Postnatal Changes in the Inactivation Properties of Voltage-Gated Sodium Channels Contribute to the Mature Firing Pattern of Spinal Motoneurons

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Submitted 16 January 2008; accepted in final form 7 April 2008

Carlin KP, Liu J, Jordan LM. Postnatal changes in the inactivation properties of voltage-gated sodium channels contribute to the mature firing pattern of spinal motoneurons. J Neurophysiol 99: 2864–2876, 2008. First published April 9, 2008; doi:10.1152/jn.00059.2008. Most mammals are born with the necessary spinal circuitry to produce a locomotor-like pattern of neural activity. However, rodents seldom demonstrate weight-supported locomotor behavior until the second or third postnatal week, possibly due to the inability of the neuromuscular system to produce sufficient force during this early postnatal period. As spinal motoneurons mature they are seen to fire an increasing number of action potentials at an increasing rate, which is a necessary component of greater force production. The mechanisms responsible for this enhanced ability of motoneurons are not completely defined. In the present study we assessed the biophysical properties of the developing voltage-gated sodium current to determine their role in the maturing firing pattern. Using dissociated postnatal lumbar motoneurons in short-term culture (18–24 h) we demonstrate that currents recorded from the most mature postnatal age group (P10–P12) were significantly better able to maintain channels in an available state during repetitive stimulation than were the younger age groups (P1–P3, P4–P6, P7–P9). This ability correlated with the ability of channels to recover more quickly and more completely from an inactivated state. These age-related differences were seen in the absence of changes in the voltage dependence of channel gating. Differences in both closed-state inactivation and slow inactivation were also noted between the age groups. The results indicate that changes in the inactivation properties of voltage-gated sodium channels are important for the development of a mature firing pattern in spinal motoneurons.

INTRODUCTION

The circuitry necessary to produce a locomotor pattern of muscle activation is already present and functional in the embryonic spinal cord of most mammals (Ren and Greer 2003; Whelan 2003). For example, rodents are capable of swimming and crawling at birth; however, the ability to raise the body off the ground and walk does not develop fully until the second or third postnatal week (Brocard et al. 1999; Jiang et al. 1999a; Westerga and Gramsbergen 1990). One important feature of postnatal neuromuscular development is the matching of motoneuron discharge rates with the twitch properties of the muscles they innervate (Vrbova et al. 1985). This matching likely ensures the force generation in limb muscles necessary for the production of overground locomotion (Martin-Caraballo et al. 2000). As the muscle twitch time decreases during postnatal development (Close 1964) a corresponding increase in the motoneuronal firing rate is required.

During the late embryonic and early postnatal periods, many types of motoneurons demonstrate an increase in maximal firing rate and duration of repetitive firing. This developmental phenomenon has been described in phrenic (Greer and Funk 2005; Martin-Caraballo and Greer 1999), oculomotor (Carrascal et al. 2006), hypoglossal (Viana et al. 1995), and spinal motoneurons (Fulton and Walton 1986; Vinay et al. 2000; Vrbova et al. 1985). This ability of motoneurons to discharge action potentials at an increasing rate has been attributed to various factors including an increase in sodium current density (Gao and Ziskind-Conhaim 1998; Garcia et al. 1998; McCabe et al. 1990), development of repolarizing conductances (McCobb et al. 1990; Viana et al. 1994; Vinay et al. 2000), afterhyperpolarization amplitude increase (Fulton and Walton 1986), the development of calcium channels (McCobb et al. 1989; Miles et al. 2004; Mynlieff and Beam 1992), or changes in modulatory inputs (reviewed in Kernell 2003; Schmidt and Jordan 2000). In addition to an increase in firing frequency, a second defining characteristic of mature spinal motoneurons is their ability to generate plateau potentials (Perrier and Hounsgaard 2000). When the conductances underlying the plateau are activated, they create a second quasi-stable potential approximately 10–20 mV more positive to the resting potential (Hounsgaard and Kiehn 1989; Hounsgaard et al. 1986). Depending on the class of motoneuron, this underlying depolarization can persist for many seconds (Lee and Heckman 1998). For a cell to fire a train of action potentials at such depolarized membrane voltages, sodium channels must resist entering an inactivated state so as to remain available for activation. In the present study we assess the biophysical properties of the sodium current generating the action potential as a potential mechanism contributing to postnatal development of the firing properties of spinal motoneurons.

In the mammalian nervous system, voltage-gated sodium currents can be generated by nine different channel subtypes (Catterall et al. 2003), each displaying some unique biophysical properties (Rush et al. 2007). The sodium channel subtype expression in mammalian spinal cord changes during the early postnatal period as demonstrated by studies examining expression at either the protein (Gordon et al. 1987; Schaller and Caldwell 2000) or mRNA level (Beekh et al. 1989; Felts et al. 1997; Garcia et al. 1998; Schaller and Caldwell 2000). Some of these changes have been directly localized to the spinal motoneurons (Schaller and Caldwell 2000) and have been supported by electrophysiological data from mice expressing a spontaneous mutation in a late-developing sodium channel.
subtypes (med mutant; Garcia et al. 1998). Taken together, these data suggest that a change in the biophysical characteristics of the sodium channels may, in part, be responsible for the change in firing properties of developing spinal motoneurons. However, this type of kinetic data is absent from the literature on motoneurons, in all likelihood due to the inherent difficulties associated with voltage- and space-clamping the sodium current in these cells (e.g., Garcia et al. 1998).

In the present study we circumvent these technical difficulties by using dissociated postnatal lumbar spinal motoneurons cultured overnight in conditions that did not promote process formation. We then tested the hypothesis that the ensemble sodium current demonstrates age-related biophysical changes consistent with the known mature firing pattern of these cells. Specifically, we assessed the ability of the maturing complement of sodium channels to resist accumulating in an inactivated state during repetitive depolarizing stimulations that mimicked repetitive firing and evaluated the tendency of these channels to undergo closed-state inactivation during prolonged depolarizations similar to the voltage change sensed by these channels during the expression of a plateau potential.

METHODS

Cell culture

Mixed spinal cord cultures were prepared from the lumbar spinal cords of BALB/c mice (P1–P12, where P0 is the day of birth). Motoneurons were identified in the cultures by prelabeling these cells through an intraperitoneal injection of the fluorescent maker Fluoro-Gold (10–20 μl of a 2% solution; Fluorochrome, Denver, CO) ≥ 24 h prior to sacrifice (Miles et al. 2005). Spinal cord slices were then placed directly on the stage of an inverted Nikon Diaphot 300 inverted microscope with a Nikon UV-2A filter set. To limit potential phototoxic damage to the cells, large, healthy-looking, round cells were first identified by using brightfield illumination, then quickly exposed to the fluorescent labeled cells. We then tested the hypothesis that the ensemble sodium current demonstrates age-related biophysical changes consistent with the known mature firing pattern of these cells. Specifically, we assessed the ability of the maturing complement of sodium channels to resist accumulating in an inactivated state during repetitive depolarizing stimulations that mimicked repetitive firing and evaluated the tendency of these channels to undergo closed-state inactivation during prolonged depolarizations similar to the voltage change sensed by these channels during the expression of a plateau potential.

Electrophysiology

After approximately 18–24 h of incubation the culture media was replaced with the external recording solution designed to isolate sodium currents. The culture dishes were then placed directly on the stage of an inverted Nikon Diaphot 300 inverted microscope with a Nikon UV-2A filter set. To limit potential phototoxic damage to labeled cells, large, healthy-looking, round cells were first identified under brightfield illumination, then quickly exposed to the fluorescent light to determine whether labeling was present (Fig. 1A). It is interesting to note that many large cells were identified in these cultures that lacked the fluorescent label and therefore recordings were not made from these cells. This observation is consistent with our previous observations (Carlin et al. 2006; Huang et al. 2000) as well as the observations of others (Thurbon et al. 1998) of large non-motoneuronal cells in the lumbar cord. Whole cell recordings were made from Fluoro-Gold–positive cells using an Axopatch 1D amplifier, DigiData1200 A/D converter, and Clampex 8 software (Axon Instruments, Union City, CA). A holding potential of −70 mV was used for all experiments. This potential was used because it was well tolerated by the cells and it likely produced a pseudophysiological amount of steady-state sodium channel inactivation, thereby increasing the physiological relevance of these data. Data were captured at 50 kHz and low-pass filtered at 5 kHz. The borosilicate glass pipettes had resistances between 1.5 and 2.8 MΩ when filled with intracellular solution. Series resistance was compensated as required so that the calculated voltage error did not exceed 6 mV. Using a flowing KCl electrode (Neher 1992), a liquid junction potential of −4 mV was determined, which made the true potentials more negative than those reported. All experiments were performed at RT (~22°C). For current traces in figures, depolarizing and hyperpolarizing capacitive transients have been either removed or truncated for clarity.

Solutions and chemicals

The intracellular solution contained (in mM): CsMeSO4 (125), TEA-Cl (30), NaCl (5), MgCl2 (1), CaCl2 (0.5), HEPES (10), EGTA...
exp(x/H)

Data analysis

Current traces were analyzed using Clampfit v.9 software (Axon Instruments). Statistical analysis and curve fitting were performed with Microsoft Excel, Microcal Origin 6.0, and SigmaPlot 9.0 (Systat) software programs. Activation curves were constructed using the calculated sodium reversal potential of 83 mV. Both the activation and steady-state inactivation conductance data were fitted with a single Boltzmann equation of the form: 

g/Gmax = Gmin + (Gmax - Gmin)/

[1 + exp((V1/2 - x)/k)],

where Gmax is the maximal conductance value, Gmin is the minimal conductance value, k is the slope factor, and V1/2 is the voltage of the half-maximal conductance value. Recovery and closed-state inactivation data were fitted with double-exponential functions of the form:

y = [A1 × [1 - exp(-x/τ1)] + [A2 × [1 - exp(-x/τ2)]],

where τ1 and τ2 are the fast and slow time constants. These curves were compared by taking the mean of the raw normalized data with the exclusion of the first point over the time period indicated in the text.

Parametric statistics were used to assess differences between age groups if the data were normally distributed (Kolmogorov–Smirnov test for normality) and groups were of equal variance. If these criteria were not met, groups were compared using the Kruskal–Wallis one-way ANOVA on ranks. Cell capacitance was determined from the voltage step to a 10-mV step that elicited the transients. Current density was obtained by dividing the cell capacitance by the peak current elicited by a voltage step to −10 mV from a 100-ms × −120-mV hyperpolarization.

RESULTS

The data from 111 Fluoro-Gold–positive postnatal spinal motoneurons were analyzed in the present study. These cells spanned the postnatal period from day 1 to day 12 and were divided into four age groups consisting of 3 days each (Fig. 1C). The mean cell capacitance (ANOVA on ranks, P = 0.41) and the current densities of the four age groups were not significantly different (ANOVA, P = 0.74; Table 1). This permitted an accurate comparison of the currents across the age groups without a differential voltage error affecting the results.

Voltage dependence of channel gating

As a first step in exploring developmental changes in sodium channel functioning we examined the voltage dependence of activation and inactivation of channel gating because these parameters can have dramatic effects on channel availability and gating at a given membrane potential. The voltage dependences of activation and fast inactivation of the sodium channels were measured using standard voltage protocols (Fig. 2, A and B). Both the activation and inactivation conductance–voltage relationships were fitted well with a single Boltzmann function. The average V1/2 values and slope (k) values of both the activation and inactivation fits were not significantly different across the measured developmental period (Table 1). Given the remarkable consistency of these parameters across the age groups, an average of the full data set was generated and is illustrated in Fig. 2C. This analysis resulted in an average V1/2 of activation of −21.3 mV (k = 4.6) and V1/2 of inactivation of −51.5 mV (k = 7.0) that describes the voltage dependence of sodium channel gating in spinal motoneurons during this postnatal period. The highly consistent data obtained from motoneurons over this developmental period indicate that a change in the voltage dependence of activation or fast inactivation does not contribute to the described developmental increase in excitability of these cells.

Frequency-dependent inactivation

For a cell to fire multiple action potentials a sufficient number of sodium channels must remain available before each spike. To assess channel availability during repetitive stimulations, such as would occur during a period of repetitive action potential firing, we assessed the sodium current amplitude during various frequencies of stimulation. To do this, cells were depolarized for 20 ms from −70 to −10 mV × 20 pulses at 1, 10, 20, and 30 Hz (Fig. 3A). It was found that the sodium current amplitude was reduced in a frequency-dependent manner, with the degree of inhibition by the 20th pulse increasing as the frequency of stimulation increased. This was consistent across the age groups (Fig. 3B). Also across all age groups and at frequencies >1 Hz, the current exhibited an initial rapid reduction in amplitude followed by a more gradual reduction. The rate of the initial reduction was almost identical across the age groups, consistent with the comparable fast-inactivation process seen across age groups (Table 1). The latter slower component

<table>
<thead>
<tr>
<th>Property</th>
<th>P1–P3</th>
<th>P4–P6</th>
<th>P7–P9</th>
<th>P10–P12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacitance, pF</td>
<td>9.55 ± 3.7 (26)</td>
<td>9.97 ± 5.5 (33)</td>
<td>9.23 ± 5.1 (28)</td>
<td>10.39 ± 4.0 (24)</td>
</tr>
<tr>
<td>Current density, pA/pF</td>
<td>188 ± 73 (17)</td>
<td>182 ± 126 (17)</td>
<td>168 ± 109 (15)</td>
<td>205 ± 131 (18)</td>
</tr>
<tr>
<td>V1/2 activation</td>
<td>−20.9 ± 4.4 (23)</td>
<td>−22.6 ± 4.7 (24)</td>
<td>−21.0 ± 4.2 (20)</td>
<td>−19.1 ± 4.1 (21)</td>
</tr>
<tr>
<td>Slope factor k</td>
<td>4.5 ± 0.8 (23)</td>
<td>4.5 ± 0.6 (24)</td>
<td>4.6 ± 0.4 (20)</td>
<td>4.1 ± 0.6 (21)</td>
</tr>
<tr>
<td>V1/2 inactivation</td>
<td>−52.9 ± 5.7 (17)</td>
<td>−52.7 ± 6.7 (16)</td>
<td>−52.0 ± 4.0 (14)</td>
<td>−50.4 ± 5.4 (19)</td>
</tr>
<tr>
<td>Slope factor k</td>
<td>6.6 ± 0.9 (17)</td>
<td>6.2 ± 0.9 (16)</td>
<td>6.4 ± 1.1 (14)</td>
<td>6.2 ± 0.9 (19)</td>
</tr>
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Values are means ± SD; numbers of cells are in parentheses. Cells were dissociated and placed in short-term (18–24 h) culture. No significant differences (ns) were detected in the values across age groups for any of the parameters.

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likely represents channels being driven into a slow-inactivated state (Blair and Bean 2003; Rush et al. 1998). As depicted in the insets in Fig. 3B, the current from the P10–P12 age group consistently had the slowest transitions into this slow-inactivated state across all frequencies.

When assessing the average channel availability over the 20 pulse stimulations, significant differences were noted between age groups. For cells in the first three age groups (comprising cells P1–P9), sodium channels demonstrated an increasing tendency to accumulate in an inactivated state, leading to a progressive reduction in channel availability during all stimulation frequencies (Fig. 3C). This trend was reversed in the oldest age group (P10–P12). As hypothesized, the most mature cells were on average significantly less susceptible to current loss compared with the younger age groups during the repetitive stimulation protocols. These results indicate that the developmental change in sodium channel availability is likely an important factor in the development of mature firing capabilities. Unexpectedly, however, we found that the youngest cells were also capable of maintaining a reasonable amount of available channels by the 20th pulse for all stimulation frequencies.

**Recovery from inactivation**

Having excluded differences in the voltage dependence of channel gating (Table 1), the ability to maintain current during repetitive stimulations is likely a consequence of either an ability of the channels to quickly recover from an inactivated state (Bean et al. 1983) or an ability to resist entering an inactivated state. These two possibilities were investigated further to understand the factors permitting the more mature cells to better follow repetitive stimulations without losing current.

The ability to recover from the inactivated state, or “reprime” (Bean et al. 1983), was assayed using a double-pulse protocol with an increasing interval (Fig. 4A). The data for the recovery process in these cells were best fitted with two exponentials (see legend of Fig. 4 for values), suggesting that even with a short 20-ms stimulation a small percentage (~20%) of channels transition into a slow-inactivated state. As can be seen from the averaged data in Fig. 4B, significant differences between age groups could be seen both in the rate at which the channels recovered and in the completeness of recovery over the 300 ms assayed. Given longer intervals, such as the 1,000-ms interval used in the 1-Hz stimulation protocol (Fig. 3B), channels in all age groups tended to become equally available (~95%). Currents elicited from cells in both the youngest (P1–P3) and oldest (P10–P12) age groups tended to have a greater proportion of channels recovering in the fast phase of recovery and tended to transition into a slow-inactivated state to a lesser degree than the two middle age groups.

These trends within the data became more obvious when we assessed the change in channel availability over various time intervals of the recovery process. A change in the channel availability during the first three time intervals (50, 100, and 150 ms) provided an indication of the rate of recovery while comparing channel availability over the last 100 ms (200–300 ms of the protocol) permitted a comparison of the tendency to remain in a slow-inactivated state. Significant differences between groups could be seen in the data of the first 50-ms interval. This interval encompasses the intervals used in both the 20-Hz (50 ms) and 30-Hz (33 ms) stimulation protocols and mainly reflects the population of channels in a fast gating mode (Morgan et al. 2000) or fast phase of recovery (Bean et al. 1983). Here the P10–P12 age group showed significantly greater recovery than that of cells of the P7–P9 age group. As the interval length was increased (100 and 150 ms), these significant differences in channel availability extended to all the younger age groups. As seen in Fig. 4C, a “U-shaped” pattern emerged over the developmental period spanned in this study, similar to the pattern in the repetitive stimulation data.

A similar pattern was also seen in the data assessing the population of channels in the slow phase of recovery: those channels that tended to remain in a slow-inactivated state (Fig. 4D). Here again the oldest age group (P10–P12) had signifi-
significantly greater channel availability compared with that of the younger age groups ($P < 0.05$, one-way ANOVA on ranks). Again the oldest and youngest age groups were similar and a U-shaped pattern was seen. The similarity between the pattern of the age-dependent changes in frequency-dependent inhibition (Fig. 2C) and both the fast and slow components of the recovery from inactivation (Fig. 4, C and D) suggested that these two parameters were related. This relationship is further explored in the following text.

Correlation analysis

Thus far the data have been arranged into age groups to examine the changes that occur during postnatal development. At this point we sought to examine the underlying factors that allowed cells to maintain available channels during repetitive stimulations. For this we grouped cells from all age groups together because some cells in all the age groups showed a remarkable ability to follow high frequencies even though on
FIG. 4. The ability of sodium channels to recover from inactivation or “reprime” changes with postnatal age. Aa: overlaid current traces demonstrating the response of the sodium current in a P12 cell to the double-pulse voltage protocol illustrated. In this protocol, the interpulse duration is increased by 10 ms during successive sweeps. Ab: the first and second pulses from the current response illustrated in Aa overlayed on an expanded timescale. B: averaged repriming data from the 4 age groups (P1–P3, n = 15; P4–P6, n = 11; P7–P9, n = 13; P10–P12, n = 18). Data are expressed as the current amplitude of pulse 2 (P2) relative the current pulse 1 (P1). Lines are double-exponential functions describing the data. Time constant values are P1–P3: \( \tau_1 = 14.4 \text{ ms}, \tau_2 = 88.5 \text{ ms} \); P4–P6: \( \tau_1 = 13.4 \text{ ms}, \tau_2 = 70.4 \text{ ms} \); P7–P9, \( \tau_1 = 17 \text{ ms}, \tau_2 = 96.2 \text{ ms} \); P10–P12, \( \tau_1 = 12.9 \text{ ms}, \tau_2 = 56.8 \text{ ms} \). In the main graph the mean data points and error bars are removed for clarity. These are shown in the two inset graphs illustrating the changes in the rate of repriming and the completeness of recovery, respectively. C: summary graph illustrating the fraction of channels recovered over 3 increasing intervals from the initial stimulus (50, 100, and 150 ms). Note that the mean values reflecting channel availability during the 3 intervals of recovery for the P10–P12 age group are significantly different from all other groups. Other significant differences between groups are illustrated in the graph (\( P < 0.05 \); ANOVA with Student–Newman–Keuls multiple pairwise comparisons). D: summary graph illustrating the age-related change in channel availability over the recovery interval of 200–300 ms. The P10–P12 age group was again significantly different from all others. Other significant differences are noted with an asterisk (*, \( P < 0.05 \); one-way ANOVA on ranks with Dunn’s multiple pairwise comparisons).
average the cells in the oldest age group were the most proficient at this task. At all frequencies studied, a strong and highly significant correlation existed between the degree of frequency-dependent inhibition (taken to be the amplitude of the 20th pulse divided by the amplitude of the first pulse) and channel availability over the initial 50-ms interval of recovery (Fig. 5A). The data for the 100-ms (0.62 < R < 0.93; P < 0.0001; n = 58 cells) and 150-ms (0.86 < R < 0.91; P < 0.0001; 58 cells) intervals showed similarly strong and highly significant correlations at all frequencies (Pearson product-moment correlation; data not shown).

On a cell-by-cell basis there were also highly significant (P < 0.0001) correlations between the degree of frequency-dependent inhibition and the current recovery over the 200- to 300-ms interval (Fig. 5B). Because this interval is entirely within the slow phase of recovery it represents the ability of channels to either transition from or resist entering a slow-inactivated state. These strong correlations between the ability of a cell to maintain channel availability during repetitive stimulations and the recovery of channels from inactivation provide evidence that the process of channel “repriming” is an important factor in the ability of the spinal motoneuron to fire multiple action potentials. This factor has been suggested to be important for permitting high firing rates in other cell types (Cummins et al. 1998, 2001; Herzog et al. 2003; Spampantano et al. 2001).

Closed-state inactivation

Because sodium channels do not need to actually open to transition into an inactivated state (Tadese and Bean 2002), we assessed the potential contribution of closed-state inactivation to the observed changes during repetitive stimulations. For this assessment the membrane potential was depolarized 20 mV more positive to the holding potential for varying periods of time before a test pulse was used to assess channel availability. The initial depolarization to −50 mV did not cause channel activation (Fig. 6A). Current amplitudes were normalized to the amplitude of the first evoked current.

As shown in the graph in Fig. 6B, the current in all of the age groups quickly (within 200–400 ms) decreased (fast component) and thereafter reached a similar rate of decay (see slope values in legend), likely that of a second slower inactivation process (slow component). Here again obvious age-related differences in channel availability could be seen at any given time point. Interestingly, with respect to closed-state inactivation it was the P4–P6 age group that demonstrated the greatest ability to maintain current under these test conditions. At present it is unclear why the current in this age group was so resistant to this particular transition. Significant differences between the age groups were seen when the average channel availability during the protocol is compared (Fig. 6C). Here the average current resistant to closed-state inactivation in the P4–P6 age group was significantly greater than that of the other three groups (P < 0.05). As well, the current in the P10–P12 group was significantly more resistant than the P7–P9 group (P < 0.05) to closed-state inactivation. With the exception of the P4–P6 data, these data demonstrated a trend similar to that of the repetitive stimulation data. With respect to this biophysical parameter, cells of the P10–P12 age group were very similar to the P1–P3 age group and more resistant than the P7–P9 age group to inactivation.

Correlation analysis

Similar to the variability in the repriming data, the ability to resist closed-state inactivation in some cells was not representative of the average of their age group. Therefore we looked at individual cells to determine whether there was a correlation between the ability to retain current during repetitive stimulations and the closed-state inactivation process (Fig. 7). At 10 Hz the correlation just reached significance (P = 0.049), whereas at 20 and 30 Hz these two parameters showed more significant relationships (P = 0.001 and 0.002, respectively). This strong correlation suggests that a component of the cell’s ability to maintain available channels is also related to the fast component of closed-state inactivation because this fast component determined the relative channel availability for depolar-
Functional changes in motoneuronal sodium currents

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With clear age-related changes in both the closed-to-inactivated state and the inactivated-to-closed-state transitions we assessed whether there were also age-related differences in the open-to-closed-state transitions. The current decay during a voltage step could be fitted with a single-exponential function and was plotted relative to membrane potential (Fig. 8). When comparing the rate of inactivation from the open state of the channel, significant differences between the age groups could not be detected at any voltage examined. There was greater scatter between the means at the more hyperpolarized voltages but, even at a membrane potential of −30 mV, this was not significant (ANOVA on ranks, \( P = 0.37 \)). Furthermore, there was not a significant correlation between the rate of open-state inactivation and the ability to follow repetitive stimulations (10–30 Hz) when assessed on a cell-by-cell basis (\( R < 0.11 \) and \( P > 0.46 \); data not shown).

FIG. 6. The isolated motoneuronal sodium current displays developmental changes in closed-state inactivation. Aa: raw traces from a P11 cell illustrating the current response to the protocol used to gauge the susceptibility of sodium channels to transition from the closed to inactivated state. Cells were depolarized from a holding potential of −70 to −50 mV for varying durations before a test pulse to −10 mV was applied to assess channel availability. Twenty overlaid sweeps are illustrated. Ab: current responses from the 1st and 20th sweep on an expanded timescale. B: averaged data for the 4 age groups as a function of time. Currents were normalized to the initial current amplitude: P1–P3, \( n = 11 \) cells; P4–P6, \( n = 9 \) cells; P7–P9, \( n = 7 \) cells; P10–P12, \( n = 12 \) cells. Mean ± SE. Lines are exponential-linear combination functions describing the data with time constants (\( \tau \)) and slopes (\( m \)) of P1–P3: \( \tau = 109 \) ms, \( m = −0.13/s \); P4–P6: \( \tau = 43 \) ms, \( m = −0.13/s \); P7–P9: \( \tau = 96 \) ms, \( m = −0.15/s \); P10–P12: \( \tau = 93 \) ms, \( m = −0.14/s \). C: summary graph comparing the average channel availability over 2 s (average of data used to construct curves in B) relative to postnatal age. Statistically significant (\( P < 0.05 \)) differences between mean values are indicated (*, ANOVA on ranks with Dunn’s pairwise comparison). Note that the P4–P6 age group was significantly different from all other groups; other significant differences are denoted with an asterisk (*). Error bars are 95% confidence intervals.

FIG. 7. The ability of channels to remain available during repetitive stimulations is correlated with the ability to resist closed-state inactivation. Scatterplot of the depolarization resistant current and the degree of frequency-dependent inactivation (20th pulse/1st pulse). Solid lines are linear regression fits of the data. All correlations were statistically significant at the \( \alpha = 0.05 \) level. At 10 Hz the correlation just reached significance (\( R = 0.34; P = 0.049 \)), but was stronger at 20 Hz (\( R = 0.50; P = 0.002 \)) and 30 Hz (\( R = 0.53; P = 0.001 \)) (\( n = 35 \) cells).

Open-state inactivation

With clear age-related changes in both the closed-to-inactivated state and the inactivated-to-closed-state transitions we assessed whether there were also age-related differences in the open-to-closed-state transitions. The current decay during a voltage step could be fitted with a single-exponential function and was plotted relative to membrane potential (Fig. 8). When comparing the rate of inactivation from the open state of the channel, significant differences between the age groups could not be detected at any voltage examined. There was greater scatter between the means at the more hyperpolarized voltages but, even at a membrane potential of −30 mV, this was not significant (ANOVA on ranks, \( P = 0.37 \)). Furthermore, there was not a significant correlation between the rate of open-state inactivation and the ability to follow repetitive stimulations (10–30 Hz) when assessed on a cell-by-cell basis (\( R < 0.11 \) and \( P > 0.46 \); data not shown).
In the present study we assessed the changes in the biophysical properties of the isolated voltage-gated sodium current in spinal motoneurons over the first 12 postnatal days. We demonstrated that the voltage-gated channels mediating this current undergo a pattern of changes such that the channels expressed in the most mature cells are better able to maintain channels in an available state during repetitive stimulations. This finding is consistent with the hypothesis that a developmental change in the biophysical properties of sodium channels contributes to the well-described developmental increase in firing rate of these cells. A developmental increase in the ability to maintain channels in an available state would work synergistically with the other known developmental changes in these cells, such as the increase in sodium current density (Gao and Ziskind-Conhaim 1998; Garcia et al. 1998; McCobb et al. 1990) and hyperpolarizing conductances (McCobb et al. 1990; Vinay et al. 2000), to enable the mature motoneurons to discharge at high frequencies for extended durations.

The increased ability of the most mature cells to maintain channels in an available state appears to be derived mainly from the ability of the channels to recover quickly from an inactivated state: first, because of the highly significant correlation that was seen between these two parameters and, second, because the developmental pattern of changes in this biophysical parameter paralleled those seen with frequency-dependent inhibition. It is important to note that the developmental change in the ability to follow repetitive stimulations was seen in the absence of an age-related change in the voltage dependence of channel gating because a hyperpolarizing shift in this parameter, relative to the holding voltage, could also result in a greater availability of channels.

The tight association between the ability to maintain available channels during repetitive stimulations and the ability to recover quickly from inactivation is not unexpected. This relationship has been demonstrated in previous studies (Bean et al. 1983; Ilyin et al. 2005) and in fact the retardation of recovery is thought to be an important component of the mechanism of action of many sodium-channel-inhibiting drugs (such as lidocaine, phenytoin, carbamazepine). But the ability to reprime quickly does not necessarily endow channels with the ability to maintain current during repetitive stimulations. For instance, when Na$_{1.2}$ and Na$_{1.6}$ channels were compared after expression in sensory neurons, the Na$_{1.2}$ isoform was found to display faster repriming kinetics than did the Na$_{1.6}$ isoform, even though it was the Na$_{1.6}$-expressing cells that were better able to maintain current during repetitive stimulations (Rush et al. 2005). Additionally, when Zhou and Goldin (2004) coexpressed these same two isoforms with the β1 subunit in Xenopus oocytes they also observed that the Na$_{1.6}$ isoform maintained significantly more current during repetitive stimulations than did the Na$_{1.2}$ isoform. However, under these experimental conditions the Na$_{1.6}$-mediated current was actually potentiated during repetitive stimulations, whereas the Na$_{1.2}$-mediated current showed the typical decline. Clearly this difference could not be due to isoform differences in repriming kinetics and, in fact, was attributed to differences in the activation process that occurred during the repetitive stimulations (Zhou and Goldin 2004).

In the present study the ability of spinal motoneurons to maintain channels in an available state seems to receive a contribution from differences in other state transitions in addition to the transition from the inactivated-to-closed state (i.e., repriming). As demonstrated in Fig. 3B, the channels in the most mature age group consistently had a slower transition rate into a slow-inactivated state compared with the other age groups (see Fig. 3B, insets). This tendency to either resist transitioning into a slow-inactivated state or recover quickly from this state was also evident during the repriming experiments (Fig. 4B).

In these latter experiments the current in the mature cells showed a greater degree of recovery over the last 100 ms of the protocol. Interestingly, the transitions into a slow-inactivated state from the closed state were very similar across the age groups (similar slope values; Fig. 5B) and therefore may represent transitions into a different slow-inactivated state. Finally, a decreased tendency to undergo the closed-to-inactivated-state transition correlated well with the ability to maintain available channels during repetitive stimulations. That is, with fewer channels participating in the fast phase of closed-state inactivation, the more channels were available for a subsequent opening. These findings suggest that differences in these inactivation transitions also contribute to the ability to follow repetitive stimulations without losing appreciable current.

The occurrence of closed-state inactivation during the repetitive stimulation protocols at first may seem unusual, but keep in mind that these cells were assessed from a holding potential of −70 mV. From the inactivation conductance–voltage graph (Fig. 2) this voltage corresponds to roughly 10% of channels being inactivated. Therefore, while holding the cell at this potential, one would expect to see transitions to an inactivated state and an increase in the rate of these same transitions as the membrane potential was pulsed to more depolarized potentials where channel openings occurred (Nuss et al. 2000).

A surprising finding in this study was that the current in the youngest cells was also very proficient at following repetitive stimulations and, with respect to other parameters, behaved in a manner similar to that of the most mature cells. A possible explanation for this will be discussed in the following section.
but this finding suggests that, at least for the very early postnatal cells, the biophysical properties of the voltage-gated sodium current do not limit the maximal firing rates. Other mechanisms such as the low sodium current density (Gao and Ziskind-Conhaim 1998; Garcia et al. 1998; McCobb et al. 1990), or the underdeveloped potassium (McCobb et al. 1990; Vinay et al. 2000), or other conductances (McCobb et al. 1989; Miles et al. 2004; Mynlieff and Beam 1992) likely play a larger role in limiting the maximal firing rate at this stage of development.

Plateau potentials

Mature spinal motoneurons generate plateau potentials (Perrrier and Hounsgaard 2000), quasi-stable potentials approximately 10–20 mV more positive to the resting potential (Hounsgaard and Kiehn 1989; Hounsgaard et al. 1986), capable of eliciting repetitive action potential discharges. In the present study we sought to identify a possible means whereby sodium channels could remain available to discharge multiple action potentials under depolarized conditions that normally promote transitions into an inactivated and therefore unavailable state. To accomplish this we tested the ability of motoneuronal sodium channels to resist transitioning from the closed state to the inactivated state using a voltage protocol that mimicked a sodium channels to resist transitioning from the closed state to an inactivated state using a voltage protocol that mimicked a slow sodium current (see Fig. 6A). In these experiments the most mature cells were better able to resist transitioning into the inactivated state than the P7–P9 age group (but it is unclear as to why the P4–P6 age group was so proficient at this particular task). Regardless, should this trend in closed-state inactivation continue, this biophysical parameter would likely become important for the expression of the mature firing properties of spinal motoneurons. This late-developing resistance to inactivation may contribute to the difficulty of demonstrating plateau potentials in spinal motoneurons of rodents until well into the second postnatal week (Jiang et al. 1999c). Furthermore, postnatal development of the resistance to closed-state inactivation would likely contribute to previous results, suggesting that nifedipine-sensitive plateau potentials play in increasing role in lumbar ventral root output during postnatal development (Jiang et al. 1999b).

Developmental changes in channel subtypes

Previous studies have demonstrated that the complement of voltage-gated sodium channels expressed in the spinal cord in general, and specifically in motoneurons, changes during postnatal development. Channel expression assessed at both protein (Gordon et al. 1987; Schaller and Caldwell 2000) and mRNA levels (Beckh et al. 1989; Black et al. 1994; Felts et al. 1997; Garcia et al. 1998; Schaller and Caldwell 2000) in the rodent spinal cord have demonstrated that around birth the sodium channel subtypes Na1.1, 1.2, 1.3, and 1.6 are all expressed to varying degrees. There is some discrepancy as to the prominent channel subtype between studies, although there is consensus that over the first two postnatal weeks there is a relative change in the expression of these channels. With respect to the ventral horn cells in particular, at birth these cells express detectable levels of Na1.1, Na1.2, and Na1.3 mRNA transcripts (Felts et al. 1997). During the following two weeks of development the levels of Na1.1 mRNA are seen to increase, the levels of Na1.3 decrease, and the levels of Na1.2 are seen to remain somewhat stable (Felts et al. 1997). The expression of Na1.6 mRNA (Felts et al. 1997; Schaller and Caldwell 2000) and protein (Schaller and Caldwell 2000) in motoneurons becomes detectable between P2 and P7 and increases until approximately the third postnatal week. These labeling studies are in good agreement with electrophysiological data obtained from the Na1.6 mutant mouse (med; Garcia et al. 1998). In this study the authors demonstrated that in the absence of the Scn8a gene encoding the Na1.6 channel, the normal postnatal increase in current density of spinal motoneurons failed to occur despite normal current density at P0. These data support the suggestion that the Na1.6 channel isoform becomes the major current contributor in these cells by the second postnatal week.

Heterologous expression and function of channel subtypes

The biophysical properties of most of the mammalian sodium channel α subunits (with or without accompanying β subunits) have been studied in relative isolation after their expression in various heterologous systems. These studies have demonstrated the fundamental properties of the four main sodium channels expressed in spinal motoneurons (Cummins et al. 2001; O’Leary 1998; Smith et al. 1998; Zhou and Goldin 2004). Of those channels that would be expressed during the first days after birth, Na1.1 and Na1.2 channels appear to have very similar properties. When expressed under similar conditions (Xenopus oocytes with the same β subunits), the voltage dependences of activation and inactivation were similar, with Na1.2 having slightly more hyperpolarized values. Both of these channels are capable of quickly recovering from inactivation (fast tau <2.2 ms) and with >80% recovering during the fast time constant (Smith et al. 1998). Again, when compared under similar expression conditions (HEK-293 cells), the voltage dependences of activation and inactivation of Na1.3 channels were found to be within 4 mV of the Na1.2 channels (Cummins et al. 2001; O’Leary 1998). The Na1.3 isoform was found to recover from inactivation threefold slower than the Na1.2 isoform at ~80 mV. On the other hand, at ~60 mV the Na1.3 channels were found to undergo closed-state inactivation more than twofold slower.

The fourth channel isoform expressed in these cells during this developmental period is Na1.6. When Na1.6 channels were coexpressed with the β1 subunit in Xenopus oocytes, these channels were so proficient at remaining in an available state that repetitive stimulations elicited a potentiation of this current (Zhou and Goldin 2004). In the same study the Na1.2 isoform showed a modest decrease in the current. Moreover, when Na1.6 was compared with Na1.2 after expression in dorsal root ganglion cells, the Na1.6 isoform was significantly better at resisting inactivation during repetitive stimulation protocols (Rush et al. 2005).

The most parsimonious explanation for the main finding of this study—the fact that the older cells can retain more current during repetitive stimulations—is a postnatal increase in the expression of Na1.6, a channel subtype that has been demonstrated to be able to retain available channels during this type of stimulation. This finding is supported by studies demonstrating that deletion of the Scn8a gene encoding the Na1.6 channel results in a decreased maximal firing rate of cells that normally...
also changes during early development. Coexpression of these during the first few days of postnatal development, Nav1.1, the channels in the Nav1.6-containing wild-type cells being 2007). These authors recognized that this was consistent with compared with that of wild-type littermates (Enomoto et al. 2001). All three fast repriming Nav1.2 channels contribute a smaller fraction to difficult task in spinal motoneurons due to the large current inactivation quicker.

Changes in $\beta$-subunit expression

The expression of the various accessory $\beta$-subunit isoforms also changes during early development. Coexpression of these $\beta$ subunits has been shown to have a strong influence on various biophysical parameters of the expressed $\alpha$-subunit-mediated currents (Isom et al. 1992). During the embryonic period high levels of the $\beta 3$ mRNA transcript are detected in the rat CNS. After P1 these levels continually decline (Shah et al. 2001). On the other hand, levels of $\beta 1$ transcripts are first detected at approximately P3 and increase until approximately P14, whereas $\beta 2$ mRNA appears at approximately the same time and the levels increase until reaching adulthood (Shah et al. 2001). All three $\alpha$-subunit isoforms that predominate during the first few days of postnatal development, Na$_{\alpha 1.1}$, Na$_{\alpha 1.2}$, and Na$_{\alpha 1.3}$, can all form complexes with $\beta 3$. With respect to Na$_{\alpha 1.2}$ (Morgan et al. 2000) and Na$_{\alpha 1.3}$ (Shah et al. 2001) the addition of this subunit increases the degree of channel availability during recovery from inactivation. Under these conditions young cells may be expected to retain a large fraction of available channels during repetitive stimulations as seen in the present study.

The preceding discussion on the postnatal changes in both the sodium channel $\alpha$ and $\beta$ isoforms, and their expected functional effects on the sodium current in spinal motoneurons, provides a possible explanation for the “U-shaped” developmental pattern observed in this study. During the earliest postnatal period the cells would express a combination of Na$_{\alpha 1.1}$, Na$_{\alpha 1.2}$, and Na$_{\alpha 1.3}$ channels in the presence of $\beta 3$ subunits. This would permit all of these channels to recover from inactivation relatively fast and likely contribute to the ability of the current to resist inactivation during the repetitive stimulations protocol. With further development, while the level of Na$_{\alpha 1.1}$ increases, that of the relatively fast repriming Na$_{\alpha 1.3}$ and $\beta 3$ combination decreases because there is a change from $\beta 3$ to $\beta 1$ and $\beta 2$. Moreover, during this period the fast repriming Na$_{\alpha 1.2}$ channels contribute a smaller fraction to the total current as the overall current density in these cells increases. These changes likely result in an overall decrease in the ability of the cell to retain current during repetitive stimulations. With the increase of the Na$_{\alpha 1.6}$ isoform during the second postnatal week, the ensemble current would again acquire the ability to retain available channels during repetitive stimulations because this $\alpha$-subunit isoform appears to be the most proficient at this task.

Limitations

An important factor in this present work was the ability to maintain voltage control over the sodium current. This is a difficult task in spinal motoneurons due to the large current density in these cells and the fact that these channels are clustered at the initial segment and in the axon, structures spatially remote from the somatic pipette during whole cell recording. In the present work we sought to circumvent these voltage- and space-clamp problems by removing the motoneuronal processes through mechanical isolation of the soma and then culturing these cells in conditions that did not promote process growth (absence of growth factors, uncoated plastic substrate). The cells were exposed to these culture conditions for a relatively short period of time with the goal of replicating the in vivo channel expression as previously described (Garcia et al. 1998). As discussed in the following text, these experimental procedures are both a strength and a possible limitation of the present work.

With the use of these experimental conditions the cells had their initial segment removed and subsequently reexpressed their sodium channels. We believe that the recorded currents are from newly expressed channels for two main reasons: 1) cells patch-clamped immediately after trituration did not demonstrate a sodium current (data not shown); and 2) given the same incubation time, cells of the various age groups demonstrated similar current densities (Table 1). It is not surprising that the cells did not demonstrate a sodium current after mechanically removing the processes because a similar result was seen when the same process-removal effect was accomplished by pulling the intact soma away from the rest of the cell in a transverse slice of spinal cord tissue (Safronov et al. 2000). In this case, the sodium current density in the soma-only situation was reduced dramatically compared with the current recorded from the intact cell. The ability to reexpress the sodium channels in isolated somata has also been demonstrated. The cell bodies of the squid giant axon are normally unexcitable, but if dissociated from the axon and cultured these cell bodies will begin to develop an inward sodium current that begins on the day of isolation and increases in density during the culture period (Brisman and Gilly 1987). Moreover, the vast majority of these newly synthesized channels are biophysically indistinguishable from the “normal” axonal channels (Gilly and Brismar 1989). To definitively determine whether the complement of newly expressed sodium channels in the present study are the same as would be expressed in the in vivo-adequate in vivo cells will require evaluation of either the protein or the mRNA; however, that the sodium current in these cells demonstrated age-related changes in inactivation properties and that these inactivation changes are consistent with the known maturation of firing properties in these cells strongly suggest that the expression of the age-appropriate channels occurred in these cells. Nonetheless, we cannot discount the possibility that an age-related change in some intracellular modulatory factor also plays a role in the observed changes.

In the present study we isolated and examined one small component of the more complex intact spinal motoneuron. We view this culture preparation as a model of an intact in vivo motoneuron and, as with any reduced preparation, there is a trade-off between gaining control in one aspect (in this case voltage control) and the loss of other aspects of the original condition. Our goal was to create an experimental condition that allowed a very accurate study of the sodium current in motoneurons and, in doing so, we may have given up the ability to assess other aspects of motoneuron functioning. Although
we did not attempt to assess action potential firing, in all likelihood the somata examined in the present study would not discharge as motoneurons would in a more intact preparation (e.g., slice, whole cord, or in vivo animal). The motoneurons examined in the present experiments lacked some of the fundamental components of spinal motoneurons such as the dense sodium channel clustering on the initial segment (Alessandri-Haber et al. 1999) and not only the dendritic membrane itself (which can have an effect on firing patterns; Mainen and Sejnowski 1996) but also the dendritic conductances important for plateau potential generation (Carlin et al. 2000; Lee and Heckman 1998). Furthermore, the overall sodium current was many fold lower under the present experimental conditions compared with that recorded from more intact spinal motoneurons (ex. Miles et al. 2005). As such, the degree to which the presently described changes in sodium channel functioning manifest themselves in the behavior of intact motoneurons during development has yet to be fully defined.

Functional consequences

In the present study we have demonstrated that the most mature spinal motoneurons possessed the greatest ability to follow repetitive stimulations due to their ability to maintain channels in a state capable of opening and passing current. This ability stems from age-related changes in various channel transitions but results mainly from an increased ability of the channels to recover quickly from inactivation. The age-related decrease in the tendency of these channels to transition into a slow-inactivated state or undergo closed-state inactivation also contributes to maintaining available channels during repetitive stimulations. The functional consequence of these kinetic changes in the native cells would be amplified by the well-described developmental increase in the sodium current density (Gao and Ziskind-Conhaim 1998; Garcia et al. 1998; McCobb et al. 1999). Therefore not only would these cells have the benefit of a larger pool from which to draw available channels, but these channels would also tend to remain in an available state. It is likely that these two factors greatly impact the ability of these cells to fire repetitively for extended periods of time.

ACKNOWLEDGMENTS

We thank Drs. G. Wu, V. Ilyin, and M. Fry for helpful comments on the manuscript, C. Gibbs for technical assistance, and M. Setterbom for assistance with figure preparation.

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GRANTS

This research was supported by Canadian Institutes for Health Research Grant 37755 to L. M. Jordan and National Institute of Neurological Disorders and Stroke Grant 1IR01 NS-4090301. J. Liu was supported by a scholarship from the National Science and Engineering Research Council of Canada.

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