Development of Ionic Currents Underlying Changes in Action Potential Waveforms in Rat Spinal Motoneurons

BAO-XI GAO AND LEA ZISKIND-CONHAIM
Department of Physiology and Center for Neuroscience, University of Wisconsin Medical School, Madison, Wisconsin 53706

Gao, Bao-Xi and Lea Ziskind-Conhaim. Development of ionic currents underlying changes in action potential waveforms in rat spinal motoneurons. J. Neurophysiol. 80: 3047–3061, 1998. Differentiation of the ionic mechanism underlying changes in action potential properties was investigated in spinal motoneurons of embryonic and postnatal rats using whole cell voltage- and current-clamp recordings. Relatively slow-rising, prolonged, largely Na+-dependent action potentials were recorded in embryonic motoneurons, and afterdepolarizing potentials were elicited in response to prolonged intracellular injections of depolarizing currents. Action potential amplitude, as well as its rates of rise and repolarization significantly increased, and an afterhyperpolarizing potential (AHP) became apparent immediately after birth. Concurrently, repetitive action potential firing was elicited in response to a prolonged current injection. To determine the ionic mechanism underlying these changes, the properties of voltage-gated macroscopic Na+, Ca2+, and K+ currents were examined. Fast-rising Na+ currents (I\textsubscript{Na}) and slow-rising Ca2+ currents (I\textsubscript{Ca}) were expressed early in embryonic development, but only I\textsubscript{Na} was necessary and sufficient to trigger an action potential. I\textsubscript{Na} and I\textsubscript{Ca} densities significantly increased while the time to peak I\textsubscript{Na} and I\textsubscript{Ca} decreased after birth. The postnatal increase in I\textsubscript{Na} resulted in overshooting action potential with significantly faster rate of rise than that recorded before birth. Properties of three types of outward K+ currents were examined: transient type-A current (I\textsubscript{A}), noninactivating delayed rectifier-type current (I\textsubscript{K}), and Ca2+-dependent K+ current (I\textsubscript{K(Ca)}). The two-fold postnatal increase in I\textsubscript{K} and I\textsubscript{K(Ca)} densities resulted in shorter duration action potential and the generation of AHP. Relatively large I\textsubscript{A} was expressed early in neuronal development, but unlike I\textsubscript{K} and I\textsubscript{K(Ca)} its density did not increase after birth. The three types of K+ channels had opposite modulatory actions on action potential firing behavior: I\textsubscript{K} and I\textsubscript{A} increased the firing rate, whereas I\textsubscript{K(Ca)} decreased it. Our findings demonstrated that the developmental changes in action potential waveforms and the onset of repetitive firing were correlated with large increases in the densities of existing voltage-gated ion channels rather than the expression of new channel types.

INTRODUCTION

Neuronal development and synaptogenesis in the rat spinal cord begins during the last week of gestation, and continues throughout the first few weeks after birth (Gao and Ziskind-Conhaim 1995; Gao et al. 1998; Kudo and Yamada 1985, 1987; Saito 1979; Seebach and Ziskind-Conhaim 1994; Takahashi 1992; Walton and Navarrete 1991; Wu et al. 1992; Ziskind-Conhaim 1990; Ziskind-Conhaim et al. 1993). Increased membrane excitability, as evident by changes in action potential waveform, is one of the fundamental characteristics of motoneuron development during embryonic and postnatal ages (Fitzgerald 1987; Huizar et al. 1975; Kellarth et al. 1971; Naka 1964; Walton and Fulton 1986; Ziskind-Conhaim 1988a). Motoneurons are excitable soon after they clustered in the ventral horn (Mandler et al. 1990; Saito 1979; Ziskind-Conhaim 1988a), but action potential properties and firing behavior markedly change during the last week of gestation and immediately after birth (Fulton and Walton 1986; Xie and Ziskind-Conhaim 1995). The increase in motoneuron excitability is functionally important not only for transforming a developmental increase in synaptic transmission (Gao et al. 1998) into an increasingly complex locomotion activity but also for triggering the onset of activity-dependent mechanisms underlying the differentiation of muscle fibers and synaptic transmission at the neuromuscular junction (reviewed by Navarrete and Vrbová 1993).

Maturation of membrane excitability in the vertebrate nervous system is a progressive sequence of events that results in changes in passive and active electrical properties. For example, the development of mammalian motoneurons is associated with a negative shift in action potential threshold, thus increasing the likelihood of initiating action potentials. Concurrently, action potential amplitude increases as its duration decreases, and prominent afterhyperpolarizing potential (AHP) is apparent (Huizar et al. 1975; Kellarth et al. 1971; Núñez-Abades et al. 1993; Viana et al. 1994; Xie and Ziskind-Conhaim 1995; Ziskind-Conhaim 1988a). Such changes represent a general developmental trend, but the pattern and time course of the expression of voltage-gated ion channels underlying these changes vary considerably in different neuronal populations (review by Spitzer 1991). Studies of mammalian and chick spinal neurons have shown that the ionic basis of the action potential is relatively constant during neuronal development, with Na+-dependent action potentials predominant from the onset of membrane excitability (Krieger and Sears 1988; MacDermott and Westbrok 1986; McCobb et al. 1990; Ziskind-Conhaim 1988a). However, in neurons of Xenopus embryos and cortical neurons of chick embryos, the appearance of short-duration action potential is associated with changes in the ionic currents underlying action potential generation. At the onset of excitability, the long-duration action potentials are Ca2+-dependent, and short-duration Na+-dependent action potentials develop shortly afterward (Baccaglini and Spitzer 1977; Blair 1983; Mori-Okamoto et al. 1983; Willard 1980).

Most studies that have characterized developmental changes in motoneuron electrical properties and the currents underlying them, have been conducted using dissociated spinal neurons
of *Xenopus* (Spitzer and Lamborghini 1976; Willard 1980), chicks (McCobb et al. 1990; O'Brien and Fischbach 1986), and mice (Heyer et al. 1981; Krieger and Sears 1988; MacDermott and Westbrook 1986; Randson et al. 1977; Rogawski and Barker 1983). The experimental benefit of cell accessibility in culture might be compromised by the concern that neuronal differentiation in culture differs from that in vivo. An alternative reliable preparation for studying changes in motoneuron membrane properties during spinal cord development in vivo is the spinal cord slice, in which neurons in various lamina of the spinal cord could be identified visually (Bardoni et al. 1997; Gao and Ziskind-Conhaim 1998; Takahashi 1978, 1990). In our study, thick spinal cord slices of embryonic and postnatal rats were used to examine the properties of a repertoire of voltage-gated ionic currents undergoing changes in action potential waveforms.

Motoneurons are generated in the thoracic spinal cord at embryonic day 11–12 (E11–12) (Normes and Das 1974) and begin to cluster in thoracic and lumbar spinal cords by E13–14 (Smith and Hollyday 1983; Ziskind-Conhaim 1988a). Dorsal-root-evoked synaptic potentials are recorded first at E15 (Kudo and Yamada 1987; Ziskind-Conhaim 1990), about the same time that motor axons form initial synaptic contacts with muscle fibers (Dennis et al. 1981). At that age, muscle fibers are multiply innervated and electrically coupled, resulting in widespread muscle contraction in response to single nerve stimulation (Dennis et al. 1981). Although motoneurons and motor axons are excitable at E15 (Ziskind-Conhaim 1988b), the frequency of spontaneous action potentials is low during embryonic development (Saito 1979; Ziskind-Conhaim 1988a), and only one action potential can be produced in response to a prolonged intracellular injection of depolarizing current (Xie and Ziskind-Conhaim 1995). The frequency of spontaneous action potentials significantly increases immediately after birth (postnatal day 1–3, P1–3), and prolonged current injection produces repetitive action potential firing at that age. To increase our understanding of the ionic currents underlying the postnatal increase in motoneuron excitability, we elected to study the correlation between changes in action potential waveforms and voltage-gated ionic currents at E15–16 and P1–3.

In previous studies, action potentials in embryonic and postnatal spinal motoneurons were recorded intracellularly using high-resistance electrodes, and the contribution of ionic currents to action potential waveform was inferred by examining the effects of blockers of voltage-gated ion channels on action potential properties (Walton and Fulton 1986; Ziskind-Conhaim 1988a). In the present study, we used whole cell voltage-clamp recording technique, a more direct experimental approach to determine the properties of a repertoire of voltage-gated ionic currents during a developmental period characterized by marked changes in action potential waveforms and repetitive firing behavior.

Preliminary descriptions of these results have been presented in abstract form (Gao and Ziskind-Conhaim 1993, 1994).

**METHODS**

**Spinal cord preparation**

Lumbar spinal cords were isolated from Sprague-Dawley rat embryos at days 15–16 of gestation (E15–16, birth is at E21–22) and from 1- to 3-day-old postnatal rats (P1–3). The procedures for lumbar spinal cord isolation and preparation of thin spinal cord slices were identical to those described previously (Gao and Ziskind-Conhaim 1995). Before electrophysiological recording, the transverse slices (150 μm) were incubated at room temperature (21–23°C) for 1 h.

Slices were transferred to a recording chamber, which was fixed on the stage of an upright microscope (Nikon Labophot). The submerged slices were perfused with extracellular solution gassed with 95% O₂-5% CO₂ and maintained at room temperature. The extracellular solution contained (in mM) 113 NaCl, 1 Mg-ATP, 0.1 GTP, 10 NaHCO₃, 1 NaH₂PO₄, and 11 glucose (pH 7.3).

**Whole cell recordings**

Motoneurons were identified as the largest cells in the lateral and medial ventral horn (Gao and Ziskind-Conhaim 1995; Takahashi 1978). Motoneurons were visualized at ×400 using Hoffman contrast optics. Recordings were carried out using a whole cell configuration of the patch-clamp recording technique (Hamill et al. 1981). Patch electrodes were pulled from borosilicate glass capillaries using a Flaming-Brown puller (Sutter Instruments). The pipettes had tip diameters of 2–3 μm. The shank and tip of the electrodes were coated with silicone elastomer (Sylgard) to reduce pipette capacitance. Unless otherwise stated, the pipette solution contained (in mM) 140 KCl, 1 Mg-ATP, 0.1 GTP, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, and 0.2 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (pH 7.2). An Axopatch-1D amplifier (Axon Instruments) was used for current and voltage recordings.

After the formation of a giga seal, the pipette resistance and capacitance were compensated electronically. After the rupture of the membrane and the establishment of a whole cell voltage-clamp configuration, 10-mV hyperpolarizing pulses were applied to obtain capacitative transient currents from which the capacitance of the cell membrane and the pipette series resistance were calculated. Series resistance was estimated by measuring the decay time constant of the capacitive current; it ranged from 5 to 10 MΩ. Electronic compensation partially reduced this resistance by 50–80%.

Motoneuron capacitance was assessed from the integral of the capacitative current. This was possible because the average membrane resistances of embryonic (947 MΩ) and postnatal (734 MΩ) motoneurons were >70-fold higher than the series resistance. Membrane capacitances varied considerably, but they significantly lower in embryonic (29.3 ± 1.0 pF, mean ± SE, n = 65) than in postnatal motoneurons (33.2 ± 1.6 pF, n = 68, P < 0.05). It is likely that the average membrane resistance of postnatal motoneurons was overestimated and the membrane capacitance was underestimated, because ~10% of postnatal motoneurons were too large for adequate voltage clamping, and recordings from these neurons were discontinued (see following section).

Leakage currents were eliminated using either on-line subtraction of a series of currents generated by small hyperpolarizing pulses (P-N4 program, pClamp 5.5, Axon Instruments) or by subtracting a linearly scaled current elicited by a 10-mV depolarizing step. Current signals were digitized at 50 kHz, and filtered at 5 kHz.

Recordings were performed only in motoneurons in which fast inward currents were produced in response to depolarizing test potentials. The adequacy of the whole cell clamp recording was tested by examining the waveforms of the fast inward and slow outward currents generated at various test potentials (Fig. 1). Recordings were discontinued in ~10% of postnatal motoneurons that were clamped inadequately as evident by the delayed activation of inward currents or the generation of several peaks of inward currents in response to a single test potential.
that voltage-gated inward currents were produced. The
maximum rates of rise and repolarization, and amplitude of
the AHP. The results are summarized in Table 1.

Motoneurons were excitable at E15, but action potential
waveforms were distinctively different from those produced
after birth (Fig. 2A). Action potential thresholds shifted
toward more negative potentials in postnatal motoneurons
(Table 1), thus increasing the probability of generating an
action potential.

Action potential amplitudes significantly increased after
birth (Fig. 2A, inset), when potentials overshooting 0 mV
were recorded in all motoneurons. In contrast, overshooting
action potentials were generated in only 40% of embryonic
motoneurons. Concurrently, action potential duration de-
clined from a mean of 9.3 ms at E15–16 to 3.4 ms at P1–3
(Fig. 2 and Table 1). The shorter duration resulted from four- to sevenfold increases in action potential maximum
rates of rise and repolarization (Table 1).

During action potential repolarization in embryonic moto-
neurons, the membrane potential remained more positive
than the potential from which the action potential was gener-
at (−70 mV), but an AHP more negative than −70 mV
was apparent in postnatal motoneurons (Fig. 2A). The mean
amplitude of the AHP was 7.5 mV (Table 1), and its duration
was 8.5 ms. Unlike action potential repolarization in spinal
motoneurons of 3- to 10-day-old postnatal rats (Walton and
Fulton 1986), the AHP recorded in our study was not pre-
ceded by afterdepolarizing potential (ADP). Moreover,
AHP waveform consisted of what appeared to be medium-
duration AHP and could not be separated into distinctive
fast- and medium-duration AHPs that characterize neurons
in the sensorimotor cortex and brain stem of neonatal and
mature mammals (Nishimura et al. 1989; Schwиндt et al.

Prolonged (150-ms) intracellular injection of depolarizing
currents produced action potentials followed by large ADPs
in embryonic motoneurons (Fig. 2B, →). Similar current
injections generated repetitive action potential firing with
intermittent AHPs in postnatal motoneurons.

Little is known about the ionic currents underlying the
increase in motoneuron excitability during spinal cord de-
velopment in embryonic and postnatal rats. In a few studies,

TABLE 1. Developmental changes in action potential properties

<table>
<thead>
<tr>
<th>Age</th>
<th>Action Potential Threshold, mV</th>
<th>Action Potential Amplitude, mV</th>
<th>Action Potential Duration, ms</th>
<th>Maximum Rate of Rise, V/S</th>
<th>Maximum Rate of Repolarization, V/S</th>
<th>AHP Amplitude, mV</th>
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<tbody>
<tr>
<td>E15–16</td>
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<tr>
<td>n = 10</td>
<td>−35.3 ± 2.1</td>
<td>69.9 ± 2.8</td>
<td>9.3 ± 1.2</td>
<td>18.3 ± 3.0</td>
<td>8.3 ± 2.0</td>
<td>0</td>
</tr>
<tr>
<td>P1–3</td>
<td></td>
<td></td>
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<tr>
<td>n = 20</td>
<td>−47.3 ± 2.6*</td>
<td>110.6 ± 4.5*</td>
<td>3.4 ± 0.2*</td>
<td>68.8 ± 6.4*</td>
<td>59.4 ± 6.7*</td>
<td>7.5 ± 1.5</td>
</tr>
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Values are means ± SE; n, number of motoneurons. Action potentials were generated during short (30 ms) intracellular injections of depolarizing
currents pulses from a holding potential (HP) of −70 mV. Afterhyperpolarizing potentials (AHPs) were not apparent in embryonic motoneurons.
* Significantly different from E15–16 motoneurons, P < 0.01.
the ionic mechanism was inferred from changes in action potential waveforms in response to blockers of voltage-gated ion channels (Walton and Fulton 1986; Ziskind-Conhaim 1988a). In the present study, a more direct experimental approach was adopted to examine the time course of differentiation and properties of voltage-gated macroscopic Na\(^+\), Ca\(^{2+}\), and K\(^+\) currents that are correlated with increased motoneuron excitability.

**Voltage-gated Na\(^+\) currents**

To isolate inward sodium currents (\(I_{\text{Na}}\)), outward K\(^+\) currents were blocked by replacing KCl in the pipette with isotonic CsCl and adding tetraethylammonium (TEA, 10–20 mM) and 4-aminopyridine (4-AP, 2 mM) to the extracellular solution. \(I_{\text{Na}}\) was separated from inward Ca\(^{2+}\) currents (\(I_{\text{Ca}}\)) by including either CdCl\(_2\) (0.5 mM) or CoCl\(_2\) (5 mM) in a low-Ca\(^{2+}\) extracellular solution (0.5 mM). Ca\(^{2+}\) was not removed from the extracellular solution, because stable recordings could not be sustained in its absence. Fast-rising inward currents were generated during depolarizing test pulses to potentials between −50 and +50 mV from a HP of −70 mV (Fig. 3A). These currents were reversibly blocked by tetrodotoxin (TTX, 1 \(\mu\)M) or by substituting extracellular NaCl with equimolar concentration of Tris [hydroxymethyl] aminomethane hydrochloride (not shown), indicating that they were mediated by activation of voltage-gated Na\(^+\) channels. TTX-resistant Na\(^+\) currents, similar to those recorded in dorsal root ganglion and hippocampal neurons (Akopian et al. 1996; Bossu and Feltz 1984; Hoehn et al. 1993) were not expressed in embryonic and postnatal motoneurons.

Similar \(I_{\text{Na}}\) waveforms were elicited in embryonic and postnatal motoneurons (Fig. 3A), but the threshold potential for \(I_{\text{Na}}\) activation was more negative in postnatal (−50 mV) than in embryonic motoneurons (−40 mV, Fig. 3B). The 10-mV potential change was similar to the 12 mV negative shift in action potential threshold (Table 1), suggesting that the latter was determined primarily by the change in threshold for \(I_{\text{Na}}\) activation. \(I_{\text{Na}}\) significantly increased after birth, which might reflect an increase in the number of Na\(^+\) channels, assuming that single channel conductance and its open probability remained unchanged. Concurrently, the time to peak \(I_{\text{Na}}\) decreased from 0.8 to 0.6 ms, but there was no change in the \(I_{\text{Na}}\) inactivation time constant (−1 ms, Table 2). Similar bell-shaped curves of \(I_{\text{Na}}\) amplitudes as a function of depolarizing test potentials were apparent in both embryonic and postnatal motoneurons, and \(I_{\text{Na}}\) peaked at −10 mV (Fig. 3B).

To determine whether the postnatal increase in \(I_{\text{Na}}\) resulted from a developmental increase in Na\(^+\) channel density, current densities were estimated by dividing peak \(I_{\text{Na}}\) amplitudes (generated at test potential of −10 mV) by motoneuron membrane capacitances. Our data demonstrated that peak \(I_{\text{Na}}\) densities increased threefold after birth, from an average of 38.9 pA/pF at E15–16 to 127.3 pA/pF at P1–3 (Table 2).

**Voltage-gated Ca\(^{2+}\) currents**

To record inward \(I_{\text{Ca}}\), extracellular Ca\(^{2+}\) concentration was elevated to 10 mM. Inward \(I_{\text{Na}}\) was suppressed by TTX (1 \(\mu\)M), and outward \(I_K\) was blocked by replacing KCl in the pipette with CsCl and adding TEA and 4-AP to the extracellular solution (see preceding section). Relatively small, slow-rising inward currents were produced by depolarizing test pulses to potentials between −40 and +50 mV from a HP of −70 mV (Fig. 4). Those currents were blocked by either CdCl\(_2\) (0.5 mM) or CoCl\(_2\) (5 mM), confirming that they were Ca\(^{2+}\)-dependent currents. \(I_{\text{Ca}}\) consisted of two components: relatively fast-inactivating and slow-inactivating currents (Fig. 4A). \(I_{\text{Ca}}\) amplitude was measured as the peak of the transient fast-inactivating current. Similar bell-shaped curves of \(I_{\text{Ca}}\) amplitude versus depolarizing test potentials were apparent in both embryonic and postnatal motoneurons. Throughout this developmental period, the
threshold for $I_{Ca}$ activation was about $-35$ mV, and the current peaked at $+10$ mV (Fig. 4B). Similar to $I_{Na}$, $I_{Ca}$ density significantly increased after birth, and at $+10$ mV, it was threefold larger than in embryonic motoneurons (Table 2). Comparison of $I_{Ca}$ and $I_{Na}$ densities demonstrated that in both embryonic and postnatal motoneurons, peak $I_{Ca}$ densities were fourfold smaller than $I_{Na}$ densities. The mean time to 50% peak $I_{Ca}$ was faster after birth, and at test potential to $+10$ mV, it changed from 1.6 ms in embryonic motoneurons to 0.8 ms in postnatal motoneurons (Table 2). The time constant of inactivation of the transient current did not significantly change after birth, and values of 23.2 and 17.2 ms were measured at E15–16 and P1–3, respectively (Table 2).

At least four functionally and pharmacologically distinct $Ca^{2+}$ currents have been described in neurons: N-, L-, T-, and P/Q-type currents (Bean 1989; Dunlap et al. 1995; Huguenard 1996; Snutch and Reiner 1992). The kinetics of these currents differ so that N-, L-, and P/Q-type currents consist of fast- and slow-inactivating components, whereas T-type $I_{Ca}$ current inactivates relatively fast (McCobb et al. 1989). Based on the relatively large, slow-inactivating $I_{Ca}$ component that was recorded in our study, we hypothesized that $I_{Ca}$ might be composed of N-, L-, and/or P/Q-type $Ca^{2+}$ currents. To determine the contribution of N-type channel to $I_{Ca}$, $I_{Ca}$ was recorded in the presence of $\omega$-conotoxin GVIA (1 $\mu$M, $n = 4$), a strong blocker of N-type $I_{Ca}$. The toxin reduced $I_{Ca}$ amplitude by $\sim70\%$, suggesting that $I_{Ca}$ was mediated primarily by N-type $Ca^{2+}$ channels. To examine the contribution of L-type channel to $I_{Ca}$, $Ca^{2+}$ currents were recorded in the presence of nifedipine (100 $\mu$M, $n = 7$) and verapamil (100 $\mu$M, $n = 7$), blockers of L-type $I_{Ca}$. Nifedipine reduced $I_{Ca}$ by 6–10%, whereas verapamil did not affect it, suggesting that the L-type current did not contribute significantly to $I_{Ca}$ generation. Unlike our finding, verapamil reduced the amplitude of the sustained $I_{Ca}$ by $\sim50\%$ in embryonic chick motoneurons (McCobb et al. 1989).

It is conceivable that the 20% of $I_{Ca}$ that was not suppressed by blockers of N- and L-type $Ca^{2+}$ channels consisted of P/Q-type current (Churchill and MacVicar 1998; Mintz et al. 1992), but its contribution was not tested using

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**TABLE 2.** Peak $Na^{+}$ and $Ca^{2+}$ current densities increased and the time to peak current decreased after birth

<table>
<thead>
<tr>
<th>$I_{Na}$</th>
<th>$I_{Ca}$</th>
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<tr>
<td>Current density, pA/pF</td>
<td>Time to peak, ms</td>
</tr>
<tr>
<td>E15–16</td>
<td>38.9 ± 3.8 (17)</td>
</tr>
<tr>
<td>P1–3</td>
<td>127.3 ± 13.7* (13)</td>
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</table>

Values are means ± SE; number of motoneurons is in parentheses. Peak $Na^{+}$ current ($I_{Na}$) was generated during depolarizing test potential to $-10$ mV (see Fig. 3), and peak $Ca^{2+}$ current ($I_{Ca}$) was elicited during depolarizing test potential to $+10$ mV (see Fig. 4). Currents were generated from a HP of $-70$ mV. * $P < 0.01$. 

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**FIG. 3.** Development of inward $Na^{+}$ currents. A: $Na^{+}$ currents ($I_{Na}$) were generated during a series of depolarizing test pulses to membrane potentials of $-30$ to $+10$ mV (in 10-mV increments), applied from a HP of $-70$ mV. $Na^{+}$ current ($I_{Na}$) rate of rise increased with increasing membrane depolarizations. Currents evoked at P1 were significantly larger than those produced at E15. B: voltage dependence of $I_{Na}$ in embryonic and postnatal motoneurons. Similar bell-shape current-voltage relationships were apparent for $Na^{+}$ currents at E15–16 and P1–3. $Na^{+}$ currents were significantly larger after birth. Threshold for $I_{Na}$ activation was about $-40$ mV at E15–16 and about $-50$ mV at P1–3. $I_{Na}$ peaked at $+10$ mV. Values are means ± SE for $n = 22–24$. 

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and postnatal motoneurons (Fig. 5B). 

\[ I_{\text{Ca}} \text{ was measured as the peak fast-inactivating current (1).} \]

**Voltage-gated K\(^+\) currents**

To record voltage-gated Ca\(^{2+}\)-independent K\(^+\) currents, inward \( I_{\text{Na}} \) and \( I_{\text{Ca}} \) were blocked by including TTX (1 \( \mu \text{M} \)) and CdCl\(_2\) (0.5 mM) or CoCl\(_2\) (5 mM) in a low-Ca\(^{2+}\) extracellular solution (0.5 mM). Outward K\(^+\) currents were produced from a HP of -80 mV by depolarizing test pulses to potentials between -40 and +50 mV. The outward currents consisted of a transient inactivating and a relatively sustained, noninactivating components (Fig. 5A).

**Transient K\(^+\) currents**. Isolation of transient K\(^+\) current was based on previous reports illustrating that this current is partially or completely inactivated at potentials more positive than -50 mV (McCobb et al. 1990; Nerbhone and Gurney 1989; Ribera and Spitzer 1990). Therefore, K\(^+\) currents were first generated from a HP of -80 mV and then from a brief prepulse to -40 mV. Activation of K\(^+\) channels from a potential of -40 mV produced only noninactivating currents (Fig. 5A). Subtraction of the noninactivating currents from total K\(^+\) currents revealed distinct transient currents that were almost completely inactivated during the 60-ms depolarizing pulses. These transient currents were partially blocked by 4-AP (60-90\%, 2 mM), suggesting that they were similar to A-type K\(^+\) currents (\( I_{\text{A}} \)).

Plotting peak \( I_{\text{A}} \) amplitudes as a function of depolarizing test pulses illustrated similar \( I-V \) relationships in embryonic and postnatal motoneurons (Fig. 5B). Relatively large transient K\(^+\) currents were generated in embryonic motoneurons (>1 nA at 0 mV), and their amplitudes did not further increase after birth. It is unlikely that the channel kinetics changed after birth, because the mean time to peak \( I_{\text{A}} \) (2.7-2.9 ms at E15-16 and P1-3, Table 3) and the decay time to 50% of peak current (9.8-13.9 ms) remained relatively unchanged.

**Noninactivating K\(^+\) currents**. Noninactivating K\(^+\) currents were produced by depolarizing test pulses from a HP of -40 mV (Fig. 6A). These currents were substantially reduced by TEA (60-75%, 30 mM), suggesting that they were similar to the delayed rectifier-type K\(^+\) current (\( I_{\text{K}} \)) (Del Negro and Chandler 1997; McCobb et al. 1990). From a HP of -40 mV, the threshold potential for activation of \( I_{\text{K}} \) channel was -10 mV, and it did not change after birth (Fig. 6B). Unlike \( I_{\text{A}} \), \( I_{\text{K}} \) amplitude increased twofold postnatally, and during a depolarizing pulse to 10 mV, for example, mean \( I_{\text{K}} \) amplitude increased from ~0.5 nA in embryonic motoneurons to ~1.0 nA in postnatal motoneurons (n = 14-17, Fig. 6B). Similar increases were apparent in peak \( I_{\text{K}} \) density (Table 3).

**Voltage-gated Ca\(^{2+}\)-dependent K\(^+\) current**. Extracellular Ca\(^{2+}\) is required to trigger Ca\(^{2+}\)-dependent \( I_{\text{K}} \left( I_{\text{K}(\text{Ca})} \right) \), but Ca\(^{2+}\) concentration in the extracellular solution was reduced (0.5 mM) to decrease the amplitude of inward \( I_{\text{Ca}} \), which peaked at about the same time as the outward \( I_{\text{K}(\text{Ca})} \) (Tables 2 and 3). Under these experimental conditions, the amplitude of inward \( I_{\text{Ca}} \) was <80 pA, and it was not subtracted from the outward current. Therefore, it is possible that \( I_{\text{K}(\text{Ca})} \) amplitude was underestimated by <80 pA. \( I_{\text{K}(\text{Ca})} \) was isolated from total K\(^+\) current by first generating a series of K\(^+\) currents from a HP of -80 mV (Fig. 7A, control), followed by the generation of Ca\(^{2+}\)-independent K\(^+\) currents in the presence of CdCl\(_2\) (0.5 mM, n = 14). \( I_{\text{K}(\text{Ca})} \) was measured by subtracting Ca\(^{2+}\)-independent K\(^+\) current from the total current (Fig. 7A).

The threshold for \( I_{\text{K}(\text{Ca})} \) was about -20 mV in embryonic motoneurons, and about -30 mV in postnatal motoneurons (Fig. 7B). Plotting peak \( I_{\text{K}(\text{Ca})} \) amplitude as a function of depolarizing test pulses demonstrated that in embryonic mo-
Large transient A-type K⁺ currents \( (I_A) \) were elicited in embryonic motoneurons, and their amplitude did not increase after birth. A: total K⁺ currents (control, left) were generated in an E16 motoneuron by a series of depolarizing pulses to membrane potential of \(-30\) to \(+50\) mV (in \(20\)-mV increments) applied from a HP of \(-80\) mV. To isolate the transient K⁺ currents from the non-inactivating delayed rectifier-type currents \( (I_K) \), the latter were produced at the same depolarizing pulses that were preceded by a short (35 ms) prepulse to \(-40\) mV (middle). At each depolarizing test potential, \( I_A \) was obtained by subtracting \( I_K \) from total K⁺ current (right). B: similar \( I_A \) voltage dependence was apparent in E15–16 and P1–3 motoneurons. Threshold for \( I_A \) activation was \(-30\) mV. Values are means ± SE for \( n = 12–14 \).

Ionic mechanism underlying action potential generation

Developmental changes in action potential waveforms and the ionic currents underlying them are summarized in Fig. 8. The action potentials and the fast-rising \( I_{Na} \) peaked at about the same time. The relatively slow-rising \( I_{KCa} \) peaked during action potential repolarization, implying that it did not contribute to the action potential rising phase. The sustained \( I_{KCa} \) and the transient \( I_A \) peaked during action potential repolarization but before the onset of AHP. Peak AHP was correlated with maximal \( I_{KCa} \), large \( I_K \), and reduced \( I_A \).

The contribution of inward and outward ionic currents to the action potential waveform and repetitive firing behavior was determined by examining the actions of specific blockers of voltage-gated ion channels on action potential properties.

**TABLE 3.** Densities of the noninactivating K⁺ current and the Ca²⁺-dependent K⁺ current increased after birth

<table>
<thead>
<tr>
<th>Age</th>
<th>( I_K ) Current density, pA/pF</th>
<th>( I_K ) Time to peak, ms</th>
<th>( I_{KCa} ) Current density, pA/pF</th>
<th>( I_{KCa} ) Time to peak, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>E15–16</td>
<td>53.3 ± 9.9 (12)</td>
<td>2.9 ± 0.2</td>
<td>18.7 ± 2.2 (14)</td>
<td>25.0 ± 2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11.6 ± 2.1 (7)</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>P1–3</td>
<td>40.9 ± 6.4 (14)</td>
<td>2.7 ± 0.1</td>
<td>36.9 ± 5.3* (14)</td>
<td>24.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>23.9 ± 4.4* (7)</td>
<td>3.3 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; number of motoneurons is in parentheses. Transient A-type K⁺ current \( (I_A) \), noninactivating delayed rectifier-type K⁺ current \( (I_K) \), and Ca²⁺-dependent K⁺ current \( (I_{KCa}) \) were produced during depolarizing test pulses to membrane potential of \(+10\) mV. * \( P < 0.01 \).
INWARD CURRENTS. Blocking Na\(^+\) channels with TTX (1 \(\mu\)M) reversibly suppressed action potential generation (\(n = 5\)). This finding suggested that during early stages of development of motoneuron excitability, action potential rising phase was dependent primarily on Na\(^+\) influx. Although Ca\(^{2+}\) channels were expressed early in motoneuron development, Ca\(^{2+}\) influx could not functionally substitute for Na\(^+\) influx in triggering an action potential. It is likely that the charge carried by the relatively slow-rising, small Ca\(^{2+}\) influx (Fig. 8) was effectively counterbalanced by the large K\(^+\) efflux. Indeed, only when both Na\(^+\) and K\(^+\) channels were blocked did depolarizing pulses generate slow-rising, prolonged (>20 ms) action potentials that were blocked by CoCl\(_2\) (not shown, but see Ziskind-Conhaim 1988a).

To evaluate the role of I\(_{\text{Ca}}\) in modulating action potential waveforms, Ca\(^{2+}\) channels were blocked by either CdCl\(_2\) (0.5 mM) or CoCl\(_2\) (5 mM). At E16, blocking I\(_{\text{Ca}}\) decreased the action potential duration from a mean of 6.4 to 4.1 ms (\(n = 5\), Fig. 9A, inset), suggesting that Ca\(^{2+}\) influx prolonged action potential duration. Blocking I\(_{\text{Ca}}\) greatly reduced the ADP recorded in embryonic motoneurons (Fig. 9A, →), indicating that the ADP was mediated by I\(_{\text{Ca}}\). After birth, the shorter duration action potential (3.4 ms, \(n = 7\)) was not affected by Ca\(^{2+}\) channel blockers (3.2 ms, \(n = 7\), Fig. 9B), probably because of the development of large K\(^+\) currents (Fig. 8) that played a more prominent role than I\(_{\text{Ca}}\) in regulating action potential duration. The most striking effect of blocking Ca\(^{2+}\) channels in postnatal motoneurons was the twofold decrease in AHP duration, which was associated with an almost twofold increase in action potential firing rate (Fig. 9B). It is likely that this effect was related to the suppression of I\(_{\text{K(Ca)}}\) (see next section).

K\(^+\) OUTWARD CURRENTS. To examine the effect of blocking K\(^+\) currents on action potential generation, CsCl was substituted for KCl in the pipette solution (Fig. 10). Suppressing K\(^+\) efflux significantly increased action potential duration in both embryonic and postnatal motoneurons. At E15–16, mean action potential duration increased fivefold in the presence of CsCl, changing from 9.3 ms (Table 1) to 45.4 ms (\(n = 4\)). After birth the effect of CsCl was markedly larger, and an increase of more than eightfold was apparent: from 3.4 to 29.3 ms (\(n = 7\); Table 1). Blocking K\(^+\) currents eliminated the AHP in postnatal motoneurons and prevented the generation of sustained action potential firing, confirming the importance of K\(^+\) currents in regulating firing behavior.

The specific contribution of I\(_{\text{K(Ca)}}\), I\(_{\text{K}}\), and I\(_{\lambda}\) to action potential waveforms and repetitive firing was studied by selectively blocking those currents. Ca\(^{2+}\)-activated K\(^+\) channels can be separated into two families of high (BK\(_{\text{Ca}}\)) and small (SK\(_{\text{Ca}}\)) conductance channels (reviewed by Sah 1996), which can be selectively blocked by charybdotoxin and apamin, respectively. Charybdotoxin (100 nM, \(n = 6\)) significantly increased action potential duration, shortened AHP duration and decreased its amplitude (Table 4). Similar to the effect of blocking I\(_{\text{Ca}}\), charybdotoxin markedly increased action potential firing rate (Fig. 11). Apamin (100 nM, \(n = 9\)) significantly reduced AHP duration but only slightly affected action potential duration and AHP amplitude. Apamin-induced shorter AHP duration was sufficient to significantly increase the rate of action potential firing. Blocking I\(_{\text{K}}\) with TEA (30 mM, \(n = 7\)) had the most profound effect on prolonging action potential duration and decreasing AHP amplitude (Fig. 11, Table 4), and these changes resulted in a substantially reduced frequency of repetitive action potentials. Blocking the transient I\(_{\lambda}\) with 4-AP (2 mM, \(n = 5\)) significantly increased action potential duration and effectively reduced the frequency of repetitive firing in most motoneurons (4/5) (Fig. 11, Table 4). These findings suggested that blocking I\(_{\text{K(Ca)}}\) had opposite effect on firing behavior than blocking I\(_{\text{K}}\) and I\(_{\lambda}\), and the effects were primarily modulated by reducing AHP duration and increasing action potential duration, respectively.
A possible complication of the whole cell technique is intracellular dialysis from the electrode, resulting in diffusion of important intracellular constituents into the pipette solution (Pusch and Neher 1988). However, continuous recordings for periods >60 min showed no evidence for time-dependent changes in action potential waveform and pattern of repetitive firing, implying that intracellular dialysis affected neither the ionic basis of action potential generation nor motoneuron membrane properties. A previous study comparing membrane properties using whole cell and high-resistance electrode recordings illustrated that intracellular dialysis did not affect membrane resting potential, input resistance, or time constant in adult dentate granule cells (Staley et al. 1992).

Development of motoneuron excitability

The changes in action potential properties and repetitive firing behavior reported in this study are similar to those documented in developing motoneurons using high-resistance intracellular electrodes, but some differences in the absolute values were apparent (Xie and Ziskind-Conhaim 1995; Ziskind-Conhaim 1988a). For example, estimated membrane resistances were 8- to 10-fold larger and action potential amplitudes were >20 mV larger when recorded with whole cell electrodes compared with high-resistance electrodes (Xie and Ziskind-Conhaim 1995, Table 1). These differences, at least in part, could be attributed to the nonspecific leak conductance associated with high-resistance electrodes (Staley et al. 1992). Another
probable factor contributing to these differences is the potential from which action potentials were generated. In this study, action potentials were elicited from a membrane potential of −70 mV, whereas in our previous studies they were generated from variable resting membrane potentials, ranging from −58 mV at E15 to −65 mV at P1–3 (Xie and Ziskind-Conhaim 1995). In embryonic motoneurons, prolonged current injections generated relatively slow-rising Na⁺-dependent action potentials and ADPs, whereas similar currents produced sustained firing in postnatal motoneurons. It is conceivable that the persistent depolarization during action potential repolarization increased the probability of prolonging Na⁺ channel inactivation, therefore decreasing the likelihood of generating more than one action potential. The inability to fire repetitively might be functionally advantageous in limiting the contraction forces generated by developing muscle fibers. During embryonic and early postnatal development, muscle fibers are multiply innervated and electrically coupled (Dennis et al. 1981), and single-nerve stimulation produces wide-spread muscle contraction. The inability to generate sustained action potential firing is correlated functionally with the expression of predominantly slow myosin heavy chains in the developing muscles (Hughes et al. 1993).

Our findings demonstrated that the ionic dependence of the action potential did not change during early ages of motoneuron development. From the onset of motoneuron excitability, action potentials were largely Na⁺ dependent, and the shorter duration action potential and the appearance of AHP were correlated with increased K⁺ currents densities. Similar findings have been reported in other developing neurons including grasshopper interneurons (Goodman and Spitzer 1981), chick ciliary ganglion neurons (Bader et al. 1983), rat sympathetic ganglion cells (Nerbonne and Gurney 1989), and mouse and chick spinal neurons (Krieger and Sears 1988; McCobb et al. 1990). In contrast, developmental changes in action potential properties resulted from changes in the ionic currents in spinal neurons of Xenopus embryos and cortical neurons from chick embryos. In those neurons,
DEVELOPMENT OF MOTONEURON EXCITABILITY

FIG. 9. Effects of blocking $I_{\text{Ca}}$ on action potential waveforms in E16 and P1 motoneurons. A: at E16, a prolonged (150 ms) current injection generated only 1 action potential that overshot the 0 potential and was followed by ADP (1). Action potential duration was reduced and the ADP was eliminated in the presence of $\text{Co}^{2+}$ (5 mM). Threshold potential was slightly more positive in the presence of $\text{Co}^{2+}$ presumably because of the surface charges generated by the high concentration of $\text{Co}^{2+}$. Action potentials were overlapped at their threshold potentials to illustrate that blocking $I_{\text{Ca}}$ reduced action potential duration (inset). B: sustained action potential firing was produced in a P1 motoneuron. Blocking $I_{\text{Ca}}$ by $\text{Cd}^{2+}$ (0.5 mM) did not affect action potential duration (inset), but it decreased AHP duration and increased the rate of action potential repetitive firing.

long-duration Ca$^{2+}$-dependent action potentials were predominant at the onset of membrane excitability, and short-duration Na$^{+}$-dependent potentials developed within a short time afterward (Baccaglini and Spitzer 1977; Blair 1983; Mori-Okamoto et al. 1983; Spitzer and Lamborghini 1976).

Inward currents

Both Na$^{+}$ and Ca$^{2+}$ channels were expressed at early stages of motoneuron development, but only Na$^{+}$ influx was necessary and sufficient for action potential genera-

FIG. 10. Blocking outward K$^{+}$ currents prolonged action potential duration and suppressed the sustained action potential firing in a P2 motoneuron. A: short-duration (30 ms) depolarizing current generated a single action potential, whereas long-duration (150 ms) current injection elicited a sustained burst of action potentials in a P2 motoneuron. B: in a different P2 motoneuron, K$^{+}$ currents were blocked by replacing KCl in the pipette solution with CsCl. Inhibition of outward K$^{+}$ currents substantially prolonged action potential duration and blocked the AHP and action potential repetitive firing.
TABLE 4. Effects of blocking various K⁺ currents on action potential duration and AHP duration and amplitude

<table>
<thead>
<tr>
<th>Blockers of K⁺ Channels</th>
<th>AP duration, ms</th>
<th>AHP duration, ms</th>
<th>AHP amplitude, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charybdotoxin (100 nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 6</td>
<td>2.4 ± 0.1</td>
<td>24.4 ± 3.1</td>
<td>24.7 ± 2.8</td>
</tr>
<tr>
<td>Apamin (100 nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 9</td>
<td>2.6 ± 0.2</td>
<td>25.9 ± 4.2</td>
<td>18.1 ± 2.2</td>
</tr>
<tr>
<td>TEA (30 nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 7</td>
<td>3.2 ± 0.3</td>
<td>40.2 ± 5.5</td>
<td>29.4 ± 3.8</td>
</tr>
<tr>
<td>4-AP (2 mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 5</td>
<td>2.8 ± 0.1</td>
<td>29.9 ± 9.7</td>
<td>18.4 ± 2.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of motoneurons. Repetitive action potentials were generated during a prolonged intracellular injection (150 ms) of depolarizing pulse from a HP of −70 mV. Action potential (AP) duration and afterhyperpolarizing potential (AHP) duration and amplitude were measured in the first AP in each firing train (Fig. 11). The high and small conductance Ca²⁺-dependent K⁺ channels were blocked by charybdotoxin and apamin, respectively. Iₖ and Iₐ were blocked by tetraethylammonium (TEA) and 4-aminopyridine (4-AP), respectively. * Significantly different from control, P < 0.01–0.05. † Significantly different from control, P < 0.003–0.005.

Developmental increases in action potential maximum rate of rise and peak amplitude were attributed to an increase in Iₜ₉ density. This is a developmental trend that has been documented in most differentiating cultured neurons (Bader et al. 1983; MacDermott and Westbrook 1986; McCobb et al. 1990; Willard 1980). In some cases, such as in sympathetic neurons, Iₜ₉ densities as well as the kinetics of Iₜ₉ activation and inactivation...
did not change during the period studied (Nerbonne and Gurney 1989).

Throughout embryonic and early postnatal development, Ca$^{2+}$ currents were relatively small compared with Na$^{+}$ and K$^{-}$ currents. It is unlikely that dominant Ca$^{2+}$ currents were expressed in motoneurons of rodents younger than E15, because such currents could not be detected in spinal neurons of E12 mice (Krieger and Sears 1988) and were apparent in motoneurons of E14 rats only when extracellular Ca$^{2+}$ was elevated (Ziskind-Conhaim 1988a). Restricting Ca$^{2+}$ influx might be related to complex cellular mechanisms regulating the fine balance of transient increase in cytoplasmic Ca$^{2+}$ that promotes neuronal differentiation and synaptogenesis (Fields and Nelson 1993; Gosh et al. 1993; Spitzer 1994) and prevents Ca$^{2+}$-induced neurotoxicity and cell death (Choi 1992).

At the onset of membrane excitability, $I_{\text{Ca}}$ seemed to play an important role in regulating action potential duration and repolarization. Our findings that the ADP was Ca$^{2+}$ dependent confirmed previous observations in spinal and hypoglossal motoneurons of neonatal rats (Umemiyia and Berger 1994; Walton and Fulton 1986). Although $I_{\text{Ca}}$ increased postnatally, it no longer affected action potential duration, probably because of the development of relatively larger counteracting outward K$^{+}$ currents. It is likely that the contribution of Ca$^{2+}$ influx to action potential waveform in postnatal motoneurons was related to the increase in $I_{\text{KA}}$, and the production of predominant AHP (Nishimura et al. 1989; Viana et al. 1993; Walton and Fulton 1986). The functional contribution of $I_{\text{Ca}}$ to various phases of the action potential might be mediated by the activation of distinct populations of voltage-gated Ca$^{2+}$ channels (Umemiyia and Berger 1994).

**Outward currents**

Each of the three types of K$^{+}$ currents examined in this study had a different role in regulating action potential duration, AHP waveform, and the pattern of motoneuron firing. Transient $I_{\text{A}}$ was the largest K$^{+}$ current expressed in embryonic motoneurons, but neither its amplitude nor its time to peak changed after birth. High ratio between $I_{\text{A}}$ and $I_{\text{KA}}$ during early neuronal development also was reported in gyrus granule cells of postnatal rats (Beck et al. 1992) and neurons from quail mesencephalic neural crest (Bader et al. 1985). However, in embryonic chick and Xenopus spinal neurons, the expression of $I_{\text{A}}$ lags behind $I_{\text{K}}$ (Desarmenien et al. 1993; McCobb et al. 1990). Time to peak $I_{\text{A}}$ was similar to the time to peak $I_{\text{Ca}}$, therefore it is reasonable to assume that its early expression contributed to the relatively short-duration action potential ($<10$ ms) in embryonic motoneurons. Although $I_{\text{A}}$ influenced action potential duration after birth, it was probably the postnatal expression of the large noninactivating $I_{\text{K}}$ that resulted in the shortening of action potential duration. The roles of $I_{\text{A}}$ and $I_{\text{K}}$ seemed to be reversed in other immature neurons such as sympathetic neurons, in which the shorter duration action potential was attributed to the increase in $I_{\text{A}}$ (Nerbonne and Gurney 1989).

Repetitive firing behavior is one of the properties that distinctly characterizes various neuronal populations, and it has been suggested that the firing pattern is determined primarily by the balance between persistent inward and outward currents (Schwindt and Crill 1982). In rat spinal motoneurons, repetitive firing began at birth and was correlated temporally with large increases in the expression of $I_{\text{KA}}$ and $I_{\text{KA}}(\text{Ca})$. $I_{\text{K}}$ and $I_{\text{KA}}$ effectively increased the rate of repetitive firing by shortening action potential duration. $I_{\text{KA}}(\text{Ca})$ significantly increased after birth, and both high and small conductance Ca$^{2+}$-dependent K$^{+}$ channels modulated the AHP duration and reduced the frequency of action potential repetitive firing. Blocking these channels increased the firing rate, similar to the elevated frequency that was apparent during the suppression of inward $I_{\text{Ca}}$. The opposite roles of K$^{+}$ currents in regulating the pattern of action potential firing are likely to provide a fine-tuning mechanism for balancing motoneuron output. Opposite modulatory actions of different types of K$^{+}$ channels on neuronal discharge have been reported in other populations of developing neurons. Unlike our findings, in rat mesencephalic trigeminal neurons, reducing $I_{\text{A}}$ increased the probability of generating a sustained firing, whereas blocking $I_{\text{K}}$ eliminated the repetitive firing in most neurons (Del Negro and Chandler 1997). The different roles of K$^{+}$ channels in regulating action potential firing behavior might be attributed to their relative amplitudes and subtle differences in their kinetic properties in various neuronal populations.

In summary, the ionic dependence of action potentials generated in spinal motoneurons of immature rats was studied from the onset of their excitability to the age of establishment of repetitive action potential firing. Our finding suggested that changes in the relative roles of a repertoire of ion channels that already were expressed in embryonic motoneurons accounted for the developmental changes in action potential waveforms and the establishment of distinctive pattern of repetitive firing behavior.

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Address for reprint requests: L. Ziskind-Conhaim, Dept. of Physiology, Medical School, 1300 University Ave., Madison, WI 53706.

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