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Patch clamp studies of motor neurons in spinal cord slices: a tool for high-resolution analysis of drug actions

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KEY WORDS spinal cord; motor neurons; ethanol; alcohols; general anesthesia; postsynaptic mechanisms

ABSTRACT

AIM: To develop a tool for detailed analysis of spinally acting anesthetic and analgesic agents. **METHODS**: Studies were done on visually identified motor neurons in 400 μm thick spinal cord slices from 14 - 23 d old rats using patch clamp techniques. Ethanol was used as a prototype general anesthetic agent. **RESULTS**: Cell bodies in the ventrolateral horn identified as motor neurons by retrograde fluorescent labeling had a mean dimension of 32 ± 5 μm ($\bar{x} \pm s$, $n = 25$). Mean resting potential was -62.8 ± 2.4 mV; input resistance was 44 ± 24 MΩ ($n = 19$). Threshold was -44 ± 7 mV, and action potential amplitude 101 ± 9 mV from baseline. Ethanol concentrations at and below 50 - 200 mmol/L decreased motor neuron excitability to the injected current; there was no effect on resting potential, but a variable reversible increase in input resistance. Ethanol reversibly depressed the excitatory postsynaptic potential, with a dose-response relationship similar to that previously observed for the population excitatory postsynaptic potential in intact spinal cord *in vitro*. Ethanol also reversibly depressed currents evoked by glutamate, reducing total charge transfer to 40 % ± 26 % of control ($\bar{x} \pm s$; $n = 4$). **CONCLUSION**: Reduction of connectivity in this relatively thick slice preparation does not significantly modify drug actions. The actions of ethanol on excitatory synaptic transmission observed in intact spinal cord are in part due to postsynaptic effects on motor neurons.

INTRODUCTION

Many therapeutically important drugs affecting the central nervous system exert their effects in part by actions on the spinal cord. These include opioid and other analgesic agents and inhaled general anesthetic agents. In addition to its intoxicating properties, ethanol can act as a general anesthetic agent, preventing movement in response to a noxious stimulus[1,2]. Prevention of nocifensive movement is the most common endpoint for comparing potencies among volatile anesthetic agents. For such agents the anesthetic concentration at this endpoint is the Minimum Alveolar Anesthetic Concentration (MAC), which just prevents movement in response to a noxious stimulus[3]. MAC is determined by anesthetic actions in the spinal cord[4-9]. Thus actions on spinal cord are directly relevant to general anesthesia.

In previous *in vitro* studies using neonatal rat spinal cord preparation, we have shown that ethanol depresses ventral root potentials evoked by dorsal root stimulation, at concentrations corresponding to anesthetic levels[10]. The responses depressed by ethanol include the monosynaptic reflex and its underlying population excitatory postsynaptic potential (pEPSP). Studies on population evoked responses in the intact *in vitro* spinal cord cannot discriminate between presynaptic inhibition of transmitter release and postsynaptic actions on motor neurons. The present studies employed patch clamp recording from visually identified motor neurons in spinal cord slices to examine the actions of ethanol with greater resolution than is possible in an intact cord. In addition, since this study is the first from our laboratory on this preparation and employs thicker slices from more mature animals than those from other laboratories, we present the anatomical and physiological characteristics of the motor neuron population.

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MATERIALS AND METHODS

Experiments were carried out in spinal cord slices from Sprague-Dawley rats 14 – 23 d of age; most were in the range of 14 – 20 d. In a protocol approved by Stanford's panel on laboratory animal care and use, the animals were anesthetized with halothane, decapitated, and spinal cords quickly removed and placed in a protective chilled oxygenated calcium-free low sodium artificial cerebrospinal fluid (ACSF). The ACSF was composed as follows (mmol/L): KCl 5, $MgSO_4$ 2, $NaHCO_3$ 26, NaH_2PO_4 1.25, *d*-glucose 10, sucrose 252. Slices 400 μm thick were prepared as previously described[11]. Briefly, slices were sectioned from the lumbar region on a Vibratome®, and removed to an oxygenated ACSF of the following composition (mmol/L): NaCl 123, KCl 4, NaH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 26, *d*-glucose 10, $CaCl_2$ 2. In this solution the slices were allowed to recover at room temperature for 0.5 – 1 h. Individual slices were transferred to a chamber on the stage of a microscope constantly superfused with oxygenated ACSF of the same composition as the recovery solution. All experiments were carried out at room temperature.

Cell bodies beneath the cut surface of the slice were viewed on a closed circuit TV monitor using infrared illumination and a 40× water immersion objective. In preliminary studies the large cell bodies in the ventral horn, most commonly seen in the ventrolateral area, were identified as motor neurons by fluorescent labeling with Evans blue dye injected into the hind limb of the rat the day before sacrifice (Fig 1 C, D). Once this identity was established fluorescent labeling was not used in the physiological and pharmacological studies because of concerns that the dye might alter glutamate receptor properties[12].

Patch pipettes were pulled on a Flaming-Brown pipette puller (Sutter Instruments) and filled with a solution of the following composition (mmol/L): NaCl 15, potassium gluconate 110, HEPES 10, $MgCl_2$ 2, egtazic acid 11, $CaCl_2$ 1, MgATP 2, pH adjusted with KOH to 7.3. Pipettes typically had a tip resistance of 3 – 5 MΩ. The patch pipette was directed towards a motor neuron cell body under visual control. Following establishment of a Gigohm seal the patch was ruptured by brief negative pressure and subsequent measurements were made in the whole cell ruptured patch configuration in either current clamp or voltage clamp mode using an Axopatch 1D patch clamp amplifier (Axon Instruments). Excitatory postsynaptic potentials (EPSPs) were evoked by electrical stimulation of the dorsal root fragment via a concentric bipolar platinum electrode with tip diameter 0.025 mm (Frederick Haer & Co), using square wave stimuli 0.1 ms in duration, 1 – 20 V nominal intensity, frequency 0.03 – 0.1 s^{-1}[13]. EPSPs were averaged in groups of 5 – 15. Responses were also evoked by direct application of glutamate (100 mmol/L) from a pipette close to the cell body at intervals of 3 min. Glutamate-evoked responses were not averaged. Ethanol was obtained from commercial sources (Gold Seal Chemical Co, Hayward, CA) as a 95 % compound and diluted into the ACSF to the desired concentration.

Ethanol from another source (Grain Processing Corporation, Muscatine, IA) was used in a few experiments and gave similar results. Ethanol was applied by superfusion in the bath by gravity feed from a container similar to that containing the control ACSF. Superfusion rates were maintained constant between control and alcohol-containing solutions, and sham experiments, in which both containers held control solutions, indicated that switching from one to the other container had no effect.

A commercially available software package (pClamp, Axon Instruments) was used to acquire data. Results were digitally stored and analyzed off-line. Input membrane resistance was calculated as the slope of the current/voltage relationship obtained by linear regression. Results were obtained from a single cell in each slice. Electrophysiological properties and response measurements were averaged across cells and expressed as $\bar{x} \pm s$.

RESULTS

Stability of recordings Cells were routinely held for periods of 1 to 2 h after rupturing the patch. All cells used in the study displayed stable responses to a constant stimulus intensity applied to the dorsal root over a 10 min control period. During this period there were no significant changes in input resistance, resting potential in current clamp mode, or holding current in voltage clamp mode.

Motor neuron properties Fig 1 shows cells in the ventrolateral horn of spinal cord slices. Fig 1 A and B show the same large cell body in the ventral horn before (A) and after (B) formation of a high resistance seal to the patch pipette. Fig 1 C and D show a large cell body visualized by infrared illumination (C) and by fluorescence (D). The animal from which this slice was prepared had received 50 μL injections of Evans Blue dye

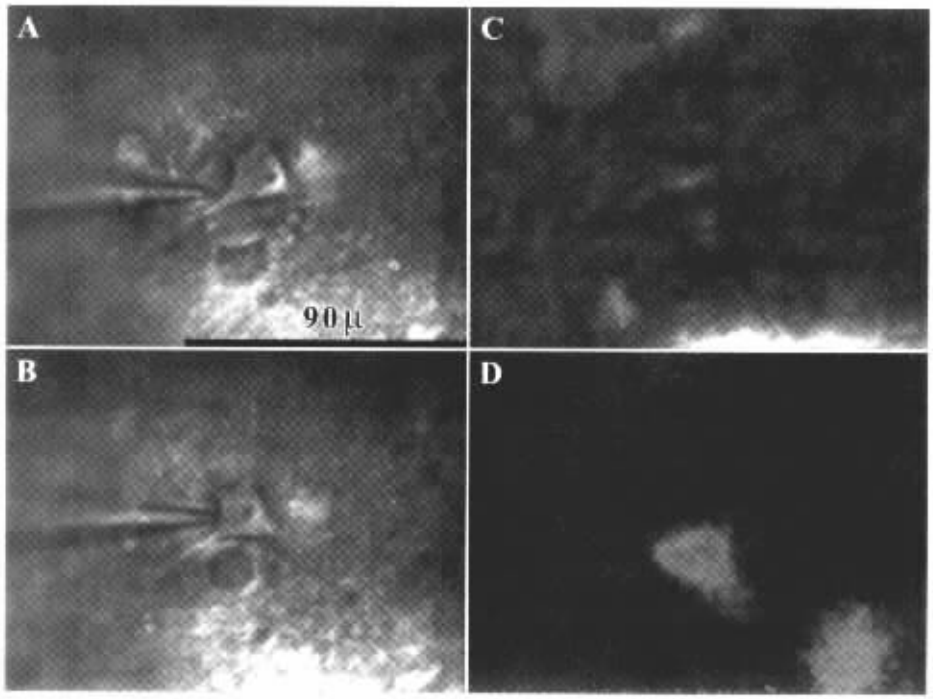

Fig 1. Motor neurons in spinal cord slice. A, B, a large cell body in the ventrolateral region of the ventral horn, viewed by infrared illumination at a depth of 30 – 50 μm beneath the cut surface of the slice. Magnification 400, calibration bar 90 μm. A patch pipette electrode is seen approaching the cell in A; B shows the cell after a high resistance seal has been formed and the patch ruptured. C and D show a slice from an animal that received an injection of Evans blue dye into the gastrocnemius muscle the day before, viewed by infrared illumination (C) and fluorescence optics (D). Fluorescence appears in the same region as the visible cell body in C, confirming that this is a motor neuron cell body. Another region of fluorescence is seen in a cell body outside of the plane of focus of the infrared image.

in the gastrocnemius muscles of both hind limbs on the day preceding sacrifice. Fluorescence coinciding with the infrared image reveals the cell to be a retrogradely labeled motor neuron. A sample of 25 cells was identified as motor neurons by fluorescent labeling. The cell bodies in this sample had the following measurements: Diameter in the longest dimension ranged from 28.46 to 52.01 μm, with a mean of 41.04 μm and standard deviation (SD) 6.20. In the shortest dimension the range was 12.94 to 39.16 μm, with a mean of 23.54 ± 6.66. Mean dimension [(short + long)/2] ranged from 24.32 to 44.33 μm, mean 32 ± 5. With respect to electrical properties, a sample of 19 cells studied in current clamp mode had resting potentials of −62.8 ± 2.4 mV. Minimum resting potential (absolute) was −59 mV, maximum −67 mV. Mean input resistance was 44 ± 24 MΩ, with a range from 11 to 101 MΩ. Threshold to impulse initiation in response to current injection was −44 ± 7 mV, ranged from −28 to −56, and action potential amplitude measured as difference from resting potential 101 ± 9 mV, ranged from 80 to 115 mV.

Responses evoked to dorsal root stimulation

In current clamp mode, electrical stimulation in the dorsal root entry area gave rise to a short-latency depolarization whose amplitude and duration increased with increasing stimulus intensity. An example is shown in Fig 2A. At the higher stimulus intensities, the depolarization was sufficient to elicit action potentials (Fig 2A). The response was abolished in ACSF with low (0.2 mmol/L) calcium and high (6.3 mmol/L) magnesium, indicating it was entirely synaptic in origin (Fig 2B).

In voltage clamp mode stimulation in the dorsal root entry region elicited an inward current whose magnitude was dependent on stimulus intensity and holding potential. An example is shown in Fig 3 A and B, with the voltage/current relationship of the same cell shown in Fig 3C. Reversal potential was not established for this current (Fig 3C) because at depolarized holding potentials an inward action potential current was superimposed on it (Fig 3B).

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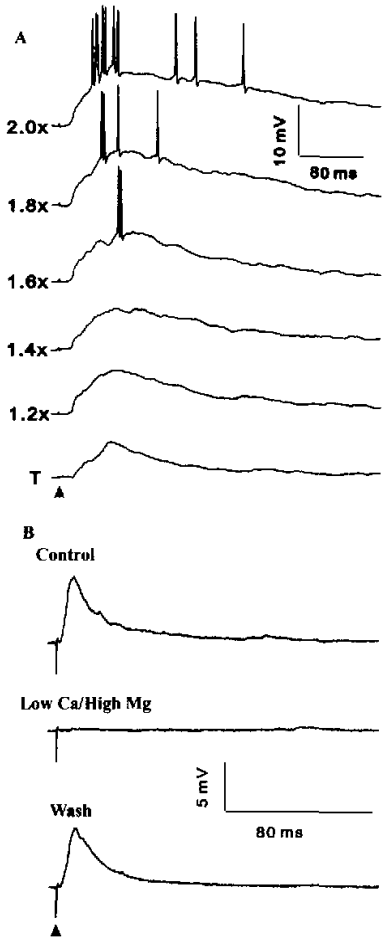

Fig 2. Excitatory postsynaptic potentials (EPSPs) evoked by dorsal root stimulation from a motor neuron in current clamp mode. A, records from a motor neuron in a slice taken from a 15-d-old animal; this cell had a resting potential of −63 mV. A stimulus was given to the dorsal root at the time indicated by the arrow, at increasing intensities in successive traces from bottom to top. T, threshold; additional stimuli are multiples of threshold intensity as indicated. The stimulus evoked an upward (depolarizing) deflection. At higher intensities the waveform became longer and more complex, and action potentials were superimposed on it. Records are averages of 5 responses. B, records from a different cell in a slice from a 14-d-old animal, resting potential −67 mV. The depolarizing response to electrical stimulation was reversibly abolished by perfusion with ACSF in which the calcium concentration was decreased to 0.2 mmol/L and magnesium concentration increased to 6.3 mmol/L. This is an evidence for a synaptic origin. Records are averages of 6 responses.

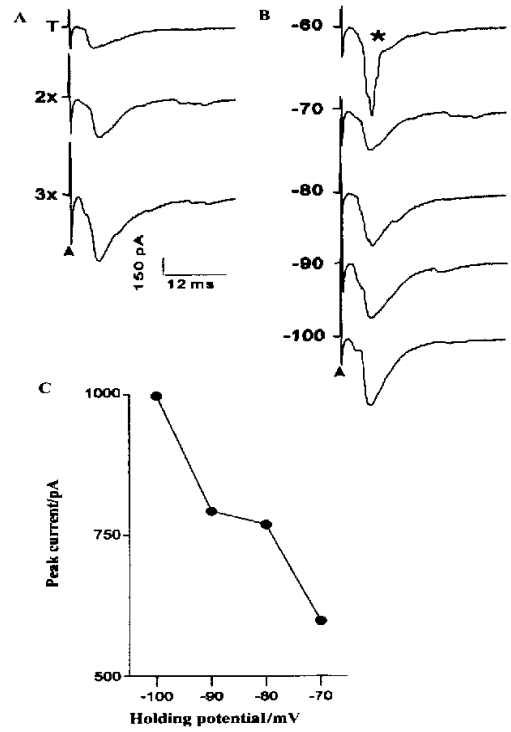

Fig 3. Inward currents evoked by dorsal root stimulation in voltage clamp mode from a motor neuron in a slice taken from a 22-d-old rat. A, responses to increasing stimulus intensity (arrow) at threshold (T) and multiples of threshold intensity (2×, 3×); holding potential was −70 mV. B, holding potential in the same cell as shown in A was varied as indicated. Response amplitude increased with hyperpolarization. At −60 mV the inward current was overridden by an action current (asterisk). Under these conditions reversal potential of the inward current could not be established. Records in both A and B are averages of 6 responses. C, voltage-current relation from a single cell, the same cell as shown in A and B, with peak current amplitude as the measured variable.

Glutamate-evoked currents Glutamate applied by brief pressure pulses to a pipette located close to the cell body evoked inward currents whose amplitude and duration increased with pulse duration. An example is shown in Fig 4A. Membrane conductance increased during the current. The inward current varied with holding potential (Fig 4B). Fig 4C shows the current/voltage relationship for the cell shown in Fig 4B. The current was near zero at a holding potential of +30 mV, the most positive potential that could be achieved without

rapid deterioration of the cell. Thus reversal potential could not be exactly determined, but the regression line of the voltage-current relationship suggests a reversal potential of approximately +25 mV.

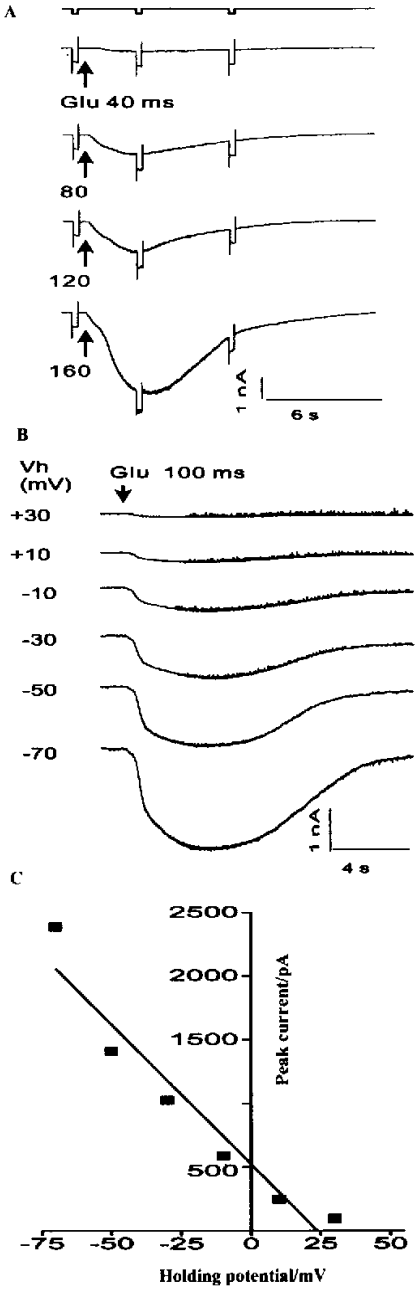

Fig 4. Glutamate-evoked inward currents in motor neurons. Glutamate was applied at the time indicated by the arrows from a pipette containing glutamate 100 mmol/L, using a pressure pulse of 200 kPa for the durations indicated. Records are single traces (not averaged) from a cell in a slice taken from an 18-d-old animal. A, increasing duration of pressure to the glutamate pipette evoked progressively larger inward currents. Brief downward square hyperpolarizing voltage steps as shown in the protocol at the top of the figure were used to monitor changes in membrane conductance; conductance increased during the inward current. These records were made in the presence of TTX (300 nmol/L). B, inward currents evoked by glutamate varied with holding potential and were nearly abolished at +30 mV. No TTX was present when these records were made. In the absence of TTX brief long-latency current fluctuations superimposed on the large inward current reflected transmitter release from glutamate-activated interneurons. Records are single responses to glutamate applications at 3 min intervals. C, voltage/current relation from a single cell, the same cell as shown in B.

Effects of ethanol on intrinsic neuronal properties Ethanol (50 – 200 mmol/L) had no significant or consistent effects on resting potential. In 5 out of 6 cells exposed to cumulative increases in ethanol from 25 – 200 mmol/L input resistance increased to a variable extent, and the increase was at least partly reversible on washing. An example is shown in Fig 5 A and B. Threshold also reversibly increased with increasing ethanol concentrations (Fig 5C). Number of impulses decreased both for a given level of current (Fig 5D) or membrane potential (Fig 5E). Number of impulses rebounded to a level above control on a washout. There were also changes in the shape of the action potential, most notably a decrease in afterhyperpolarization (Fig 5A). Since the absolute values of threshold and number of impulses vary among cells, it was not useful to attempt to average the ethanol-induced changes in these intrinsic properties for a sample of 6 cells.

Ethanol sensitivity of the EPSP In preparation of the spinal cord slice considerable connectivity is lost, particularly in the anteroposterior direction but also in the same-segment connections that lie outside the plane of the slice. The ability to examine the intact isolated spinal cord and to compare it to spinal cord slice offers the unique possibility of testing whether the drug sensitivity is altered in the more highly dissected preparation. Excitatory postsynaptic potentials were evoked by dorsal root stimulation in 6 cells from different slices. Cumulative dose-response curves to ethanol were constructed and compared to the dose-response curve of the population

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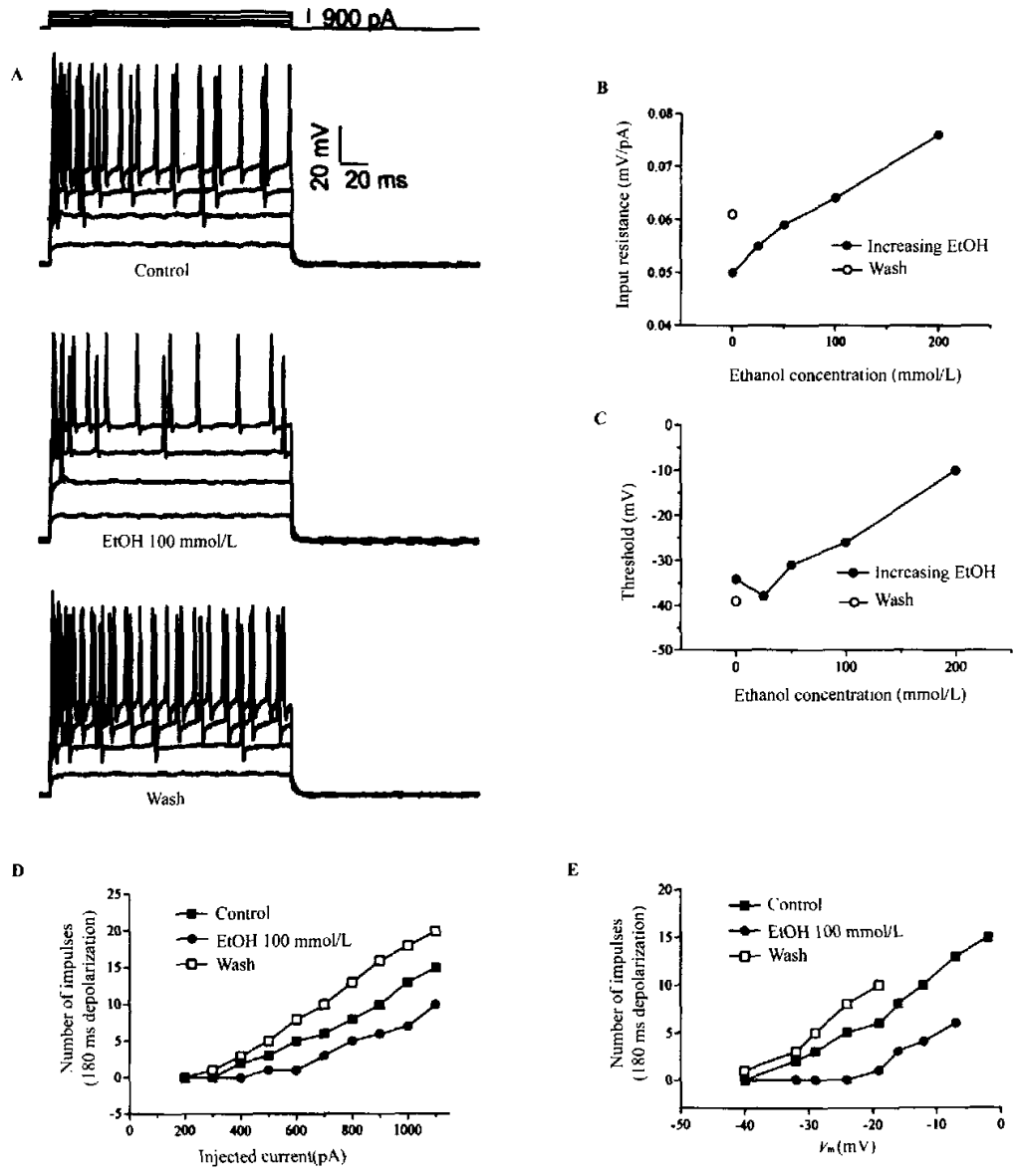

Fig 5. Effects of ethanol on intrinsic neuronal properties. A, in current clamp mode depolarizing current injections were given as shown in the protocol at the top of the figure, evoking a series of impulses in the cell. Ethanol reversibly decreased the number of impulses and reduced the afterhyperpolarization; there was an accompanying increase in membrane resistance. This cell was treated with ethanol at increasing concentrations for 15 min each to create a cumulative dose-response curve. B shows the reversible increase in input resistance. C, in the same cell ethanol reversibly increased the threshold to first impulse initiation. D, E, at 100 mmol/L ethanol reversibly decreased the number of impulses evoked by a given amount of injected current (D) or at a given level of depolarization (E). There was an overshoot on wash. Graphs B-D are from a single cell, the same cell as shown in A.

EPSP in intact cord taken from our previous studies[10]. Linear regression analysis showed that the curves were not different from each other (Fig 6).

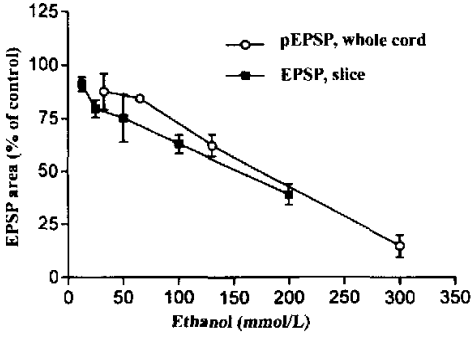

Fig 6. Comparison between sensitivity of the EPSP to ethanol in intact isolated spinal cord and in spinal cord slices. Results in whole cord are means of 4 – 7 individual preparations each exposed to a single ethanol concentration, and are taken from a previous publication[10]. Results for spinal cord slices are cumulative dose-response curves from 6 motor neurons, each in a different slice. Error bars are SEM. The additional dissection and loss of anteroposterior connections in the slice preparation did not significantly alter sensitivity to ethanol.

Actions of ethanol on glutamate-evoked currents In the presence of tetrodotoxin (300 nmol/L), ethanol 100 mmol/L reversibly decreased glutamate-evoked currents (Fig 7). Peak glutamate currents were significantly depressed to 80.2 % ± 9.11 % of control ($n = 4$, $P < 0.05$) and total charge transfer to 40.1 % ± 26.2 % of control ($P < 0.05$).

DISCUSSION

Comparison with other studies on motor neurons A number of studies have been carried out on the properties of motor neurons in rat spinal cord with either intracellular electrodes[11,13-16] or patch clamp techniques[17,18]. The electrical properties of motor neurons in the present study largely is in agreement with those reported by others. The input resistance is higher than that reported in some studies with intracellular electrodes[14,15], but is in agreement with those reported in others[11,13,16]. Higher resistance in patch clamp measurements compared to intracellular measurements may be due to the tight seal between membrane and electrode in the patch clamp configuration[17]. Resistances of motor neurons in the present study were lower than that reported in another study using patch electrodes[17]. The most important contributory difference is probably the age of the animals, 14 – 23 d in the present study versus 2 – 3 d in the other. The age difference is also reflected in the size distribution of the cell bodies, which in 14 – 23 d old animals are much larger than and almost do not overlap diameters in the 2 – 3 d old animals[17]. Although a decrease in electrical coupling between motor neurons with age[19] might tend to increase membrane resistance, input resistances of both spinal[14] and hypoglossal[20] motor neurons have been reported to decrease with age, perhaps because of an increase in ion channel density.

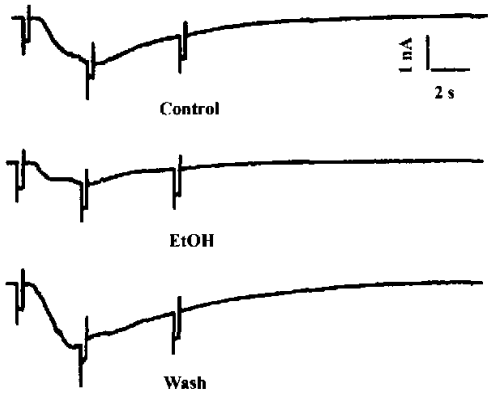

Fig 7. Ethanol reversibly depressed glutamate-evoked currents in the presence of TTX. Ethanol 100 mmol/L was applied for 15 min (EtOH) followed by 30 min wash with drug-free ACSF (Wash). Downward deflections are brief hyperpolarizing voltage steps to monitor input resistance.

Effects of ethanol on motor neuron properties General anesthetic agents have been reported to hyperpolarize neurons in spinal cord[21,22]. However in the hippocampus effect of volatile anesthetic agents varies, only one of the three tested produce hyperpolarization associated with a conductance increase[23]. The present study in spinal cord motor neurons showed no change in resting potential in either direction, and many cells exposed to ethanol, particularly for long periods, showed a decrease in conductance. There was an increase in threshold for impulse initiation and a decrease in the number of action potentials in a train, which could be due to number of causes. A study in motor neurons from younger rats (1 – 3 d old) showed a similar decrease in ability to sustain repetitive firing in the presence of

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ethanol 70 mmol/L; the decrease may be related to inhibition of voltage-gated calcium currents[24]. The decrease in afterhyperpolarization after each impulse points to inhibition of a voltage-dependent potassium channel. Ethanol-induced increases in afterhyperpolarization and reduction in spike frequency in hippocampus have been suggested to be due to an increase in calcium-dependent potassium conductance[25], and such a change has been suggested to be a common mechanism of anesthetic action[26]. In motor neurons there appears to be no long-lasting afterhyperpolarization of the type ascribed to $I_{K(Ca)}$ at least under the recording conditions in the present study.

Effects of ethanol relation to general anesthesia Ethanol produces general anesthesia as measured by abolition of a motor response to a noxious stimulus in 7 d old rats at concentrations close to the higher end of the range (200 mmol/L) used in the present study. There is a pronounced age-dependence, such that mature animals are anesthetized at 100 mmol/L[1,2]. The present study therefore employed concentrations relevant to general anesthesia in the animals from which the spinal cord slices were prepared, over the age range which brackets the age of the animals in the study (14 – 20 d). The anesthetic endpoint used in the behavioral studies of ethanol anesthetic potency, abolition of movement in response to a noxious stimulus, or MAC[3], is a function of anesthetic actions in the spinal cord[4–9]. We have previously reported similar depressant effects of ethanol and volatile anesthetics on the circuitry that mediates the monosynaptic reflex in isolated intact spinal cord from 2 – 7 d old rats, at anesthetically relevant concentrations over the same range as used in the present study[10,27]. In the present study in slice preparation we observe changes in intrinsic motor neuron properties and a depressant effect of ethanol on glutamate-evoked currents in the presence of tetrodotoxin. These results lead to the conclusion that the depressant effects of ethanol on excitatory synaptic transmission observed in intact cord are at least in part due to the actions on the motor neurons themselves in addition to any actions on elements presynaptic to them.

Actions of ethanol on synaptic transmission in slices as compared to intact cord The additional resolution afforded by slice preparations comes at the cost of paring away much of the connectivity that can be obtained in the intact organ. The neonatal spinal cord offers the unique capability of examining both the intact cord and the slice preparation *in vitro*, and thus of testing the relevance of drug actions in slice preparations to the situation in whole cord. The nearly superimposed dose-response curves for ethanol depressant actions on the population EPSP in whole cord and EPSPs in individual motor neurons in slice suggest that none of the important mediators of drug actions on this response, for instance tonic inhibitory interneurons, are lost in this relatively thick slice preparation. We have used the present study as a basis for more detailed analysis of the postsynaptic actions of both ethanol[28] and clinical volatile general anesthetic agents[29].

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脊髓切片中运动神经元的膜片箝技术研究：一种分析药物作用的高效工具

关键词 脊髓；运动神经元；乙醇；醇类；全身麻醉；突触后机制

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