

# Development of Potassium Conductances in Perinatal Rat Phrenic Motoneurons

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**Martin-Caraballo, Miguel and John J. Greer.** Development of potassium conductances in perinatal rat phrenic motoneurons. *J Neurophysiol* 83: 3497–3508, 2000. Prior to the inception of inspiratory synaptic drive transmission from medullary respiratory centers, rat phrenic motoneurons (PMNs) have action potential and repetitive firing characteristics typical of immature embryonic motoneurons. During the period spanning from when respiratory bulbospinal and segmental afferent synaptic connections are formed at *embryonic day 17 (E17)* through to birth (gestational period is ~21 days), a pronounced transformation of PMN electrophysiological properties occurs. In this study, we test the hypothesis that the elaboration of action potential afterpotentials and the resulting changes in repetitive firing properties are due in large part to developmental changes in PMN potassium conductances. Ionic conductances were measured via whole cell patch recordings using a cervical slice-phrenic nerve preparation isolated from perinatal rats. Voltage- and current-clamp recordings revealed that PMNs expressed outward rectifier ( $I_{KV}$ ) and A-type potassium currents that regulated PMN action potential and repetitive firing properties throughout the perinatal period. There was an age-dependent leftward shift in the activation voltage and a decrease in the time-to-peak of  $I_{KV}$  during the period from *E16* through to birth. The most dramatic change during the perinatal period was the increase in calcium-activated potassium currents after the inception of inspiratory drive transmission at *E17*. Block of the maxi-type calcium-dependent potassium conductance caused a significant increase in action potential duration and a suppression of the fast afterhyperpolarizing potential. Block of the small conductance calcium-dependent potassium channels resulted in a marked suppression of the medium afterhyperpolarizing potential and an increase in the repetitive firing frequency. In conclusion, the increase in calcium-mediated potassium conductances are in large part responsible for the marked transformation in action potential shape and firing properties of PMNs from the time between the inception of fetal respiratory drive transmission and birth.

## INTRODUCTION

Phrenic motoneurons (PMNs) commence functioning prenatally to drive the diaphragm for the generation of fetal breathing movements. Prior to the inception of inspiratory synaptic drive transmission from medullary respiratory centers, rat PMNs have action potential and repetitive firing characteristics typical of immature embryonic motoneurons (Martin-Caraballo and Greer 1999). However, during the period spanning from when respiratory bulbospinal and segmental afferent synaptic connections are formed at *embryonic day 17 (E17)* (Allan and Greer 1997a,b) through to birth (gestational period is ~21 days), a pronounced transformation of PMN electrophysiological properties occurs. The changes include a 50% reduction in action potential duration,

a ~10-mV increase in action potential amplitude, a twofold increase in repetitive firing rates, and the expression of well-developed afterpotentials (Martin-Caraballo and Greer 1999). The underlying changes in ionic conductances responsible for these marked transformations in PMN properties were previously unknown. In this study, we test the hypothesis that the elaboration of action potential afterpotentials and the resulting changes in repetitive firing properties are due in large part to developmental changes in PMN potassium conductances.

Potassium conductances influence the shaping of action potentials, neuronal repetitive firing patterns, and the summation of synaptic inputs in neural cells (reviewed by McLarnor 1995). Several types of potassium channels have been identified based on their electrophysiological and pharmacological properties (Rudy 1988). The most widely distributed potassium channels are the delayed outward rectifier ( $I_{KV}$ ), transient A-type ( $I_A$ ), and calcium-activated potassium ( $I_{KCa}$ ) conductances. Other potassium conductances that have a more limited expression in neuronal systems include the inward rectifier, muscarine-activated, and ATP-sensitive potassium channels. The delayed rectifier and A-type potassium conductances regulate the timing of action potential formation and the repetitive firing pattern of neuronal cells (Dekin and Getting 1987; Spigelman et al. 1992). Calcium-activated potassium conductances are important in generating afterhyperpolarizing potentials that ultimately influence neuronal firing properties. The expression pattern of these various classes of potassium conductances are developmentally regulated (Gao and Ziskind-Conhaim 1998; McCobb et al. 1990; Mienville and Barker 1997; O'Dowd et al. 1988; Spigelman et al. 1992). At very early stages of neuronal development, there are pronounced increases in the expression of delayed rectifier and A-type potassium conductances (McCobb et al. 1990; O'Dowd et al. 1988; Spigelman et al. 1992). In this study, we found those conductances to be well established in PMNs prior to the inception of fetal respiratory synaptic drive and to change little during the latter stages of embryonic development. However, we found that calcium-activated potassium channel expression increased dramatically after the inception of respiratory synaptic drive. Further, the potassium currents associated with these channels underlie many of the changes in PMN action potential characteristics and firing properties observed perinatally. A preliminary account of this work has appeared previously (Martin-Caraballo and Greer 1997, 1998).

## METHODS

Electrophysiological experiments were carried out using a cervical spinal slice-phrenic nerve preparation as previously described (Mar-

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tin-Caraballo and Greer 1999). Embryos (*E16–E18*) were delivered from timed-pregnant Sprague-Dawley rats anesthetized with halothane (1.2–1.5% delivered in 95% O<sub>2</sub>–5% CO<sub>2</sub>) and maintained at 37°C by radiant heat, following procedures approved by the Animal Welfare Committee at the University of Alberta. To determine the timing of pregnancy, the day in which a morning test revealed the appearance of sperm plugs was labeled as *E0*. Fetal age was confirmed by comparing the crown-rump length of the embryos with previously published values by Angulo y González (1932). Newborn rats [*post-natal day 0 to 1 (P0–P1)*] were anesthetized by inhalation of metofane (2–3%). Embryos and newborns were decerebrated, and the brain stem–spinal cord with the phrenic nerve attached was dissected in artificial cerebrospinal fluid (ACSF) at 27 ± 1°C. The spinal segment was cut with a vibratome (Pelco, Redding, CA) into a single slice containing the C<sub>4</sub> segment (*E18, P0–P1*) or C<sub>3</sub>–C<sub>4</sub> segment (*E16*) with the phrenic nerve and the dorsal root ganglia attached (≈750 μm thick). The dorsal roots were then cut to prevent reflex-mediated synaptic activation of PMNs in response to antidromic stimulation of the phrenic nerve used for identification of PMNs. The spinal cord slice was transferred to a silicone elastomer (Sylgard)-coated recording chamber and pinned down (at the dorsal border of the white matter) and continuously perfused with ACSF solution at 27 ± 1°C (perfusion rate 2 ml/s, volume of the chamber 1.5 ml). The slice was left to equilibrate for at least 1 h before recording, and data were typically acquired for up to 5 h after slice preparation.

### Whole cell recordings

Recording electrodes were fabricated from thin wall borosilicate glass (1.5 mm external and 1.12 mm internal diameter purchased from A-M Systems, Everett, WA). The pipette resistances were between 3 and 4 MΩ. To decrease capacitive transients, pipette tips were coated with Sigmacote (Sigma Chemical, St. Louis, MO) and the level of fluid submerging the slice was minimized during recordings. The electrode was advanced with a stepping motor (PMC 100, Newport; Irvine, CA) into the PMN pool located in the medial zone of the ventral horn close to the border between the white and gray matter. To avoid clogging of the recording electrode, positive pressure (≈30 mmHg) was applied while entering the tissue to a depth of at least 100 μm from the slice surface. Pressure was then removed while advancing within the PMN pool. Once the pipette made contact with a cell, negative pressure was used to form a gigaohm (>1 GΩ) cell-pipette seal and gentle suction then applied to rupture the patch membrane. Seal formation and membrane breakthrough were monitored by observing the response to a hyperpolarizing current step (0.3 nA). Whole cell recordings were initially established in the ACSF solution and performed with an AxoClamp 2B amplifier (Axon Instruments; Foster City, CA). The liquid junction potential between the pipette and external solutions was ≈10 mV. The appropriate correction was applied to the reversal potential of the fast and medium duration afterhyperpolarizing potentials (fAHP and mAHP, respectively). Data were filtered at 30 kHz, digitized with an A/D interface, and analyzed with the use of pCLAMP (Axon Instruments) and Origin (Northampton, MA) software.

Seal formation and whole cell recordings were carried out in current-clamp mode. Once the whole cell configuration was established, motoneurons were identified as belonging to the PMN pool by antidromic stimulation of the phrenic nerve via a suction electrode. Rectangular pulses of 0.5 ms duration and 0.5 Hz frequency were delivered with a pulse generator (Master 8, AMPI, Jerusalem, Israel) while pulse amplitude was manually controlled with a stimulus isolation unit (Iso-Flex, AMPI; Jerusalem, Israel). Stimulation amplitudes were ≤0.2 mA. To minimize current spread, the ground wire for antidromic stimulation was wrapped around the tip of the suction electrode. Antidromic action potentials were recorded on tape with a video cassette recorder (Sony, Tokyo) or captured with pCLAMP software for subsequent analysis. The anatomical location of anti-

dromically activated PMNs within the ventromedial zone of the ventral horn (Allan and Greer 1997a) was also confirmed with successful intracellular fills with Lucifer yellow in ≈31% of PMNs analyzed. Neurons not responding to antidromic stimulation were not analyzed. Presumptive glial cells, characterized by having resting membrane potentials more negative than –70 mV and incapable of firing action potentials in response to antidromic or orthodromic stimulation, were also encountered but not analyzed.

Electrophysiological properties of PMNs were studied in *E16* (*n* = 48), *E18* (*n* = 50), and *P0–P1* (*n* = 58) PMNs. Action potentials and firing patterns were recorded at varying holding membrane potentials in the current-clamp configuration. Independent action potentials were evoked by antidromic stimulation of the phrenic nerve or by orthodromic injection of depolarizing step of current (0.5 ms duration). Antidromic or orthodromic action potential duration was measured at half-maximal amplitude. The duration of the afterhyperpolarizing potential (AHP) was measured from the falling phase of the action potential to the point in the mAHP membrane potential trajectory that returned to the holding membrane potential. The mAHP amplitude was measured at the point of maximum hyperpolarized voltage deflection relative to the holding membrane potential. The fAHP amplitude was measured from the resting membrane potential to the negative deflection between the repolarizing phase of the action potential and the following afterdepolarizing potential. Repetitive firing properties were investigated following injection of 1-s-long depolarizing pulses of increasing amplitude. Firing frequency was determined as the number of action potentials per 1-s stimulus at the lowest intensity of current necessary for evoking repetitive firing. The effects of various drugs on firing frequency were studied at the lowest stimulation current (threshold current) necessary to elicit repetitive firing. All data values are presented as means ± SE. The *n* value represents the number of PMNs from which a particular measurement was made. Significant differences between values before and after a pharmacological treatment within a given age were calculated by using paired Student's *t*-test, whereas differences among various age groups were tested using ANOVA (with a Student-Newman-Keuls post hoc test).

On completing the examination of PMN action potential and repetitive firing properties in current-clamp mode, we switched to the single electrode voltage-clamp configuration (discontinuous mode) to record potassium currents. The switching rate was <40 kHz, the output bandwidth was set at 1 kHz, and the clamp gain was ≈0.8 nA/mV. Head stage output was monitored with a separate oscilloscope to monitor the voltage transients prior to sampling. Outward rectifier and A-type potassium conductances were measured in calcium-free solution in the presence of TTX (0.5–1 μM) to eliminate calcium- and sodium-mediated conductances, respectively. For recording outward rectifier potassium currents, a 200-ms depolarizing prepulse (to –40 mV) was applied from a holding potential of –70 mV, whereas a 200-ms hyperpolarizing prepulse (to –110 mV) was applied to maximally activate A-type potassium currents. Leak and capacitive currents were subtracted from the scaled control currents by a P/4 protocol.

The steady-state activation and inactivation of the transient component and the activation of the noninactivating component were plotted as a function of membrane potential. For the inactivation plots, the current at a given membrane potential was normalized to maximal current. For activation, conductances (*G*) were calculated from currents as  $G = I/(V_c - V_r)$  where *V<sub>c</sub>* is the command potential and *V<sub>r</sub>* is the calculated reversal potential for potassium (–97 mV). The plots of normalized current or conductance versus membrane potential were fitted with a Boltzman function in the form  $I/I_{max}$  (or  $G/G_{max}$ ) =  $1/[1 + \exp((V_{1/2} - V)/k)]$ , where *V* is the step potential, *V<sub>1/2</sub>* is the potential at half-maximal normalized value, and *k* characterizes the steepness of the activation or inactivation curves.

### Intracellular and extracellular solutions

ACSF contained (in mM) 128 NaCl, 3 KCl, 0.5 NaHPO<sub>4</sub>, 1.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 23.5 NaHCO<sub>3</sub>, and 30 glucose, pH 7.4 when bubbling with 95% O<sub>2</sub>-5% CO<sub>2</sub>. In the calcium-free solution, calcium ions were replaced by an equimolar concentration of cadmium or cobalt chloride, and NaHPO<sub>4</sub> was removed to avoid precipitation. The standard pipette solution contained (in mM) 130 potassium gluconate, 10 NaCl, 1 CaCl<sub>2</sub>, 10 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), 10 HEPES, 5 Mg ATP, 0.3 NaGTP, pH 7.3 with KOH. A solution containing lower levels of the calcium chelator BAPTA was used when examining PMN afterpotentials to maximize the underlying calcium-mediated potassium conductances. The composition of the pipette solution with a lower calcium buffer capacity was similar to that of the standard solution except for the following changes: BAPTA was decreased to 0.1 mM, calcium was not added, potassium gluconate was 126 mM, and phosphocreatine (10 mM) was added. The pipette solution utilized for blocking potassium conductances contained (in mM) 110 cesium methanesulphonate, 30 tetraethylammonium chloride (TEACl), 10 BAPTA, 10 HEPES, 5 Mg ATP, and 0.3 NaGTP, pH 7.3 with tetraethylammonium hydroxide (TEAOH).

The osmolarity of all the external and internal solutions were ~325 and ~315 mOsm, respectively, as measured with a freezing point osmometer (Advanced Instruments; Needham, MA).

### Drugs

Stock solutions of drugs were prepared as 100–1,000 times concentrates. All drugs were added into the perfusate by switching to reservoirs containing the appropriate test solution. A waiting period of ≥5 min was used to allow for equilibration before data were collected. The following drugs were used: TTX, TEACl, apamin, 4-aminopyridine (4-AP), cytochrome-c (Sigma), iberotoxin, lidocaine-ethyl bromide (QX 314, RBI, Natick, MA); ω-agatoxin (Peninsula Laboratories, Belmont, CA); and ω-conotoxin GVIA (Alomone Laboratories, Jerusalem, Israel). The perfusion medium of the spinal slice with ω-conotoxin GVIA or ω-agatoxin also contained cytochrome-c (0.05%) to avoid nonspecific binding to plastic tubing.

## RESULTS

### Developmental changes in spike properties of phrenic motoneurons

Phrenic motoneurons undergo considerable changes in their passive and active properties during the *E16*–*P1* period (Martin-Caraballo and Greer 1999). As shown in Fig. 1, these include obvious changes in action potential duration, amplitude, and afterpotentials. In *E16* PMNs, the main spike was followed by a slowly decrementing afterdepolarization (ADP) that is transformed to a hump-ADP by birth. The fAHP and mAHP, which were absent at *E16*, developed by birth. As described below, a combination of current- and voltage-clamp experiments in conjunction with pharmacological channel blockers were used to dissect out the role of various potassium conductances during this period of PMN electrophysiological transformation.

### Effects of blocking multiple potassium conductances

The role of potassium conductances in regulating action potential duration was initially studied using TEA and cesium-filled recording electrodes to block multiple potassium conductances. Upon general blockade of potassium conductances, a long-duration spike was evident at all ages studied (Fig. 2A).

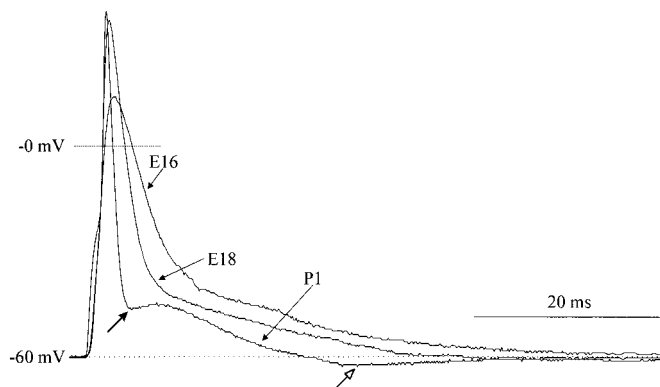


FIG. 1. Developmental changes in the action potential of phrenic motoneurons (PMNs) from embryonic day 16 (*E16*) to birth. Superimposed action potentials at the 3 ages studied: *E16*, *E18*, and postnatal day 1 (*P1*) motoneurons. Antidromic action potentials were elicited following stimulation of the phrenic nerve and recorded with a low 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA; 0.1 mM)-intracellular pipette solution. Filled arrow points to the fast duration afterhyperpolarization (fAHP), whereas the empty arrow indicates the medium duration AHP (mAHP).

The conductances underlying the long-duration component were examined by selectively blocking sodium and calcium conductances with intracellular QX 314 (1 mM) or following incubation in calcium-free medium, respectively. As shown in Fig. 2B (*left panel*), stimulation with 10 consecutive depolarizing pulses resulted in the disappearance of the fast, sodium-dependent spike due to the use-dependent blockade of sodium channels with QX 314 (Connors and Prince 1982). The long-duration component, which remained after sodium current blockade, was attenuated following incubation in calcium-free medium (Fig. 2B, *right panel*). The duration of the calcium-dependent long-duration component decreased dramatically with age. The times for the plateau to attenuate to half-maximal amplitude were as follows: *E16* = 320 ± 45 ms (mean ± SE, *n* = 8); *E18* = 126 ± 23 ms (*n* = 6, *P* ≤ 0.05 vs. *E16*); *P0* = 41 ± 10 ms (*n* = 7, *P* ≤ 0.05 vs. *E16*).

### Effects of blocking potassium currents with tetraethylammonium (TEA)

The maturation of outward potassium conductances play an important role in shaping the action potential waveform and repetitive firing patterns in several differentiating neuronal systems (McCobb et al. 1990; Spigelman et al. 1992). Externally applied TEA (10 mM) was used to block outward potassium conductances to evaluate their role in the spike repolarization of PMNs. At all ages tested, TEA significantly increased the action potential duration (Table 1). However, the effect of TEA on prolonging the action potential decreased significantly with age (Table 1). This is consistent with the reduction of action potential duration with age found after inhibition of all potassium conductances with intracellular TEA and Cs<sup>+</sup> (see above). The effect of TEA on the regulation of the repetitive firing of PMNs was more difficult to quantify since motoneurons became very excitable. In *E16* and *E18* PMNs, TEA treatment evoked complex spikes, such as plateau potentials, whereas in neonatal PMNs, TEA application increased the firing frequency by ~28% (11 ± 1 vs. 15 ± 1 Hz, *n* = 4; Fig. 3).

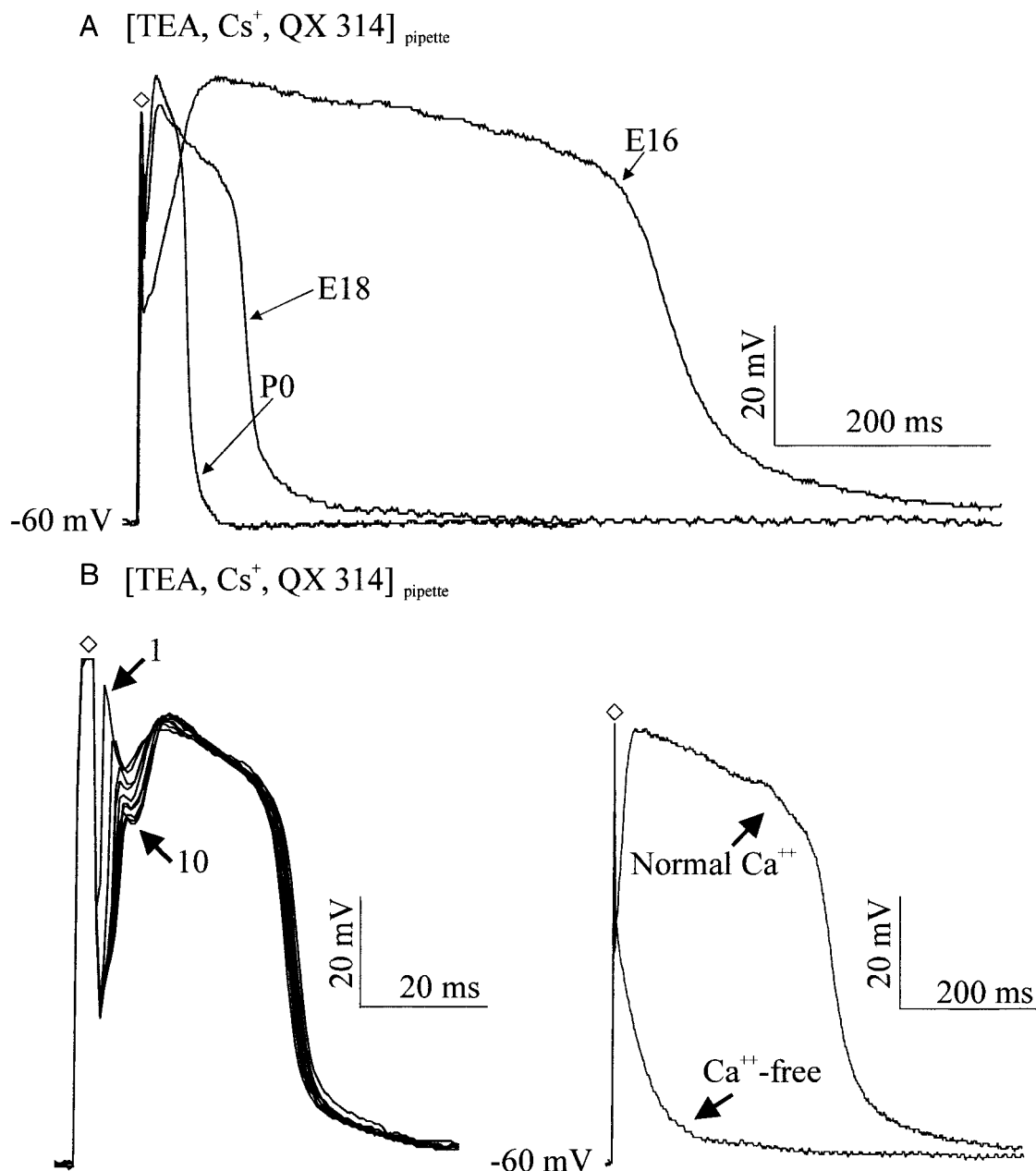


FIG. 2. *A*: block of multiple of potassium conductances revealed plateau potentials in PMNs that varied in duration depending on age. Plateau potentials were generated following intracellular current injection through the recording pipette filled with a solution containing QX 314 (1 mM), TEA<sup>+</sup> (110 mM), and Cs<sup>+</sup> (30 mM) to block sodium- and potassium-mediated conductances. The diamond in this and following figures represents the stimulation artifact following 0.5-ms current injection. The duration of the calcium-dependent spike decreased with age. *B*: recordings illustrating that plateau potentials are calcium dependent and insensitive to sodium channel blockade. Recordings were from *P0* (left) and *E16* (right) PMNs with a pipette solution containing TEA<sup>+</sup> (110 mM), Cs<sup>+</sup> (30 mM), and QX 314 (1 mM). *Left panel*: 10 consecutive spikes (arrow 1–10) and the QX 314 use-dependent inhibition of the fast sodium transient (arrow) with no effect on the long-duration component. *Right panel*: the plateau was eliminated following incubation in calcium-free medium.

#### Effects of blocking A-type potassium conductances with 4-AP

The effects of TEA on the action potential duration were less pronounced at hyperpolarized holding potentials (Table 1). Thus we tested whether this was due to the presence of an A-type potassium current that has a transient character and is inactivated at depolarized potentials (McCobb et al. 1990; Spigelman et al. 1992). Bath application of the A-type current inhibitor 4-AP (1–3 mM) prolonged the duration of the spike without significantly changing action potential amplitude at all

ages tested (Fig. 4A, Table 1). Following 4-AP treatment, the action potential duration tended to increase with age; however, this increase was not statistically significant (Table 1). Further, the prolongation of action potential duration by 4-AP was enhanced when action potentials were elicited from hyperpolarized holding potentials (Fig. 4A).

In addition to its contribution to action potential repolarization, the 4-AP-sensitive potassium conductance was involved in regulating the firing behavior of PMN at all ages studied.

TABLE 1. Change in PMN AP duration following block of potassium conductances with TEA and 4-AP

	$V_h$ , mV*	Normalized AP Duration, %†		
		E16	E18	P0
TEA (10 mM)	-50	375 ± 75‡ (3)	ND	ND
	-60	217 ± 29‡ (3)	190 ± 20‡ (5)	132 ± 8‡,§ (4)
	-75	190 ± 10‡ (3)	ND	ND
4-AP (3 mM)	-60	349 ± 84‡ (5)	471 ± 170‡ (3)	633 ± 159‡ (3)

Values are means ± SE; number of cells is in parentheses. PMN, phrenic motoneuron; AP, action potential; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; ND, no data available. \* Membrane holding potential. † AP duration at half-maximal amplitude was expressed as a percentage of the control response without any treatment. ‡  $P \leq 0.05$  vs. control (no treatment, 100%). §  $P \leq 0.05$  vs. E16.

When PMNs fired from hyperpolarized holding potentials, a delayed excitation was observed (Fig. 4B). The delay excitation was defined as a lag between the onset of depolarization and the onset of firing. Delayed excitations were observed in 47% of E16 (8 of 17), 54% of E18 (13 of 24), and 21% (4 of 19) of neonatal PMNs. The ramplike depolarization characteristic of delayed excitation was blocked by 4-AP ( $\geq 1$  mM; Fig. 4B) and insensitive to TTX (0.5–1  $\mu$ M,  $n = 6$ ; not shown) or incubation in calcium-free medium ( $n = 6$ ; not shown). Despite the removal of the delayed excitation by 4-AP, the firing frequency of PMNs was reduced. This is likely explained by the concomitant increase in action potential duration caused by 4-AP (Fig. 4B).

Role of calcium-activated potassium conductances in controlling firing properties and afterpotential formation

Previous results indicate that calcium ions contribute to shape the action potential in developing PMNs (Martin-Carballo and Greer 1999). In particular, incubation in calcium-free medium eliminates the mAHP in E18 and neonatal motoneurons and results in the widening of action potentials postnatally. Thus we investigated whether these calcium-dependent effects were mediated by calcium-activated potassium conductances.

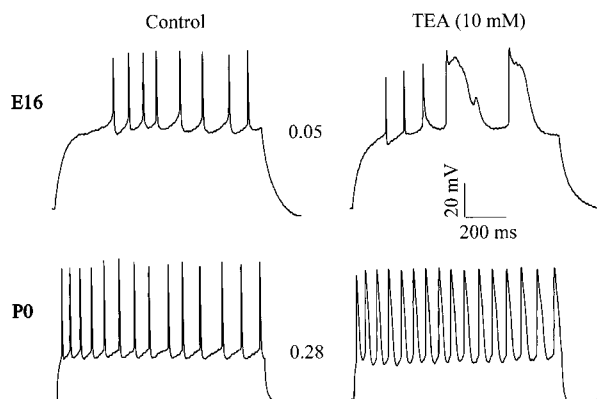


FIG. 3. Effect of externally applied TEA (10 mM) on the firing pattern of PMNs. Repetitive firing was evoked following intracellular current injection for 1 s. The amount of applied current, in this and following figures, is stated between the representative traces (0.05 and 0.28 nA). Holding potential is -60 mV.

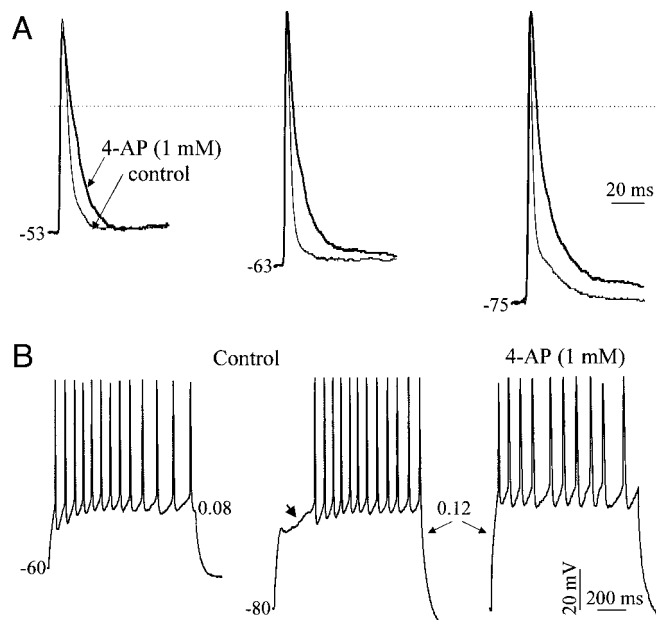


FIG. 4. Effect of 4-aminopyridine (4-AP, 1 mM) on the action potential duration and firing pattern of one representative E18 PMN. A: 4-AP prolongs the duration of the action potential, and this effect was enhanced at hyperpolarized holding potentials (-75 mV). Dashed line indicates 0 mV. B: hyperpolarizing holding potentials generated a delayed excitation (short arrow in middle panel). Treatment with 4-AP (1 mM) prevented the expression of the delayed excitation (right panel). Firing of PMNs was determined in the current-clamp configuration following injection of 1-s-long depolarizing pulses at various holding potentials. Delayed excitation was present at all ages tested.

To investigate the role of calcium-activated potassium conductances in the regulation of repetitive firing properties, we tested the effect of calcium-free medium (Figs. 5 and 6). The diminution of calcium conductances had little effect on the repetitive firing properties of PMNs at E16. However, the removal of calcium from the medium had pronounced effects on the steady-state firing frequency of PMNs at E18 and P0. When measured at the threshold current required to elicit repetitive firing, the change in steady-state frequency in control versus calcium-free medium were as follows: E16, 10 ± 1 versus 11 ± 2 ( $n = 4$ ); E18, 8 ± 1 versus 19 ± 1 ( $n = 7$ ,  $P \leq 0.05$  vs. control); P0–P1, 15 ± 1 versus 21 ± 1 ( $n = 6$ ,  $P \leq 0.05$  vs. control). Further, the spike-frequency adaptation, which starts to develop by E18 and becomes quite pronounced by P0, is reduced when calcium-activated potassium conductances are diminished (Fig. 6, bottom panel).

ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF THE mAHP. The expression of a mAHP in PMNs is first observed in a minority of E18 motoneurons (Martin-Carballo and Greer 1999). By birth, 80% (16 of 20) of PMNs express a clear mAHP (Fig. 7A). Thus we restricted our analyses of the pharmacological and electrophysiological properties of mAHPs to neonatal PMNs.

The amplitude of the mAHP was voltage dependent, increasing and decreasing at depolarizing and hyperpolarizing holding potentials, respectively (Fig. 7A). The estimated reversal potential of the mAHP was  $-98 \pm 5$  mV ( $n = 8$ ), close to the calculated reversal potential for potassium ions ( $-97$  mV). The duration of the mAHP was not significantly different at holding

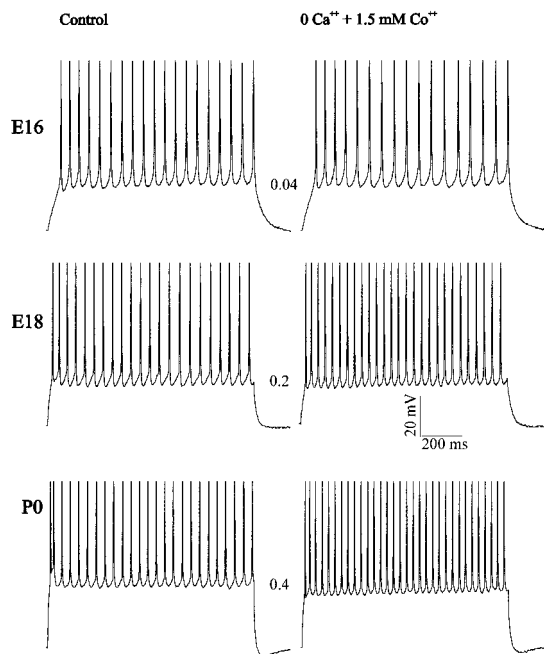


FIG. 5. Effect of calcium-free medium on the repetitive firing properties of PMNs. Repetitive firing of PMNs was determined in the current-clamp configuration following injection of 1-s-long depolarizing pulses and recorded with a low-BAPTA pipette solution. Incubation in calcium-free medium increased the firing frequency of PMNs at *E18* and *P0*, but not at *E16* (*E16*,  $n = 4$ ; *E18*,  $n = 7$ ; *P0–P1*,  $n = 6$ ). Amount of injected current is shown between traces.

potentials of  $-50$  ( $52 \pm 7$  ms),  $-60$  ( $50 \pm 10$  ms), and  $-75$  mV ( $46 \pm 8$  ms;  $n = 8$ ).

As previously reported, the mAHP is sensitive to incubation in calcium-free medium (Martin-Caraballo and Greer 1999). As shown in Fig. 7*B*, the mAHP was blocked by the N-type calcium channel blocker  $\omega$ -conotoxin GVIA (also see Table 2). Inhibition of the mAHP by  $\omega$ -conotoxin GVIA induced a 35% increase in the firing frequency of neonatal motoneurons (Table 2). Inhibition of L-, P-, and T-type calcium conductances with nimodipine ( $10 \mu\text{M}$ ,  $n = 3$ ),  $\omega$ -agatoxin ( $500$  nM,  $n = 3$ ), and nickel ( $200 \mu\text{M}$ ,  $n = 3$ ), respectively, had no effect on PMN mAHPs (not shown). Further experiments demonstrated that the mAHP was blocked by apamin ( $1 \mu\text{M}$ ), an inhibitor of the small-conductance calcium-activated potassium channels (Fig. 7*C*, Table 2). Collectively, these data demonstrate that the mAHP is generated as a result of the entry of calcium via N-type calcium channels followed by the activation of small-conductance calcium-dependent potassium channels. Functionally, the blockade of mAHPs with apamin increased PMN firing rate by 68% (Fig. 9*A*, Table 2).

**ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF THE fAHP.** A fast AHP was not observed in *E16* or *E18* PMNs. However, by *P0–P1*, 29% (8 of 29) of PMNs had a clear fast AHP (Fig. 8*A*). The amplitude of the fAHP was enhanced by hyperpolarizing holding potentials. Further, the membrane potential reached by the peak fAHP varied with the holding potential (Fig. 8*A*). The amplitude of the fAHP, as a

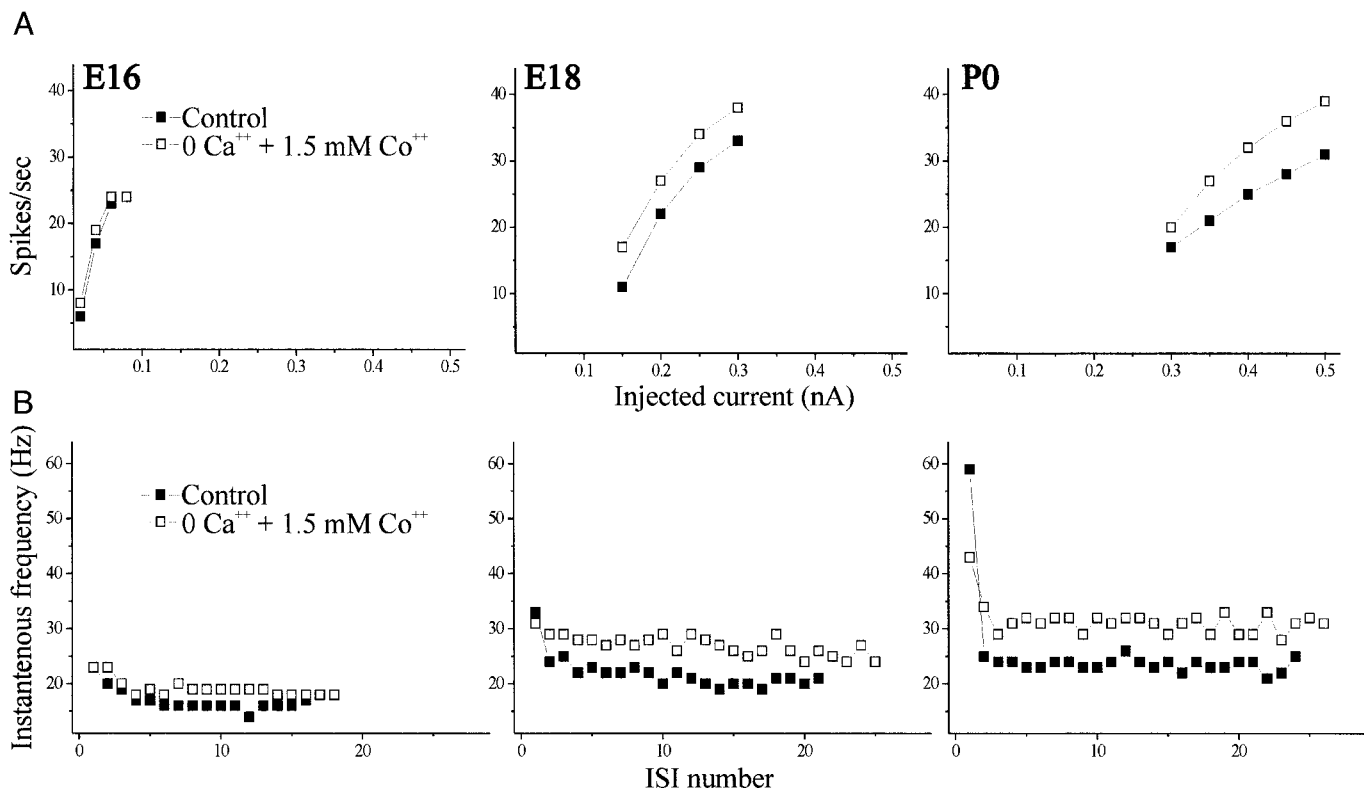


FIG. 6. Plots of the firing frequency of PMNs as a function of injected current or interspike interval number. *A*: frequency-current plots of PMNs in control and following incubation in calcium-free medium. The y-axis represents the number of spikes generated following injection of a constant amount of current for 1 s. *B*: plots of instantaneous firing frequency [1/interspike interval (ISI) duration] vs. the ISI number. Spike frequency adaptation was more pronounced in *E18* and *P0* motoneurons and was diminished in calcium-free medium. Data used to generate plots in *A* and *B* are from the same PMNs represented in Fig. 6.

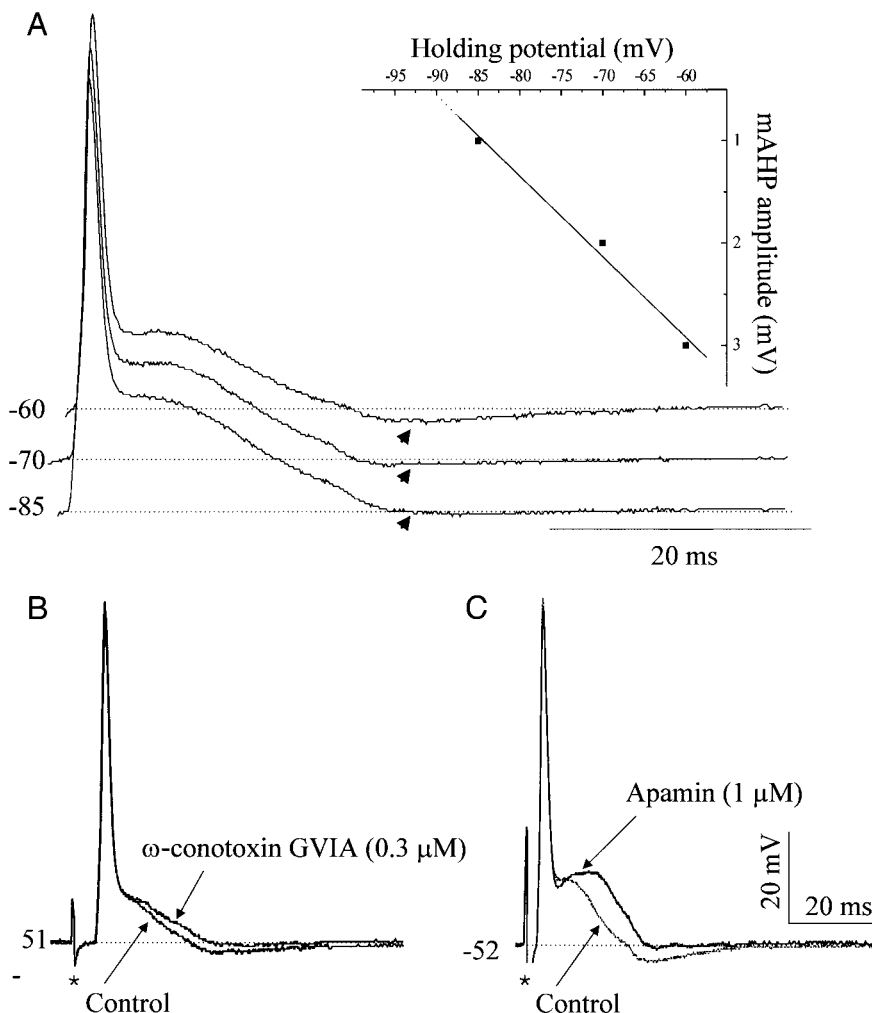


FIG. 7. Electrophysiological and pharmacological properties of the medium-duration AHP in a P0 PMN. A: voltage dependence of the mAHP amplitude. Note that the mAHP was enhanced by depolarizing holding potentials (arrowhead). *Insert*: plot of the mAHP amplitude vs. holding potential. Extrapolation of the linear regression to the y-axis revealed the reversal potential of the mAHP. In this particular neuron the reversal potential was -91 mV. B: the blocker of N-type calcium channels, ω-conotoxin GVIA (0.3 μM) inhibited the expression of the mAHP and increased the afterdepolarizing potential. C: the blocker of calcium-activated potassium channels (small conductance), apamin (1 μM) eliminated the mAHP and enhanced the afterdepolarizing potential. In this and subsequent figures, antidromic action potentials were elicited following stimulation of the phrenic nerve and recorded with a low BAPTA (0.1 mM)-intracellular pipette solution. Artifact due to antidromic stimulus is indicated by an asterisk in this and following figures.

function of voltage, was fitted by a linear regression, and the reversal potential of the fAHP was estimated by extrapolation to be  $-50 \pm 2$  mV ( $n = 8$ ; Fig. 8B).

Potassium channel blockers were applied to determine the ionic conductances underlying the generation of the fAHP in neonatal PMNs. As shown by the data regarding mAHP characteristics, blockade of the small-conductance calcium-activated potassium conductance with apamin did not block the fAHP (Fig. 7C). The potential roles of other major calcium-activated potassium currents, the maxi-type, were

assessed by two methods. First, TEA at doses (1 mM) sufficient for blocking the maxi-type calcium-activated potassium channel yet low enough not to cause significant block of the delayed rectifier, was applied (Viana et al. 1993). The addition of 1 mM TEA resulted in the disappearance of the fAHP ( $n = 3$ , Fig. 8C) and an increase in the duration of the action potential by ~50% ( $1.9 \pm 0.2$  vs.  $3.7 \pm 0.4$  ms,  $n = 3$ ) without any significant change in action potential amplitude ( $79.7 \pm 1.4$  vs.  $79.7 \pm 1.2$  mV). Second, the selective blocker of maxi-type calcium-acti-

TABLE 2. Modulation of neonatal PMN AP and firing properties following blockade of calcium-activated potassium conductances

	n	Control				Treatment				
		AP amplitude, mV	AP duration, ms*	mAHP amplitude, mV	Firing frequency, Hz	AP amplitude, mV	AP duration, ms	mAHP amplitude, mV	Firing frequency, Hz	Change in firing frequency, %
Apamin (1 μM)	7	77 ± 3	2.2 ± 0.2	2.2 ± 0.2	15.8 ± 1.1	74 ± 3	2.2 ± 0.2	0.2 ± 0.1†	26.2 ± 3.0†	+68 ± 29†
ω-Conotoxin GVIA (0.3 μM)	4	73 ± 4	2.3 ± 0.2	1.8 ± 0.3	15.7 ± 1.0	71 ± 3	2.4 ± 0.2	0.1 ± 0.1†	20.0 ± 1.8	+35 ± 10†
Iberotoxin (0.3 μM)	6	80 ± 4	1.8 ± 0.1	2.6 ± 0.6	14.7 ± 1.1	77 ± 3	2.2 ± 0.1†	2.6 ± 0.7	15.7 ± 1.0	+11 ± 4†

Values are means ± SE; n is number of cells. mAHP, medium duration afterhyperpolarization; for other abbreviations, see Table 1. \* AP duration was calculated at half-maximal amplitude. † P ≤ 0.05 vs. control (no treatment).

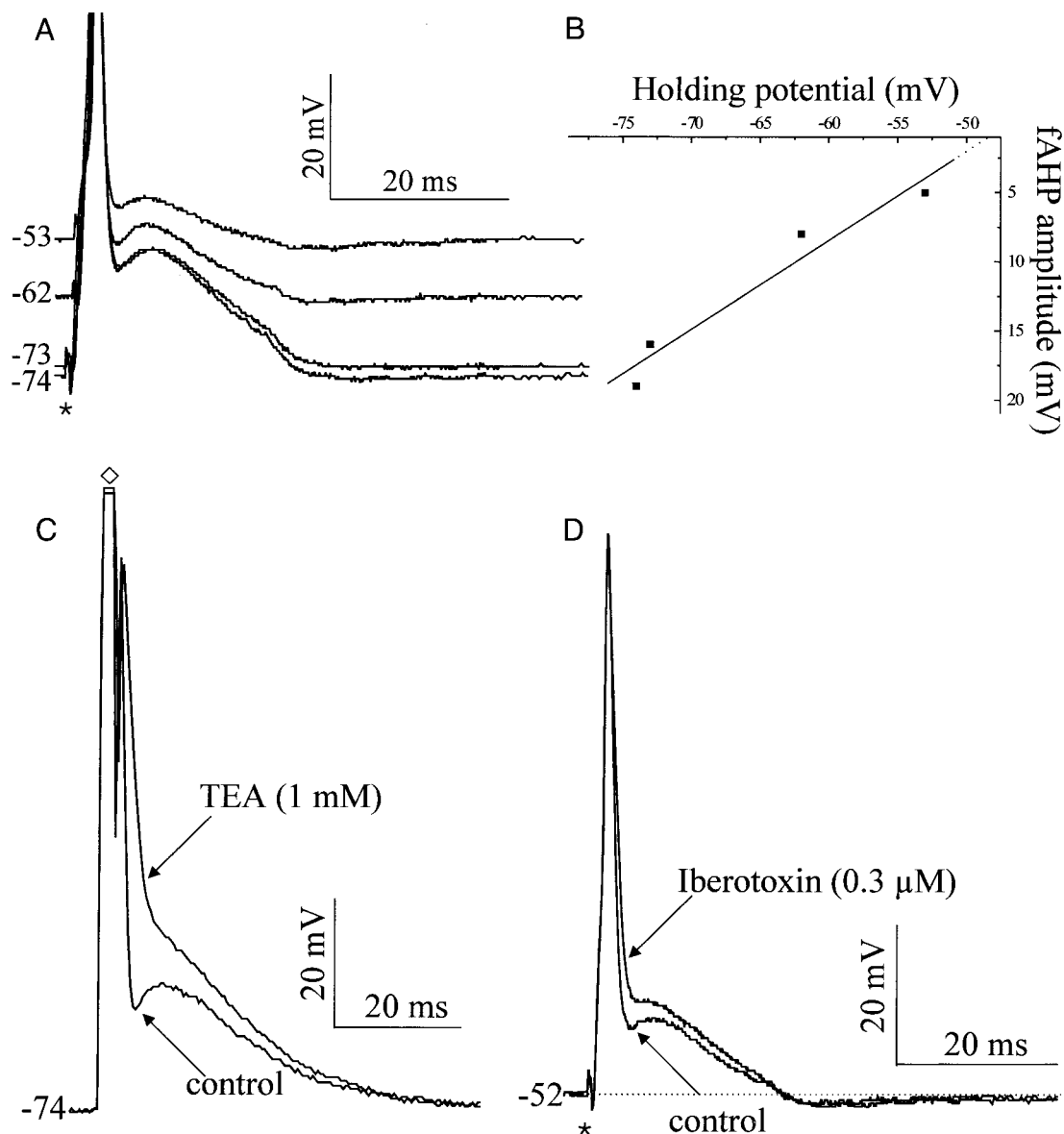


FIG. 8. Electrophysiological and pharmacological properties of the fast AHP in a P0 PMN. *A*: the amplitude of the fAHP was voltage dependent as hyperpolarizing potentials tended to enhance the fAHP. *B*: plot of the fAHP amplitude versus holding potential. Extrapolation of the linear regression to the y-axis revealed the reversal potential of the fAHP, which in this particular neuron was  $-48$  mV. *C*: TEA (1 mM) prolonged the action potential duration and prevented the expression of the fAHP. *D*: the blocker of calcium-activated potassium channels (maxi-type), iberotoxin ( $0.3 \mu\text{M}$ ), eliminated the expression of the fAHP while increasing action potential duration. The mAHP, which was enhanced by biasing the resting potential to a depolarized level, was not effected by iberotoxin.

vated potassium channels, iberotoxin ( $0.3 \mu\text{M}$ ), was added to the bath (Galvez et al. 1990). The effects of iberotoxin were similar to that of low concentrations of TEA: both prolonged the duration of action potentials and eliminated the fAHP (Fig. 8*D*, Table 2). Thus these data suggest that maxi-type calcium-activated potassium channels are involved in the generation of the fAHP in neonatal PMNs. Functionally, despite increasing action potential duration, the blockade of the maxi-type calcium-activated potassium conductance by iberotoxin resulted in only a slight increase in the firing frequency of neonatal PMNs (Fig. 9*B*, Table 2). It should be noted that we were not successful in elucidating the calcium current subtype responsible for activating the

maxi-type calcium-activated potassium conductance. There was no significant effect on fAHP amplitude or duration with the addition of  $\omega$ -conotoxin GVIA (Fig. 7*B*, Table 2), nimodipine ( $10 \mu\text{M}$ ,  $n = 3$ ),  $\omega$ -agatoxin ( $500 \text{ nM}$ ,  $n = 3$ ) or nickel ( $200 \mu\text{M}$ ,  $n = 3$ ; data not shown).

#### Voltage-clamp analysis of voltage-dependent potassium currents

To further characterize potassium-mediated conductances in perinatal PMNs, recordings were made in voltage-clamp mode. Voltage-clamp experiments revealed that by *E16* outward potassium currents expressed two voltage-dependent compo-

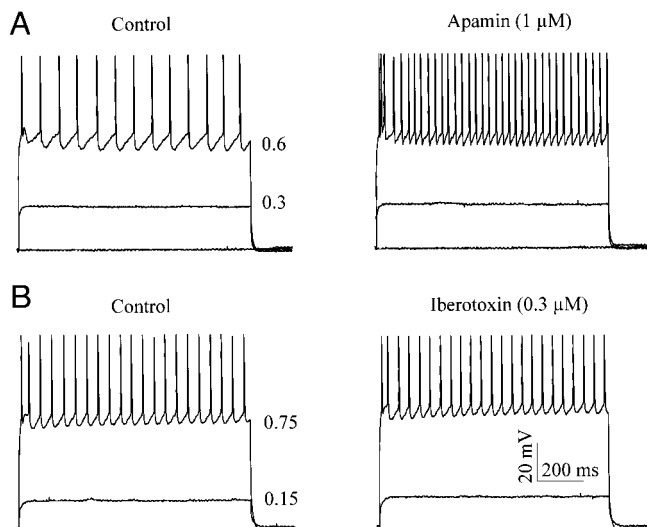


FIG. 9. Effect of calcium-activated potassium conductances in regulating the firing pattern of 2 representative *P0* PMNs. *A*: apamin treatment greatly increased the firing frequency of a *P0* motoneuron without any significant change in the input resistance of the PMN. *B*: iberotoxin ( $0.3 \mu\text{M}$ ) slightly increased the firing frequency of PMNs. Change in input resistance was determined from the voltage response to a depolarizing current injection (0.15 or 0.3 nA), subthreshold for repetitive firing. Action potential amplitudes have been truncated.

nents: a transient, fast inactivating and a noninactivating conductance (Fig. 10*A*). Based on previous results in other developing neurons (McCobb et al. 1990; Spigelman et al. 1992), the noninactivating and transient components of potassium currents were classified as being the delayed outward rectifier ( $I_{KV}$ ) and A-type ( $I_A$ ) potassium conductances, respectively. The transient component was revealed when potassium currents were recorded following a hyperpolarizing prepulse (to  $-110$  mV for 200 ms; Fig. 10*Aa*). Holding the membrane potential at more depolarized potentials (at  $-40$  mV for 200 ms) resulted in the inactivation of the transient component (Fig. 10*Ab*) and the generation of a noninactivating potassium conductance. The absolute value of the transient component was isolated by subtraction of the noninactivating current generated by a depolarizing prepulse from total current generated by the hyperpolarizing prepulse (Fig. 10, *Aa* and *Ab*). Activation and inactivation of the transient components were plotted as a function of voltage and fitted to a Boltzman equation (Fig. 10*C*).

It was evident from the kinetics of the current recordings that there were space-clamp problems that effected the accuracy of current measurements. These problems were particularly pronounced in neonatal PMNs that have elaborate dendritic trees and increased soma diameter relative to embryonic PMNs (Allan and Greer 1997b). For instance, the slow time-to-peak ( $19.0 \pm 2.6$  ms;  $n = 16$ ) and the more depolarized activation  $V_{1/2}$  value ( $-5 \pm 3$  mV;  $n = 13$ ) of the  $I_A$  conductance at *P0* compared with data from other motoneurons (McCobb et al. 1990; Safronov and Vogel 1995) likely resulted from space-clamp problems. Thus these limitations precluded us from systematically quantifying age-dependent changes in the density or kinetics of potassium-mediated conductances. Further, we were unable to reliably record calcium-activated potassium currents in *P0–P1* PMNs. However, there was a striking change in the time-to-peak for the noninactivating component

that could not be attributed to an artifact of the space-clamp problem. Between *E16* and *P0–P1*, there was an  $\sim 55\%$  decrease in the time-to-peak ( $133 \pm 26$ ,  $n = 23$  vs.  $57 \pm 21$  ms,  $n = 26$ ) and an  $\sim 8$ -mV leftward shift in the voltage for half-maximal activation ( $V_{1/2}$ ,  $19 \pm 1$ ,  $n = 10$ ,  $P \leq 0.05$ , vs.  $11 \pm 2$  mV,  $n = 11$ ) without any significant change in the steepness of the activation curve ( $k$ ,  $14 \pm 1$  vs.  $15 \pm 1$  mV; Fig. 11). If anything, these age-dependent differences in  $I_{KV}$  kinetics would have been underestimated due to space-clamp problems.

At all ages tested, the  $I_{KV}$  and  $I_A$  had similar pharmacological sensitivities. The transient A-type potassium conductance was completely eliminated following incubation with 3 mM of 4-AP without significant changes in the outward rectifier potassium conductance (not shown). Treatment of the noninactivating steady current with 10 mM TEA greatly diminished the expression of  $I_{KV}$  in PMNs (not shown).

## DISCUSSION

The present findings demonstrate that the outward rectifier and A-type potassium currents are involved in regulating PMN action potential and repetitive firing properties throughout the perinatal period spanning from *E16* through to *P0–P1*. The most profound change in potassium conductances during this period resulted from the marked increase in calcium-activated potassium channel expression following the inception of inspiratory drive transmission. The emergence of these conductances appear to be in large part responsible for the marked transformation in action potential shape and firing properties of PMNs prior to birth.

### Calcium-dependent potassium conductances

PMN action potential shape and duration were not affected by calcium-dependent potassium conductances until after the inception of inspiratory drive transmission (i.e., post *E17*). This was despite the fact that there was a substantial calcium conductance during the action potential in *E16* PMNs. Thus there is likely an age-dependent increase in the expression of calcium-activated potassium channels in PMNs during the perinatal period.

**MAXI-TYPE CALCIUM-DEPENDENT CURRENT.** Block of the maxi-type calcium-dependent potassium conductance with iberotoxin caused a significant increase in action potential duration in neonatal PMNs, implicating a calcium-dependent potassium conductance in spike repolarization. A similar conductance has been implicated in spike repolarization in neonatal lumbar and hypoglossal motoneurons (Takahashi 1990; Viana et al. 1993) and the shaping of the locomotion pattern in *Xenopus* tadpoles (Sun and Dale 1998). Functionally, besides decreasing action potential duration, the presence of calcium-activated potassium conductances will prevent the large accumulation of calcium ions during alternating bursts of activity such as respiration and locomotion.

The maxi-type calcium-dependent potassium conductance also regulates the expression of the fAHP observed in a subpopulation of neonatal PMNs. The fAHP was found in the transition between the repolarizing phase of the action potential and the afterdepolarizing potential of PMNs. The nature of fAHP generated in PMNs differed somewhat from that re-

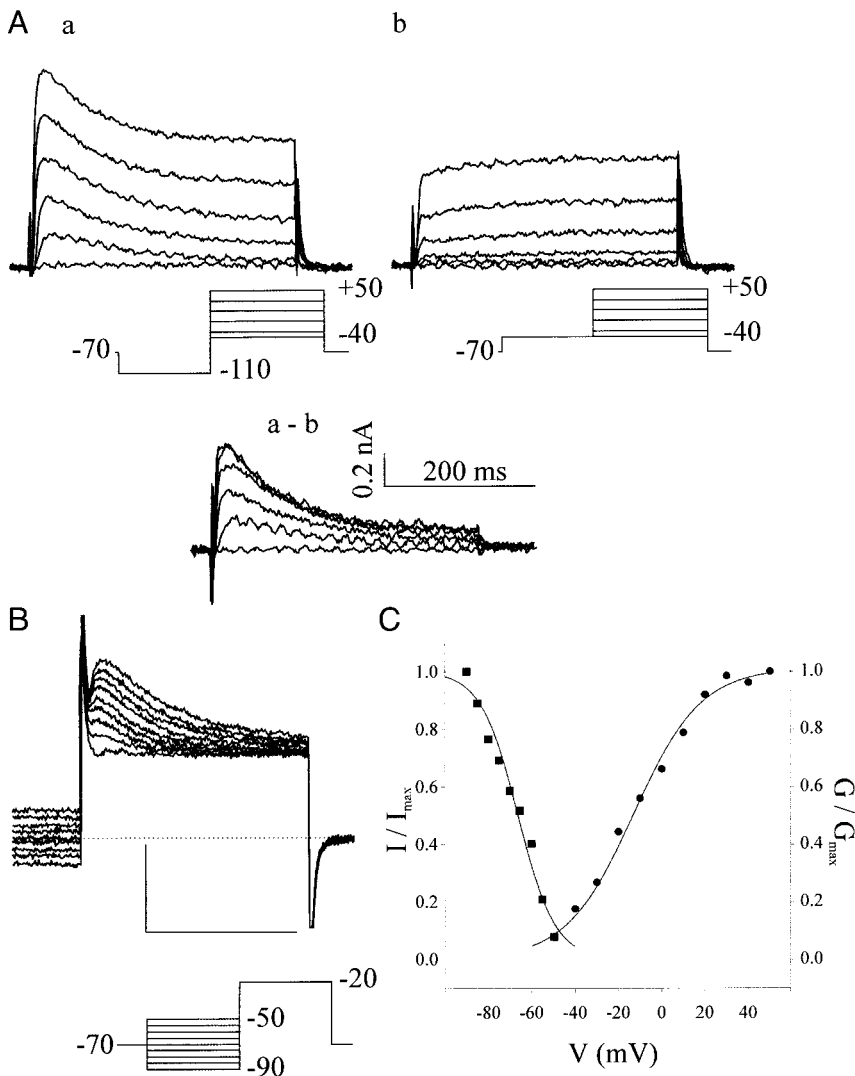


FIG. 10. Voltage-clamp recordings of potassium currents in a *E16* PMN. *Aa*: potassium currents generated following a hyperpolarizing prepulse potential to  $-110$  mV. Note the expression of a transient component (with fast activation and inactivation kinetics). *Ab*: potassium currents generated following a depolarizing prepulse potential to  $-40$  mV. Note the presence of a steady component only. *Aa* and *Ab*: subtraction of the potassium currents generated with the 2 previous protocols revealed the transient component alone. *B*: steady-state inactivation of the transient component is voltage dependent. Potassium currents were generated by a fixed depolarizing pulse to  $-20$  mV preceded by hyperpolarizing prepulses to various levels (from  $-90$  to  $-50$  mV). *C*: plots of the steady-state activation and inactivation of the transient component as a function of membrane potential. The activation and inactivation values of the transient potassium currents were determined from data shown in *Aa*, *Ab*, and *B* and fitted with a Boltzman function.

ported for some other neurons. First, the reversal potential of the fAHP found in PMNs appears to be more depolarized than that in hypoglossal and sensory neurons (Schwindt et al. 1988;

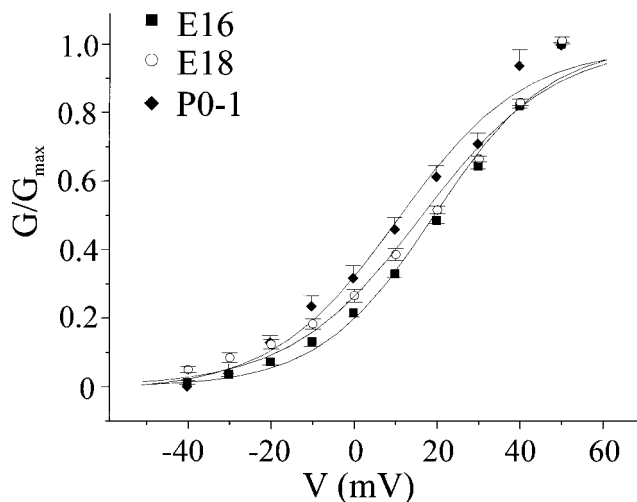


FIG. 11. Developmental changes in the activation of the  $I_{KV}$  as a function of voltage. Steady-state potassium conductances were determined following the protocol in Fig. 10*Ab* and fitted with a Boltzman function (see METHODS).

Viana et al. 1993). Second, the membrane potential reached by the peak of the fAHP in PMNs changed with the amount of current injected into the cell to hold the membrane at various holding potentials. This was not the case in cat sensory neurons (Schwindt et al. 1988). Thus there may be a mixed cationic conductance responsible for the fAHP in PMNs that differed from previously examined neurons.

**SMALL-CONDUCTANCE CALCIUM-DEPENDENT CURRENT.** Block of the small-conductance calcium-dependent potassium channels in PMNs with apamin did not have any effect on spike repolarization. However, there was a marked suppression of the mAHP and an increase in the repetitive firing frequency of PMNs. The activation of small-conductance potassium conductances has also been implicated in spike frequency adaptation in other mammalian motoneurons (Gao and Ziskind-Conhaim 1998; Schwindt and Crill 1981; Viana et al. 1993; Walton and Fulton 1986). A detailed study of the ontogeny of calcium conductances in PMNs is currently under investigation and will provide information regarding the interaction between calcium and potassium conductances. However, the data from the current study demonstrates that the calcium ions responsible for generating the mAHP in PMNs entered via  $\omega$ -conotoxin GVIA-sensitive calcium channels, suggesting that the small-

conductance calcium-activated potassium channels are localized near the N-type calcium channels in PMNs. This is similar to the findings from studies of hypoglossal motoneurons (Vianna et al. 1993). However, given that the ADP was not blocked by  $\omega$ -conotoxin GVIA, it is likely that the calcium influx necessary for generating the ADP and mAHP are separate.

#### *Outward rectifier and A-type potassium conductances*

Transient, 4-AP-sensitive  $I_A$  and slowly activating, noninactivating TEA-sensitive  $I_{KV}$  conductances are functional in PMNs as early as *E16* and continue to be expressed into the postnatal period. The actions of  $I_{KV}$  and  $I_A$  for regulating action potential duration and repetitive firing properties of PMNs were examined. Blockade of all potassium conductances with intracellular TEA and  $Cs^+$  or externally applied TEA resulted in a prolongation of action potential duration, the prolonged influx of calcium, and the generation of plateau potentials that were particularly pronounced in embryonic PMNs. This is likely due to the significant role of calcium entry in shaping the action potential in embryonic PMNs. We observed an age-dependent leftward shift in the activation voltage and a decrease in the time-to-peak of  $I_{KV}$  similar to those found for *Xenopus* spinal neurons (Burger and Ribera 1996; Gurantz et al. 1996). The leftward shift in the activation of  $I_{KV}$  combined with the fact that the action potential in neonatal PMNs can reach a larger overshooting potential than in embryonic neurons, will result in a faster repolarization of the action potential as delayed rectifier potassium channels activate faster and at lower voltages (Martin-Caraballo and Greer 1999). Repetitive firing properties were affected by a delayed excitation mediated by a  $I_A$  conductance. The degree to which the  $I_A$ -mediated delayed excitation influences PMN firing during the inspiratory phase of the respiratory cycle would vary depending on the magnitude of the membrane hyperpolarization during the preceding expiratory phase. Thus although  $I_A$  conductances are expressed in PMNs throughout the perinatal period, the  $I_A$ -mediated effects on firing will change as a function of inhibitory synaptic drive development (Gao and Ziskind-Conhaim 1995; Singer et al. 1998; Wu et al. 1992) and changes in resting membrane potential [becomes more hyperpolarized with age (Liu and Feldman 1992; Martin-Caraballo and Greer 1999)].

**FUNCTIONAL SIGNIFICANCE.** Collectively, the changes in potassium conductances result in a reduction in PMN action potential duration, resulting in increases in the rate and range of repetitive firing frequencies attainable at birth. There are also parallel changes in the contractile properties of the diaphragmatic musculature (Martin-Caraballo et al. 2000). Prenatally, the kinetics of muscle twitches are slow, and thus fused tetanic contractions are achieved at a relatively low frequency. Diaphragmatic muscle twitch contractions are considerably faster and the tetanic frequency higher by birth. The net result of the concomitant age-dependent changes in PMN and diaphragmatic muscle properties is that the full-range of potential diaphragm force recruitment can be utilized and problems associated with diaphragm fatigue are minimized.

There is also an increased complexity to PMN action potential shape as a result of changes in potassium-activated conductances. A significant component of state-dependent regula-

tion of neuronal excitation by neuromodulators is mediated by modifications of ionic channel function and the subsequent alterations in action potential and repetitive firing properties (for review see Jones and Kaczmarek 1996; Levitan 1999). Thus with the development of an increased palette of ionic conductances in PMNs, access to potential modulatory control mechanisms is readily available to meet the varied demands of breathing after birth.

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