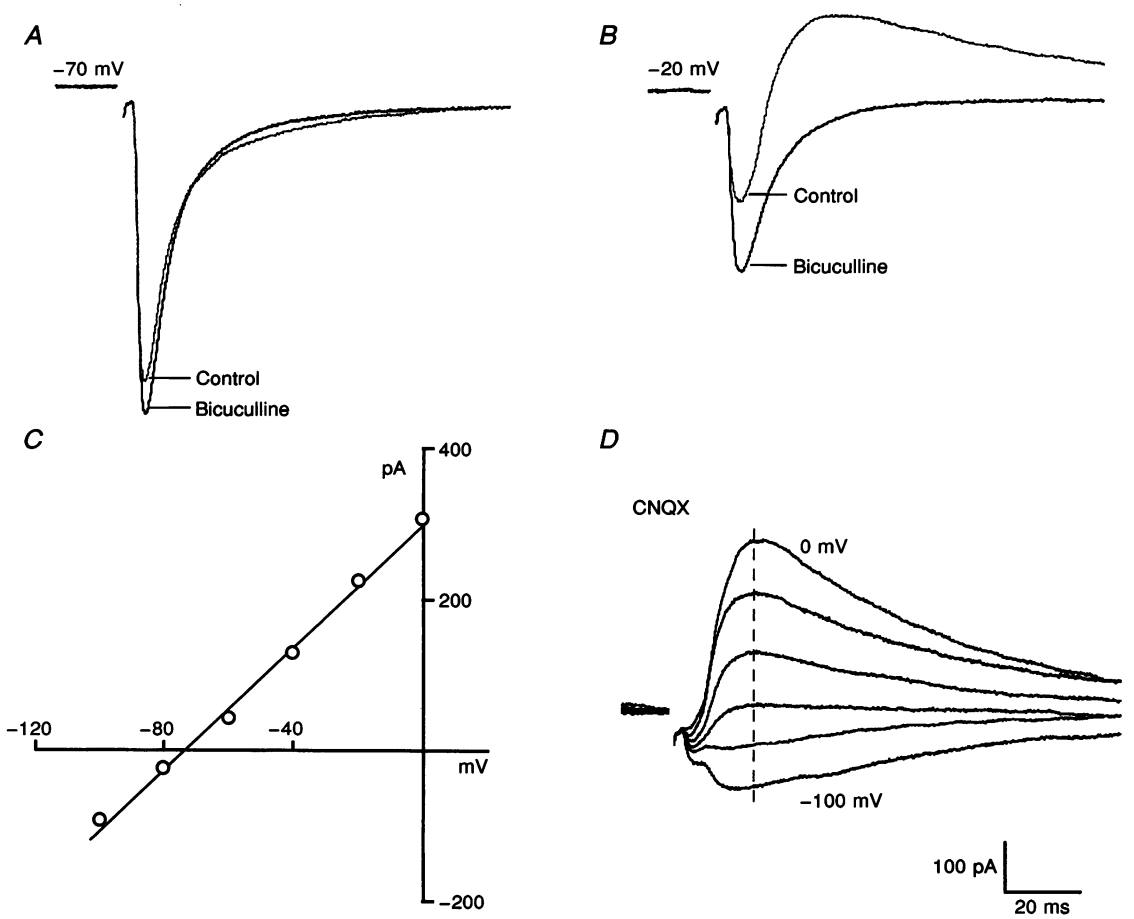

Figure 4. The GABAergic component of the synaptically evoked response in axons

A, evoked responses from another cell recorded at −70 mV before (control) and in the presence of bicuculline (10 μM). The response in bicuculline decays more rapidly (decay time constant (t) = 8·1 ms for control response; $t = 7·7$ ms in bicuculline). *B*, at −20 mV the early inward component is followed by an outward conductance (same axon as in *A*). Following the application of bicuculline (10 μM) the late outward response is abolished and the peak inward current is larger. *C*, the current–voltage plot of the peak of the evoked response for the same neurone (as in *A* and *B*) after wash-out of bicuculline and addition of CNQX (10 μM). The CNQX-insensitive portion of the response demonstrated a reversal potential of −75 mV in this neurone. *D*, individual responses used to create the graph in *C*. The measurements were made at the time point marked by the dashed line. The responses were evoked at potentials between −100 and 0 mV at 20 mV intervals.

In all cells tested ($n = 27$) application of CNQX (10 μM) reduced the response amplitude at −70 mV. The peak inward current was reduced to 24·6 ± 5·4% of control ($n = 27$; see Fig. 5*A*) but appeared unaffected by AP5 in Mg^{2+}-containing artificial cerebrospinal fluid (ACSF). However, Mg^{2+} attenuates the amplitude of NMDA receptor-mediated responses at resting membrane potentials (Mayer, Westbrook & Guthrie, 1984; Nowak, Bregestovski, Ascher, Herbet & Prochiautz, 1984). Thus, after obtaining a synaptic response in Mg^{2+}-containing saline, wash-out of Mg^{2+} augmented the response (in 6 of 7 cells; Fig. 5*B*). The inward current was partialy blocked by application of CNQX (10 μM) and more completely blocked by the addition of AP5 (100 μM). In two of five cells a small inward conductance remained in CNQX and AP5 at a holding potential of −70 mV (Fig. 5*B*). In the remaining three cells an outward conductance remained. This conductance was most probably mediated by $GABA_A$ receptor activation in both sets of cells.

In the presence of bicuculline and in Mg^{2+}-containing saline, the early component of the synaptic response was studied in isolation of other synaptic components. Current–voltage plots of the response were computed. The response demonstrated approximately linear voltage dependency and a reversal potential of 0·3 ± 9·1 mV ($n = 3$; rise time as fast as 0·85 ms; Fig. 5*C* and *D*). This was not, however, significantly different than that for the early component of the mixed response and was similar to the reversal potentials of the extrapolated evoked responses.

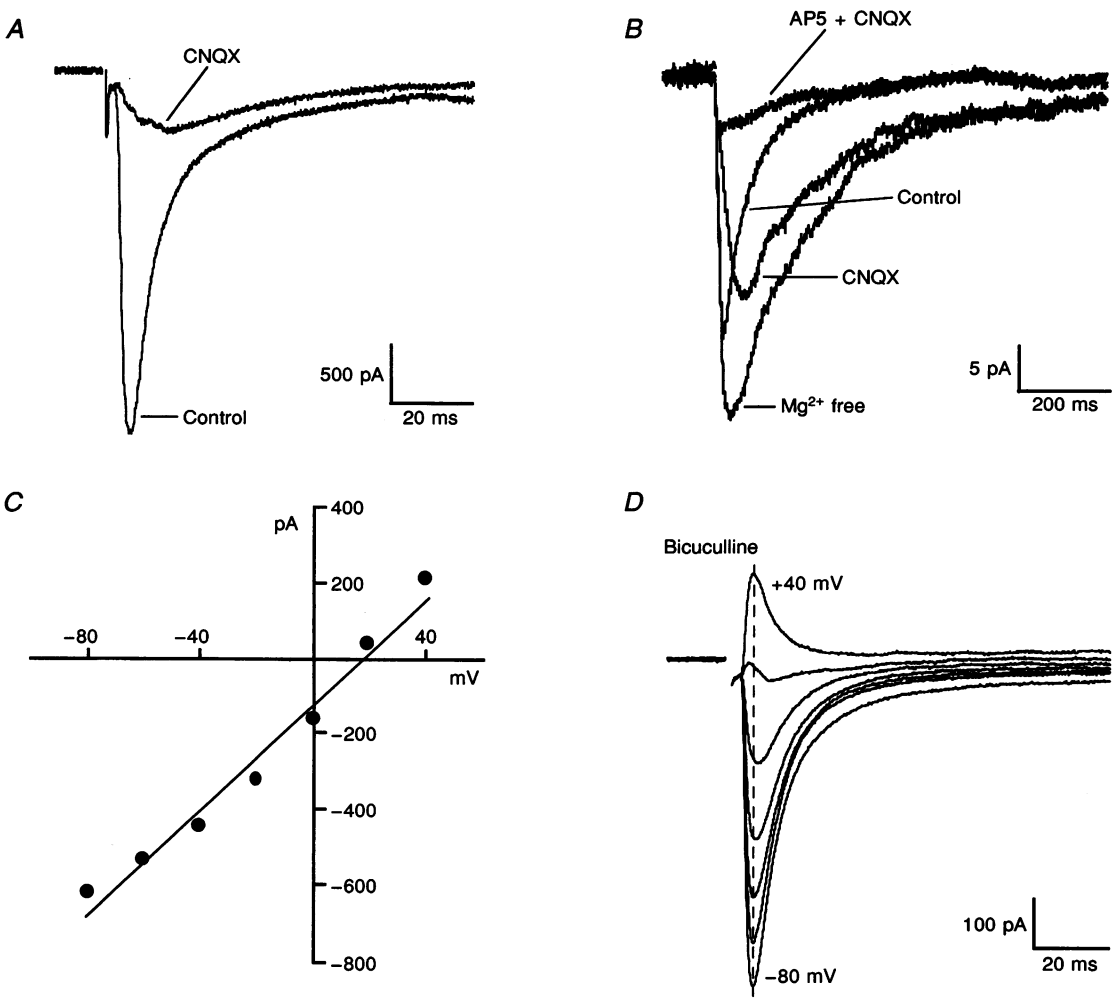

Figure 5. The glutamatergic component of the synaptically evoked response in axons

A, an evoked response recorded before and after the application of CNQX (10 μM; holding potential, −70 mV). *B*, the control stimulus was in normal ACSF containing 1·8 mM $MgCl_2$. Following wash-out of Mg^{2+} the response was increased in amplitude and duration. The addition of CNQX (10 μM) reduced the amplitude of the response revealing a component with a slower time to peak. Finally the addition of AP5 (100 μM) reduced the response still further (holding potential, −70 mV). *C*, a current–voltage plot of the peak of an evoked response recorded in the presence of bicuculline (10 μM) and normal Mg^{2+} (same axon as in Fig. 4). The response shows an approximately linear current–voltage relationship with a reversal potential of 20 mV. *D*, the individual responses from which the graph in *C* was calculated. The peak responses were calculated at the time point marked by the dashed line. The responses were evoked at potentials between −80 and +40 mV at 20 mV intervals.

The axons possess both AMPA and NMDA receptors

The concept that axons receive inputs mediated by excitatory amino acid receptors is novel. It was considered important to demonstrate that these receptors were present on the axonal membrane. Under whole-cell patch clamp conditions, in addition to the evoked responses recorded, the axons clearly receive spontaneous activity in the absence of any excitant applied to the spinal cord, electrical or chemical (Fig. 6*A*). The addition of NMDA (100 μM) to the superfusate can initiate fictive locomotion in the lamprey spinal cord. Such an application led to an increase in spontaneous synaptic activity (Fig. 6*A*; $n = 8$). To demonstrate that receptors were present on the axons, agonists were applied in small volumes by pressure ejection immediately above the recording site in the bath. Pressure application of AMPA (250 μM in the pipette) invariably led to an inward conductance at −70 mV with an increase in the frequency of superimposed spontaneous activity. In the presence of TTX, an ejection of 250 μM AMPA caused a slow monotonic inward current ($n = 4$; Fig. 6*B*). Pressure ejection of 500 μM NMDA had no discernible effect in Mg^{2+}-containing saline ($n = 4$). In a further four cells the response to pressure ejection of NMDA was tested in low-Mg^{2+} saline. Pressure application of NMDA then led to an inward conductance at −70 mV. This was characterized by a monotonic slow inward current with a subsequent increase in spontaneous synaptic activity. Application of NMDA in low-Mg^{2+} saline in the presence of TTX led to only a slow monotonic inward component (Fig. 6*C*). A small increase in noise was seen but is not readily visible in the figure. The small change in NMDA-induced noise despite the large conductance of these channels is probably due to space clamp errors inherent in recording from these large structures. In contrast, a similar application of kainate (250 μM; $n = 2$) led to no current activation in these axons, a result somewhat at odds with known properties of postsynaptic AMPA receptors in the mammalian system which are sensitive to kainate. It is, however, difficult to extrapolate results obtained from diffusion of low concentrations of agonist into the spinal cord with data obtained with rapid perfusion of cells or receptors. The rate of desensitization of the receptors to a low concentration of agonist may be higher for kainate than AMPA. Although kainate does not desensitize AMPA receptors as efficiently as AMPA in most studies (cf. Mayer & Vyklicky, 1989), it has been shown to markedly desensitize receptors in dorsal root ganglion cells (Huettner, 1990).

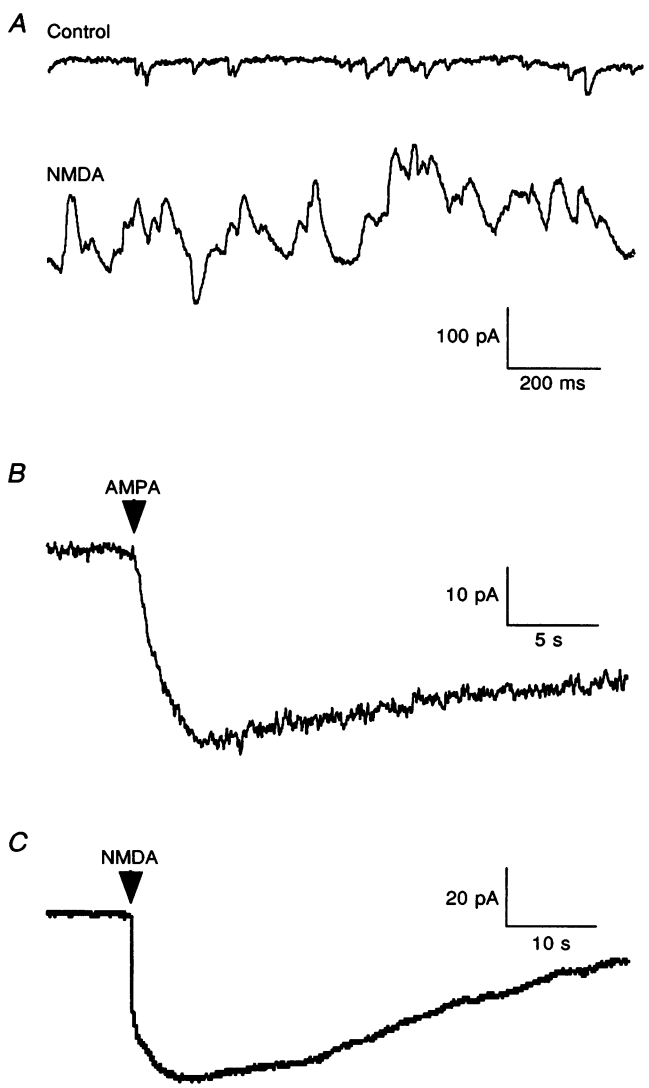

Figure 6. The effect of excitatory amino acid receptor agonist application to the spinal cord
A, spontaneous responses recorded from a reticulospinal axon in the isolated spinal cord. The top trace shows spontaneous activity in normal Ringer solution. The bottom trace shows the activity in response to bath application of 100 μM NMDA. This application of NMDA will initiate fictive locomotion in the lamprey spinal cord (Cohen & Wallén, 1980). *B*, recordings from another reticulospinal axon in the isolated spinal cord demonstrating an inward current response to a 1 s application of 250 μM AMPA in the presence of 1 μM TTX. *C*, pressure application of NMDA over the recording site led to an inward current in the axon in low-Mg^{2+}-containing ACSF. In normal Mg^{2+} solution no current was recorded in response to an NMDA puff ($n = 4$; not illustrated; recordings in 1 μM TTX).

Two components of spontaneous activity are visible

In normal saline, in four axons, the membrane potential could be sufficiently depolarized to investigate the voltage dependence of the spontaneous events (see Fig. 7). The recorded spontaneous activity comprised two distinguishable components of activity. At resting membrane potential (−70 to −80 mV) activity was characterized by inward spontaneous events with an extended time course (Fig. 7*C*). Sampling and averaging of these events showed that rise times ranged from 5·0 to 18·8 ms (mean, 12·4 ± 3·1 ms; although note that the precise start of the postsynaptic current (PSC) could not be determined because no stimulus timing was available for the spontaneous activity, which will cause problems in alignment of responses for averaging) and

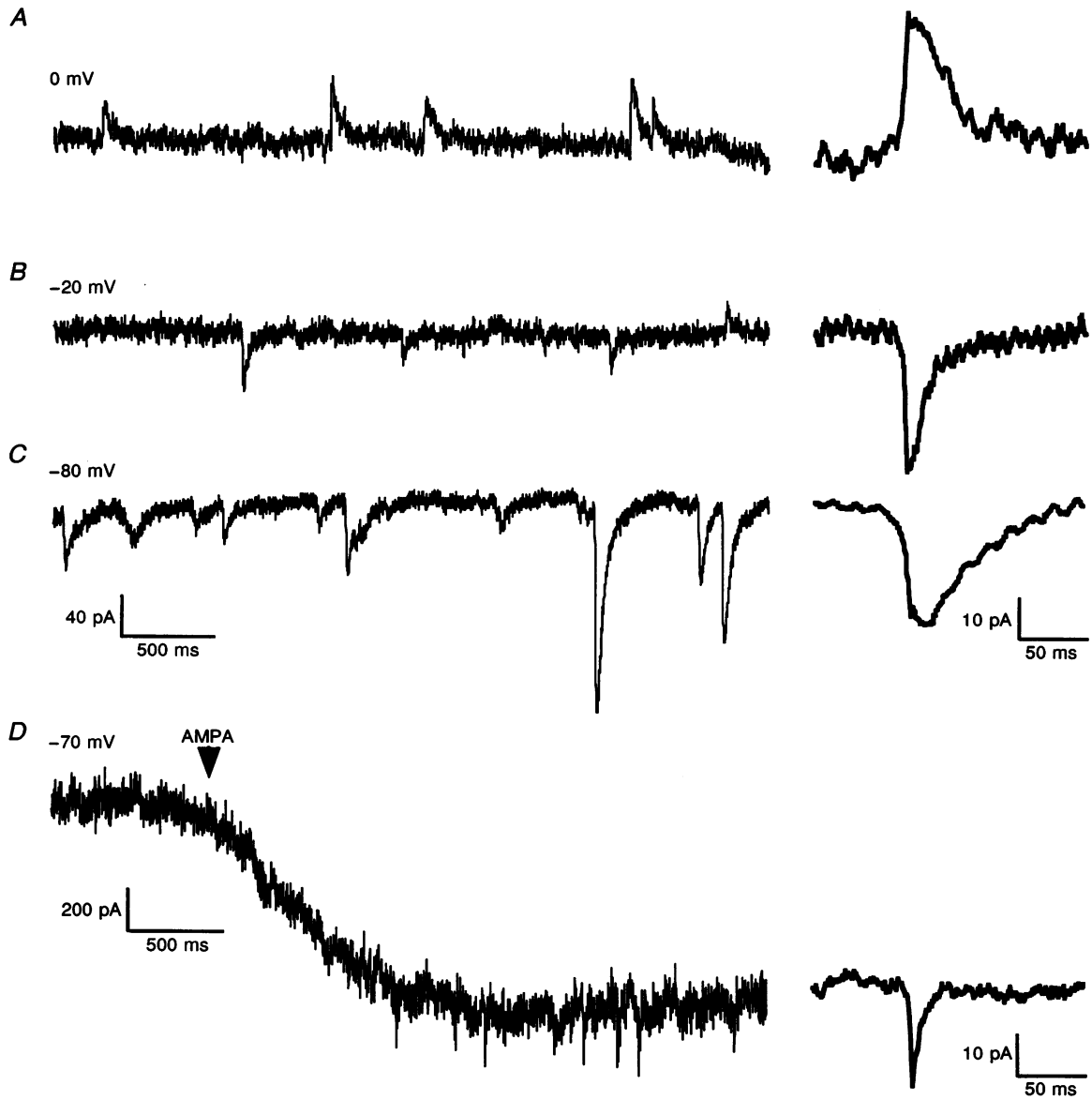

Figure 7. Spontaneous currents recorded in axons

A, at a holding potential of 0 mV the axon received numerous spontaneous outward currents. These currents were selected and averaged from traces recorded at 0 and −20 mV to show their duration (right-hand trace). *B*, at −20 mV spontaneous inward currents and outward currents were visible. The inward currents were sampled and averaged from traces recorded at −20 and −40 mV to show their duration (right-hand trace). This time course was significantly faster than the outward currents in *A*. *C*, at a holding potential of −80 mV the axons received numerous spontaneous inward currents. Selection and averaging of these currents indicated that their time course was not significantly different from the outward currents recorded at 0 mV. *D*, in the presence of bicuculline (10 μM) all spontaneous activity was lost. Pressure ejection of AMPA over the spinal cord led to a slow inward current with superimposed fast inward responses. Sampling and averaging of these fast responses (right-hand trace) indicates that they have a time course similar to the events recorded at −20 mV in *B* above.

decay times that ranged from 15·1 to 70·3 ms (mean, 54·4 ± 13·2 ms). Depolarization of the axon under voltage clamp to 0 mV revealed outward events with kinetic properties not significantly different from those seen at hyperpolarized potentials (Fig. 7*A*). At −20 mV both outward and inward spontaneous activity was observed (Fig. 7*B*). Sampling and averaging of the inward events at −20 mV revealed fast spontaneous currents. Rise times ranged from 6·3 to 8·3 ms (mean, 7·4 ± 0·6 ms) and decay times ranged from 19·9 to 32 ms (mean, 25·0 ± 3·9 ms).

As the reversal potential of one of these components was at negative membrane potentials and because a component of the evoked response was $GABA_A$ receptor mediated, experiments were performed to test if the spontaneous activity showed a GABA component. Bicuculline (10 μM) was applied to the spinal cord and essentially all spontaneous activity was eliminated. However, the component of the spontaneous activity with a reversal potential at positive membrane potentials was unlikely to be GABA mediated. It is likely that this effect of bicuculline was acting both directly on the axons and indirectly on the spinal network to reduce spinal excitation. The spinal cord was therefore transiently excited by a short application of AMPA. Pressure application of AMPA over the recording site led to a slow monotonic inward component. Superimposed on this response were a number of synaptic responses similar to those shown at more depolarized potentials in the absence of bicuculline (Fig. 7*D*). These responses were sensitive to TTX and had

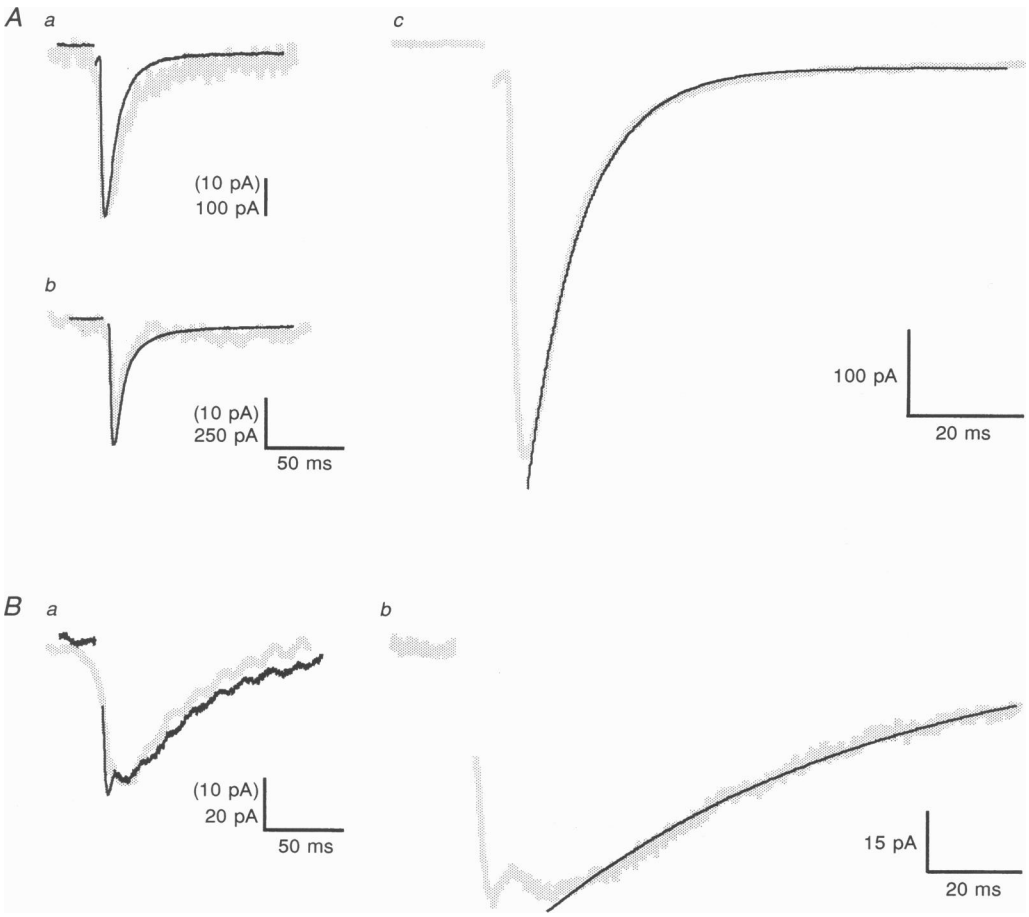

Figure 8. Comparison of evoked and spontaneous currents

A a, overlays of averaged spontaneous activity from Fig. 7 (grey) and evoked activity in the presence of bicuculline (black) from the same neurone. The recordings were made at membrane potentials of −20 to −40 mV. The spontaneous and evoked responses show a very similar time course. *A b*, similar responses were recorded at −70 to −80 mV. The spontaneous activity (grey) was recorded in the presence of bicuculline and after the pressure application of AMPA (see Fig. 7*D*). The evoked component (black), recorded in the presence of bicuculline, again showed a similar time course. *A c*, the evoked component in the presence of bicuculline at −40 mV (grey) with a single exponential fit of the decay phase overlaid (black). The time course of the decay showed a rate constant (τ) of 9·6 ms. *Ba*, overlay of the averaged spontaneous activity at −80 mV (grey) and the evoked activity in CNQX recorded at −80 mV. Again the time constants are similar. *B b*, the evoked component in CNQX (grey) with an overlaid single exponential fit of the decay phase. The time course of the decay showed a rate constant (τ) of 75·2 ms. The scale bars for the overlaid evoked and spontaneous activity show magnitude of evoked responses and spontaneous (in parentheses).

kinetic properties similar to the spontaneous activity with a positive reversal potential (rise time, 5·93 ± 0·87 ms; decay time, 8·96 ± 1·26 ms; $n = 3$).

Spontaneous and evoked responses show similar properties

To determine if the spontaneous activity and the evoked responses were from similar sources the kinetic properties of the spontaneous responses, obtained at different holding potentials, were compared with the kinetic properties of the pharmacologically isolated components of the evoked responses obtained from the same axons. Single exponential curves were fitted to the decay phase of the events in order to make these comparisons. The spontaneous inward conductances recorded at −80 mV (from Fig. 7*C*) were compared with the evoked bicuculline-sensitive responses obtained in the presence of CNQX (from Fig. 4*D*; the same axon was used for both evoked and spontaneous recording). The time constant for the decay of the evoked response in CNQX (59·93 ± 4·87 ms; $n = 15$) was not significantly different from the time constant of the spontaneous events recorded at −80 mV (41·30 ± 2·69 ms; $n = 12$). Figure 8*Ba* shows these responses overlaid. At −40 to −20 mV the spontaneous activity was mostly limited to fast inward responses. The averages of these spontaneous inward responses (from Fig. 7*B*) were compared with the isolated CNQX-sensitive evoked activity at a holding potential of −40 mV taken from Fig. 5*D*. Again these were similar (Fig. 8*A a*). Spontaneous activity recorded in the presence of bicuculline but after excitation with AMPA (from Fig. 7*D*) was compared with the CNQX-sensitive component of the evoked response obtained at −80 mV (from Fig. 5*D*) in Fig. 8*A b*. The decay time constant for these evoked responses was 9·13 ± 0·10 ms ($n = 15$) which is not significantly different from the decay time constant for spontaneous events recorded in the presence of bicuculline (8·14 ± 0·75 ms; $n = 15$).

The significant difference between the decays of the slow and the fast events, whether spontaneous or evoked, and the near order of magnitude difference in decay provide supporting evidence that two different classes of synaptic input impinge on these axons.

Calculation of gap junction resistance

To demonstrate that the electrical component of axo-dendritic synapses does not underlie the responses recorded in these axons by transmission of electrical potentials from surrounding dendrites it was important to demonstrate the net conductance of these electrical synapses. Patch clamp recordings of spinal interneurones were made and micro-

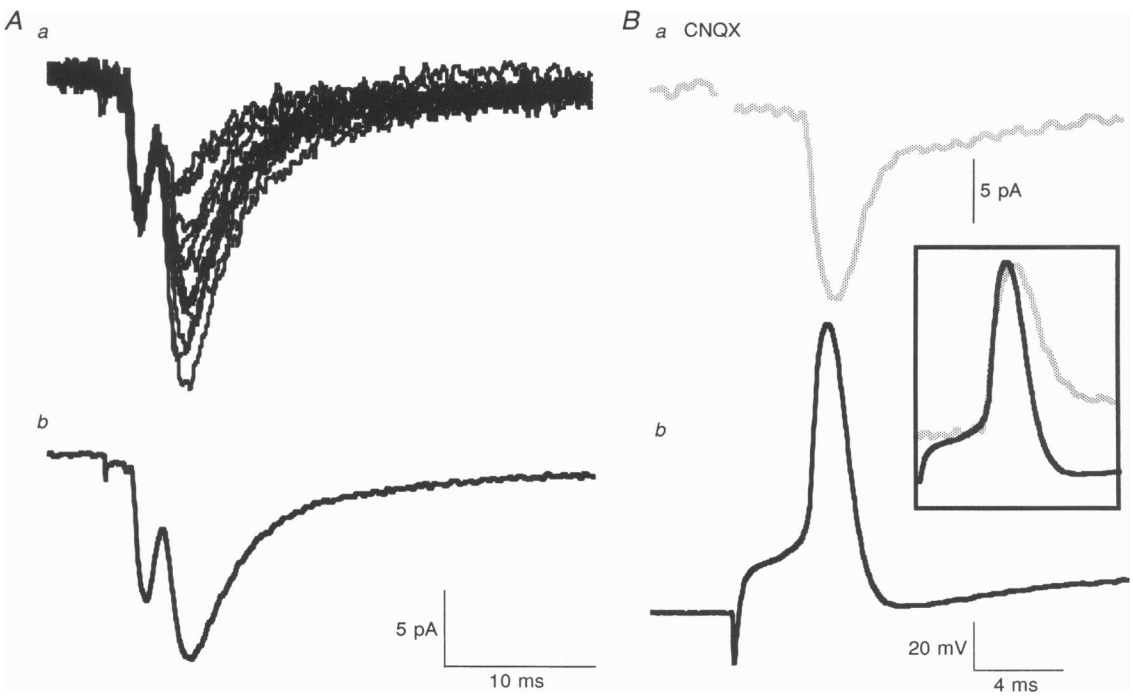

Figure 9. Measurement of gap junction resistance

A a, responses recorded in a spinal motoneurone following stimulation of a presynaptic reticulospinal axon recorded as a pair. The responses are to 19 sequential stimuli of the axon applied as depolarizing current steps through the presynaptic recording microelectrode sufficient to evoke a single presynaptic action potential. *A b*, average of the 19 responses shown in *A a*. *Ba*, the response to axonal stimulation after the addition of CNQX to the superfusate. The remaining component is the electrical component of the synaptic response. *Bb*, the presynaptic response to depolarizing current application. The inset shows the presynaptic action potential shown in *Bb* and the electrical component of the synaptic current in *a* scaled, inverted and superimposed with the same timebase. Note that there is essentially no filtering of the electrical component.

electrode recordings made from paired presynaptic terminals of reticulospinal axons within the dendritic tree of the postsynaptic neurones. Subthreshold depolarization of the axon (approximately 10 mV) generated a small measurable current in the postsynaptic cell (0·12 pA mV^{-1} of presynaptic depolarization). Axo-dendritic synaptic currents were then evoked by stronger current injection into the presynaptic axons (Fig. 9). The evoked postsynaptic currents showed two distinct components: an early invariant component (electrical; Rovainen, 1974), that was insensitive to glutamate receptor antagonists and a later invariant component that was largely eliminated by the application of CNQX. To make an accurate estimate of the conductance of the gap junctions underlying the electrical component it was important to ensure that the invariant electrical component was not significantly filtered by the electrical properties (space clamp) of the postsynaptic neurone. This could be confirmed by a direct comparison between the rise time of the electrical component and of the presynaptic action potential. No measurable slowing of the 10–90% rise time of the electrical component was seen with respect to the rise time of the action potential (measured values of $0·64 \pm 0·03$ ms for the presynaptic action potential and $0·65 \pm 0·03$ ms for the postsynaptic electrical synaptic response). The scaled electrical component and presynaptic action potential have been overlaid to demonstrate this (inset to Fig. 9*B*). The mean electrical component amplitude was $13 \pm 4·0$ pA ($n = 3$). This was evoked by a 120 mV presynaptic spike. The time course of the electrical component was not significantly altered by space clamp error in the postsynaptic neurone. Consequently the conductance of the gap junction underlying the electrical component may be estimated (108 pS, actual range was 175–66 pS).

DISCUSSION

The use of whole-cell patch recordings in the giant axons of the lamprey has enabled a resolution of recording sufficient to discern individual synaptic inputs to the axon. Functionally, such recordings are electrically equivalent to recording from the presynaptic terminal. These axons are known to form *en passant* presynaptic elements which release glutamate along the length of the spinal cord and possess no arborization (Rovainen, 1974; Shupliakov, Brodin, Cullheim, Ottersen & Storm-Mathisen, 1992). The ability to record spontaneous activity with patch electrodes, as opposed to microelectrodes, may be due to both the increased fidelity of the recording and the lack of a shunt conductance around the patch electrode seal.

Stimulation of the lamprey spinal cord evokes a depolarization in reticulospinal axons that is capable of initiating an action potential in the absence of the dendrites or soma. A number of sources of such depolarization might be hypothesized. (1) The axons receive synaptic glutamatergic and GABAergic inputs from other neurones in the spinal cord. (2) The axons possess *en passant* presynaptic terminals which excite spinal neurones, both from release of glutamate and by excitation via gap junctions at electrical synapses. It is possible that stimulation of the spinal cord leads to excitation of the dendrites of spinal neurones and depolarization of the axons via the electrical synapses. At the level of single synaptic inputs, for example spontaneous activity, this is unlikely since there is a large impedance mismatch between the dendrite and the axon. The individual dendritic events would be an insufficient current source to affect the membrane potential of the much larger presynaptic axon. The electrical synapses between axons and individual dendrites show resistances of approximately 10 GΩ (100 pS). To generate the mean recorded spontaneous inward current at −20 mV (30 pA of current in the axon) the dendrite would need to develop a potential difference with respect to the axon of approximately 300 mV. However, the sum of depolarization of multiple postsynaptic cells might depolarize the recorded axon. (3) Stimulation of the spinal cord may release significant quantities of potassium into the extracellular space which would cause a non-specific depolarization of all neuronal tissue in the vicinity of the potassium release. Such an effect has not been recorded for somatically recorded events although during fictive locomotion phasic variations of up to 0·4 mM potassium has been recorded in the lamprey grey matter (Wallén, Grafe & Grillner, 1984). This may be sufficient to cause a noticeable depolarization of the axons.

By utilizing the good electrical access and low electrode shunt conductance characteristic of patch clamp recording, we have demonstrated that evoked depolarizing inputs to lamprey axons demonstrate reversal potentials and that the axons receive spontaneous inputs. The evidence presented in this study indicates that these evoked and spontaneous currents are mediated by the same synaptic processes. Within the accuracy of the recording possible under these circumstances, the spontaneous inputs show similar kinetic properties and reversal potentials to the pharmacologically isolated components of the evoked responses. The range of rise and decay rates recorded for the evoked responses presumably reflect the variable proportions of glutamate- and GABA-mediated components, both of which activated inward conductances at the membrane potentials used in most experiments (−70 mV). It seems likely that at −70 mV the activity appeared dominated by the longer GABAergic components simply because these events were much more visible than the faster glutamate-mediated events.

Taken together, the presence of spontaneous excitatory activity and the ability to reverse the evoked activity, indicate that the activity is due to synaptic activation of receptors on the axons themselves. Neither K^+-induced non-specific depolarization nor stimulation through gap junctions from spinal dendrites could show reversal. Individual spontaneous responses in postsynaptic spinal neurones are unlikely to provide enough current flow through electrical

synapses from dendrites to these axons to show significant axonal currents, particularly as these gap junctions are believed to be rectifying (Ringham, 1975).

We may demonstrate this using the calculated value for gap junction resistance (Fig. 9) and using accepted values of intracellular resistivity (r_i) and dimensions of the synaptic contact. A conservative estimate for r_i of 300 Ω cm (Spruston, Jaffe, Williams & Johnston, 1993) was used to make this calculation. Synaptic contacts are approximately 2 μm diameter spine-like structures approximately 4 μm in length (not illustrated). The calculated net resistance of this structure to the main branch of the dendrite is 3·8 MΩ. Thus, to clamp the postsynaptic terminal through the dendrite, one would necessarily need to control the voltage in a large component of the postsynaptic neurone. The resistance of the gap junctions is very large compared with the impedance of the somata in the spinal cord, indicating that depolarization of the axon under voltage clamp could not control the postsynaptic membrane potential. If the postsynaptic neurone had an input impedance as high as 1 GΩ (this is four times higher than any recorded impedance using patch clamp) then every 10 mV of postsynaptic depolarization would require 100 mV of depolarization at the presynaptic axon in the absence of postsynaptic voltage-operated conductances. Thus, the reversal of axonal responses strongly indicates that the synaptic inputs recorded in the axons are directed to the axons themselves. This is, of course, a necessary property of the synapse. If the resistance between the spine and soma were similar to the gap junction, the capacitance–resistance circuit of the structure would filter postsynaptic responses to negligible amplitudes.

Individual spontaneous events are also unlikely to cause enough K^+ release to allow a response to be recorded in the axon. Furthermore, when these inputs evoked action potentials the after-hyperpolarization was retained, ruling out extracellular K^+ as a mediator of the depolarization because there was clearly no significant change in the equilibrium potential for potassium (E_K) (Fig. 1; Matthews & Wickelgren, 1978). Both evoked and spontaneous inward currents are due to the activation of glutamatergic and GABAergic synapses. $GABA_A$-mediated responses show a reversal potential slightly positive to the membrane potential and are blocked by bicuculline (see Fig. 4). Glutamate receptor-mediated responses are faster, show a positive reversal potential and are blocked by CNQX and AP5 (see Fig. 5). Supporting evidence that the responses are mediated by axonal synaptic glutamate receptors is provided by the agonist activation of NMDA and AMPA receptors on these axons in the presence of TTX (see Fig. 6). The lack of an increase in noise associated with the application of NMDA may simply reflect an inability to gain an adequate space clamp of the entire axon. This phenomenon may also account for the degree of variability in rise times of the evoked responses and is a probable consequence of recording from a tube (the axon) at a single point. If a synapse is near to the recording electrode it will by recorded from accurately with little space clamp error. Synapses at more distant sites are subject to a failure in space clamp.

The location of inputs to reticulospinal axons is currently under investigation. Previous electron microscopic investigation has indicated that the axons possess axo-axonic synaptic inputs, although the source of these inputs is not known (Wickelgren, 1977). The number of candidate cell groups that could be responsible for these synaptic responses is somewhat limited. There exists three classes of glutamic acid decarboxylase- (GAD)-positive GABAergic interneurones in the spinal cord. Two of these are in the grey matter of the spinal cord and one represents a group of liquor-contacting cells around the spinal canal (Brodin, Dale, Christenson, Storm-Mathisen, Hökfelt & Grillner, 1990); these three cell types are the only likely source of GABAergic input. The glutamate responses may be activated by one of a number of glutamatergic terminals in the spinal cord. These include dorsal root ganglion primary afferents, dorsal cells (Brodin, Christenson & Grillner, 1987), excitatory interneurones (Buchanan, 1982; Buchanan & Grillner, 1987) and other reticulospinal neurones (Buchanan, Brodin, Dale & Grillner, 1987). The invariant latency between stimulus and the initiation of the evoked response indicates that the synapses are distributed along the length of the segment of axon in the spinal cord. In this way it is the distance between the stimulating electrode and the axon that dictates the latency regardless of the location of the recording patch electrode.

It has been demonstrated previously that vertebrate spinal axons receive inhibitory GABAergic input. This and other forms of presynaptic (monoaminergic) inhibition are common in the vertebrate CNS. GABAergic input to presynaptic terminals of primary afferents in the spinal cord evokes a depolarization of the terminal activated, at least partly, by a $GABA_A$ receptor-mediated increase in permeability of the terminal to Cl^-. As demonstrated in this study and earlier (Eccles *et al.* 1963; Nicoll & Alger, 1979; Alford *et al.* 1991), the reversal potential of this conductance is marginally depolarized with respect to the resting membrane potential of the terminal. The depolarization evoked by GABAergic inputs to terminals has therefore been considered inhibitory because it acts to prevent further depolarization and possibly spiking at the terminal by shunting the terminal input impedance. $GABA_B$ receptors acting at these terminals are also believed to inhibit release by reducing Ca^{2+} entry following spiking at the terminal (Dutar & Nicoll, 1988).

Output from reticulospinal axons is not, however, modulated by the application of $GABA_B$ receptor agonists. While $GABA_A$ receptor-mediated input to these axons has not previously been investigated, it seems likely that this is an inhibitory presynaptic response given the reversal potential

of the synaptic event. In addition, spinal axons receive excitatory synaptic inputs mediated by glutamate receptors. This is in contrast to previously identified effects of glutamate receptor activation on axons and presynaptic terminals. Kainate has been demonstrated to depolarize but to inhibit action potential conductance of rat dorsal horn axons (Agrawal & Evans, 1986). Synaptic excitation directed to vertebrate presynaptic axons and terminals has not previously been demonstrated in any form and the concept that excitation directed to the axon which can evoke spikes in that axon in the absence of dendritic or somatic input violates the principle of dynamic polarization proposed by Ramon Y Cajal and generally accepted as a guiding idea in neuroscience.

The significance of this axo-axonic excitatory input is unclear, but these synapses may be common in vertebrates. Ionotropic glutamate receptors (AMPA and NMDA) are believed to be present on mammalian presynaptic terminals (Kovalev & Hetey, 1987; Shimizu *et al.* 1990). Cyclothiazide, an agent that reduces desensitization of AMPA receptors, has been claimed to unmask presynaptic AMPA-mediated effects (Barnes *et al.* 1994). Interestingly, in this regard the application of cyclothiazide has been shown to significantly increase the amplitude of AMPA-mediated synaptic responses in culture (Diamond & Jahr, 1995), a phenomenon that may be consistent with presynaptic AMPA receptors. Antidromic spiking of cat sensory neurones has been previously reported under conditions in which their input could only originate from the central nervous system rather than their receptive field in the periphery (Dubuc *et al.* 1986, 1988; Gossard *et al.* 1989). This latter result implies that primary afferent depolarization (PAD) in mammals is capable of initiating spiking at the level of the presynaptic terminal. Such a result is in contradiction to the notion that PAD represents a GABAergic inhibitory conductance shunt of the terminal (Eccles *et al.* 1963) and it is possible that a component of PAD is excitatory. Indeed, excitatory inputs to axons have been identified in lamprey reticulospinal neurones (Wickelgren, 1977), and goldfish VIIIth nerve afferents (Pereda, Bell & Faber, 1995), though in both cases this has been ascribed to electrotonic conduction from postsynaptic dendrites through synaptic gap junctions (Matthews & Wickelgren, 1978).

In the spinal cord the capacity to directly stimulate the reticulospinal axon implies that these axons could contribute to network activity in the absence of inputs from the brain. Excitation directed to reticulospinal axons which themselves excite spinal neurones might lead to feed-forward excitation of the spinal locomotor pattern generator. The inputs to reticulospinal axons sum to initiate action potentials; thus synaptic integration occurs in the axon, which acts as a postsynaptic device. This, combined with synaptic inhibition that has been previously reported in axons (Eccles *et al.* 1963; Alford *et al.* 1991), indicates a role for the axon and presynaptic terminal in signal processing that is essentially similar to dendritic processes. The activation of NMDA receptors implies a role involving Ca^{2+} homeostasis in the synaptic terminal because input synapses have been identified in lamprey axons adjacent to their own output synapses (Matthews & Wickelgren, 1978).

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Acknowledgements

We express our gratitude to Dr N. T. Slater, Dr J. C. Houk and N. E. Schwartz for their help in this study and comments on the manuscript. This study was supported by the National Institute of Neurological Disorders and Stroke, grant NS31713.

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Received 31 May 1996; accepted 7 November 1996.