A-Current Expression is Regulated by Activity but not by Target Tissues in Developing Lumbar Motoneurons of the Chick Embryo

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INTRODUCTION

Neuronal populations can be distinguished by their morphological, physiological, and biochemical characteristics. The intrinsic electrophysiological properties of neurons represent an especially important phenotypic trait, because they control the resting level of excitability, the transmission of impulses, and the integration of synaptic inputs. These intrinsic properties are determined by the functional expression of a specific ensemble of ionic channels.

The mature complement of neuronal ion channels is not present at the stage of terminal mitosis. Rather, channel expression is a dynamic and developmentally regulated process. The developmental expression of some types of channels proceeds according to an internal developmental program (Dourado and Dryer 1992; Spitzer and Lamborghini 1976), whereas expression of others appears to require neurotrophic factors and cell-cell interactions can regulate developmental expression of $I_A$ (Dourado and Dryer 1994; Dourado et al. 1994; McFarlane and Cooper 1993; Raucher and Dryer 1994). Therefore we have readdressed the issue of $I_A$ development in LMNs to determine cellular processes that regulate these channels. We have confirmed the greater than threefold increase in $I_A$ density between E6 and E11 initially reported by McCobb et al. (1990). In addition, we show that $I_A$ expression and gating properties are regulated by ongoing electrical activity in ovo and in vitro. More surprisingly, the expression, kinetics, and voltage dependence of $I_A$ in LMNs do not appear to be regulated by interactions with target tissues.

METHODS

Motoneuron labeling and dissociation

Labeling, dissociation, and culture of chick LMNs were performed as described previously (Martin-Caraballo and Dryer 2002a,b). Briefly, LMNs were retrogradely labeled in ovo with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). DiI was injected into thigh and foreleg muscles 1–3 days before spinal cord dissociation. The ventral horns were excised into a Ca$^{2+}$- and Mg$^{2+}$-free solution, mildly trypsinized at 37°C (at E6, 0.1% trypsin for 15 min; at E11, 0.2% trypsin for 15 min), and transferred to a cell culture medium consisting of Eagle’s minimal essential medium (BioWhit-taker), 10% heat-inactivated horse serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The ventral horns were dissociated by trituration and plated onto poly-D-lysine–coated glass coverslips. Expression of $I_A$ in acutely dissociated LMNs was examined <4 h after spinal cord dissociation by whole cell recording. For experiments involving spinal cord-muscle co-cultures, E11 hindlimb muscles were removed and placed in a 0.5-mg/ml solution of type II collagenase for 15 min. After dissociation and plating, myotubes were incubated for 2 days before the addition of dissociated E6 LMNs.

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LMN-muscle co-cultures were maintained for 5 days in the presence or absence of 1 μM TTX.

**Spinal cord slice preparation**

Spinal cords were removed while immersed in a cold oxygenated sucrose saline consisting of (mM) 110 sucrose, 60 NaCl, 3.0 KCl, 1.25 NaH₂PO₄, 28 NaHCO₃, 7 MgCl₂, 0.5 CaCl₂, 5.0 dextrose, and 0.6 ascorbate. They were placed on a preamed 1-in block of 1% agarose and covered with a 7% solution of ultra-low gelling temperature agarose (Sigma). The tissue was cooled for 5 min to allow the agarose to harden, and 300- to 500-μm slices of the lumbar region of the spinal cord were made using a vibratome. Iₐ expression was measured from LMNs 2–3 h after slice preparation.

**Whole cell recordings**

Dissociated LMNs were identified during patch-clamp recordings using an Olympus IX70 inverted stage microscope equipped with epifluorescent optics and rhodamine filters. Recordings were made from Dil-containing cells of diameter ≥14 (at E6) or ≥16 μm (at E11) with pyramidal-shaped somata. LMNs in slices could be observed directly and were identified by their distinctive clustering into distinct motor columns in the ventro-lateral horns. Iₐ was evoked by a series of depolarizing voltage steps, −40 to +30 mV, following a 500-ms prepulse at −120 mV in normal external saline. Currents were normalized for cell size by computing soma surface area for each cell as described previously (Dourado and Dryer 1992). We have previously shown that current densities calculated in this way yield results indistinguishable from estimates of cell surface area based on capacitance measurements (Martin-Caraballo M., and Dryer SE, unpublished observations). Data were analyzed using PC clamp software (Axon Instruments). For whole cell recordings from dissociated LMNs, the external saline solution was (mM) 145 NaCl, 5.4 KCl, 0.8 MgCl₂, 5.4 CaCl₂, 10 tetrathylammonium chloride, 5 glucose, and 13 HEPES (pH 7.4 with NaOH). The pipette saline solution was (mM) 120 KCl, 2 MgCl₂, 10 HEPES-KOH, and 10 EGTA (pH 7.4). For whole cell recordings from slices, the external saline solution was (mM) 125 NaCl, 2.5 KCl, 1.0 MgCl₂, 2.0 CaCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, 10 tetrathylammonium chloride, and 25 dextrose (pH 7.45). The pipette saline solution was (in mM) 120 KCl, 2 MgCl₂, 10 HEPES-KOH, and 10 EGTA (pH 7.4). All external recording solutions contained 600 mM TTX to block voltage-activated Na⁺ currents. To stain LMNs in slice preparations, 7 mM biocytin was added to the pipette recording solution and was introduced into the cell during whole cell recording. After recording, slices were fixed in 4% paraformaldehyde and processed using the ABC method (Vector Laboratories) using diaminobenzidine as the chromophore to visualize filled cells. Activation and inactivation curves were constructed and fitted with the Boltzmann equation as described in Wisgirda and Dryer (1993). Throughout this study, V₁/₂ is the voltage at which conductance is half-maximal, and k is a constant related to the steepness of the fitted Boltzmann curve. Curve fitting was carried out using Origin v7 software (Origin Lab, Northampton, MA). Statistical analyses were carried out with Statistica software (Statsoft, Tulsa, OK). In those experimental designs in which multiple experimental groups were compared with a single control group, these consisted of one-way ANOVA followed by Tukey’s post hoc test for unequal n, with P < 0.05 regarded as significant. When a single experimental group was compared with a single control group, we used Student’s unpaired t-test. Throughout, numbers in parentheses above bar graphs indicate number of cells tested. At least five embryos were used in each group.

**In ovo drug application and surgical manipulation**

In ovo drug applications were performed as described previously (Martin-Caraballo and Dryer 2002a,b). Briefly, DiI was injected into the hindlimb at E5, followed by drug application onto the vascularized chorioallantoic membrane ~18 h later. The following drugs were applied daily from E6 until E10: d-tubocurarine (1 mg/day), mecamylamine (0.28 mg/day), and muscimol (0.1 mg two times per day). Drugs were prepared in a physiological saline containing (in mM) 139 NaCl, 3 KCl, 1 MgCl₂, 3 CaCl₂, and 17 NaHCO₃. Hindlimb removal was performed on E6 embryos, 16–18 h after DiI injection. The entire leg was amputated using a battery-operated electrocautery unit (Harvard Apparatus, South Natik, MA). Embryos were allowed to develop to E11, at which time LMNs were dissociated. LMNs were incubated for 2–4 h before electrophysiological recording.

Mecamylamine, muscimol, TTX, 5,8,11,14-eicosatetraycic acid (ETYA), anisomycin, collagenase, and trypsin were obtained from Sigma (St. Louis, MO), ciliary neurotrophic factor (CNTF) was obtained from R & D Systems (Minneapolis, MN), and d-tubocurarine was obtained from Alexis (San Diego, CA). Culture supplements and sera were from BioWhittaker (Walkersville, MD). Limbless mutant chicks (homozygous l and heterozygous Ll) were obtained from a strain maintained at the University of Connecticut Health Center (Farmington, CT).

**RESULTS**

**Developmental expression of A-currents in LMNs**

The characteristics of Iₐ in cultured chick LMNs were originally described by McCobb et al. (1990). In that study, dissociated LMNs were cultured for 24 h in the presence of complex whole embryo extracts prior to electrophysiological measurements. Those extracts contain a host of potential trophic molecules that may not be normal constituents of the normal motoneuron milieu. Therefore we have extended these earlier observations by examining Iₐ expression using whole cell recordings from cell preparations that have not been exposed for significant periods of time to tissue extracts. These recordings were made from spinal cord slices and from acutely dissociated LMNs. Iₐ were evoked by a series of depolarizing steps applied immediately after a 500-ms hyperpolarizing prepulse to −120 mV from a holding potential of −60 mV. Recordings were made in the presence of 10 mM tetrathyammonium (TEA) to block other outward currents (Fig. 1B). Mean current density and the time constant of Iₐ inactivation (τᵢᵢᵥₐᵩiᵩ) were calculated from currents evoked by the step pulse to −10 mV. This test voltage was used because residual delayed rectifier current, some of which persists in 10 mM TEA at more positive potentials, cannot be detected at this command potential. Recordings were made from LMNs on E6 and E11. These stages bracket the onset and completion of programmed LMN cell death and synapse formation with target tissues in the hindlimb.

LMNs can be readily identified in slice preparations on the basis of their position in the ventral horn and their large size. Biocytin staining indicates that the dendritic branching pattern at E11 is more complex than at E6 (Fig. 1A), as was described on the basis of silver impregnation methods in the 19th century (Cajal 1897). Moreover, in most cases, it is possible to visualize the labeled motoneuron axons leaving the spinal cord through the ventral horn. Iₐ was detectable at both developmental stages, although current density was considerably greater at E11. The extensive branching of the LMNs in slice preparations raised the possibility of inadequate spatial control of membrane voltage; therefore we also recorded from acutely dissociated LMNs that had been retrogradely labeled with DiI.
of I at E6 and E11 as indicated. The voltage protocol is shown above.

B: spinal cord slice preparation. E11 LMNs have a slightly larger cell body and more extensive dendritic branching compared with E6 LMNs. A (LMNs).

10 mV. There was a robust increase in –

compiled from many cells. Data were from currents evoked by a step pulse to /H9270

P and a compilation of mean current densities (H11021

P) of cells recordings (H11005

fi 0.0001). In this and subsequent figures, number of cells tested is
gures, number of cells tested is

tiveness of LMNs in spinal cord slices (more extensive dendritic branching compared with E6 LMNs.

E6 and E11 were indistinguishable from that observed in slice preparations (Fig. 1C). In both cases, we observed a greater than threefold increase in I A current density between E6 and E11. However, there was no change in the time course of I A inactivation at the two embryonic stages. It bears noting that I A in LMNs is blocked by 5,8,11,14-eicosatetraynoic acid (ETYA), a nonmetabolizable analog of arachidonic acid (Fig. 1D). This result, along with the kinetics and voltage dependence of the macroscopic currents, strongly suggests that the endogenous A-current in LMNs is carried by members of the Kv4 family of voltage-gated K + channels, as is observed in other native cell types and with cloned channels (Colbert and Pan 1999; Dryer et al. 1998; Holmqvist et al. 2001; Kehr 2001; Keros and McBain 1997; Ramakers and Storm 2002; Villarroel and Schwartz 1996).

Target tissues do not regulate A-current expression in developing LMNs

In chick LMNs, the developmental expression of large-conductance Ca 2+ -activated K + channels (I KCa ) depends in part on interactions with target tissues in the hindlimb (Martin-Caraballo and Dryer 2002a,b). Target tissue interactions also appear to regulate I A kinetics in developing cholinergic parasympathetic neurons (Dourado et al. 1994; Raucher and Dryer 1994). We used three different in vivo approaches to examine the role of target tissues in I A regulation in developing LMNs. In one set of experiments, DiI was injected into chick hindlimbs at E5, and eggs were returned to the incubator for 1 day to allow time for retrograde transport of the dye back to cell bodies in the ventral horn. At that time, one hindlimb was surgically removed (at E6), and the embryos were allowed to develop in ovo until E11. The spinal cord was removed, divided down the middle, and LMNs from each side were dissociated separately and used for electrophysiology. We have previously shown that this procedure causes a large reduction in the normal developmental expression of macroscopic I KCa in LMNs ipsilateral to the lesion (Martin-Caraballo and Dryer 2002a). However, we observed that I A current density, the voltage dependence of activation or inactivation, and the time course of channel inactivation (τ inactivation ) in developing LMNs were indistinguishable in LMNs isolated ipsilateral or contralateral to the excised hindlimb (Fig. 2). These results provide strong evidence suggesting that target tissues do not play a significant role in the regulation expression of LMN I A channels.

A second set of experiments utilized the limbless mutant (Fig. 3). Limbless is an autosomal recessive mutation in chick that affects limb bud formation. Mutants that are homozygous at this locus (ll) develop limb buds at the normal embryonic stage, but the limb buds fail to undergo further development and degenerate shortly after their initial formation (Carrington and Fallon 1988; Prahlad et al. 1979). In contrast, embryos heterozygous at this locus (Ll) develop normal hindlimbs and therefore serve as a useful control (Fig. 3A). LMNs were identified by injecting DiI into limb buds at E4. The lumbar
ventral spinal cords from l1 and lI embryos were excised at E11 and dissociated, and I_A was recorded acutely from fluorescently labeled LMNs. Typical examples of recordings from lI and ll LMNs are shown in Fig. 3, C and D, and mean results from many cells are compiled in Fig. 3, C and D. Note that the evoked currents are indistinguishable in these two strains, and the absence of target tissues had no effect on I_A current density, voltage dependence, or kinetics in developing LMNs (Fig. 3, C and D).

A third set of experiments was designed to assess the effect of increasing contacts between LMNs and target tissues. Previous studies have shown that chronic treatment with α-tubocurarine, a skeletal muscle nicotinic receptor antagonist, increases LMN access to target-derived trophic factors as a result of increased sprouting of motoneuron axons along the surface of the muscle (Oppenheim et al. 2000; Tang and Landmesser 1993). We have previously shown that this treatment also increases the expression of whole cell I_KCa in developing chick LMNs, whose expression depends on target-derived factors (Martin-Caraballo and Dryer 2002a,b). However, daily in ovo treatments with α-tubocurarine had no effect on I_A density, kinetics, or voltage dependence compared with LMNs from vehicle-treated embryos (Fig. 4, A and B; Table 1). This result provides an additional line of evidence that I_A and I_KCa are differentially regulated in LMNs.

**Regulation of LMN A-currents by electrical activity in vivo and in vitro**

We have previously shown that the normal developmental expression of I_KCa channels in chick LMNs is regulated by ongoing electrical activity, as well as by target tissue interactions (Martin-Caraballo and Dryer 2002a). Here we present in vivo and in vitro evidence indicating that I_A is also regulated by ongoing electrical activity in LMNs. In one set of experiments, embryos were treated chronically with the GABA_A agonist muscimol, with the neuronal nicotinic antagonist mecamylamine, or with vehicle. Both drugs reduce synaptic activation of LMNs in vivo, leading to an increase in apoptotic cell death (Millner and Landmesser 1999; Usiak and Landmesser 1999), and a decrease in the developmental expression of I_KCa (Martin-Caraballo and Dryer 2002a). As with the experiments already described using the neuromuscular nicotinic agonist α-tubocurarine, drugs were applied directly onto the chorioallantoic membrane on each day, starting at E6 and continuing through E10. Each of these drug treatments completely eliminated spontaneous embryonic movements as reported previously (Martin-Caraballo and Dryer 2002a). Spinal cord cells were dissociated at E11, and I_A expression measured by whole cell recording from LMNs immediately thereafter. We observed that muscimol and mecamylamine both caused a >80% reduction in I_A density compared with vehicle-treated controls (Fig. 4A). These treatments also caused a marked and statistically significant increase in the rate of inactivation in that portion of I_A that remained after blockade of the normal developmental expression of I_KCa channels in chick LMNs.
of activity (Fig. 4B). The voltage dependence of $I_A$ activation and inactivation were not affected by these treatments (Table 1). These data strongly suggest that ongoing electrical activity is required for normal expression and/or maintenance of $I_A$, but that activity at the peripheral neuromuscular junction is not required.

A similar phenomenon can be observed in dissociated cell culture (Fig. 5). In these experiments, retrogradely labeled E6 spinal cord neurons were co-cultured with hindlimb myotubes. Under these conditions, LMNs can be maintained in vitro for long periods of time without addition of trophic factors to the medium. The medium in one set of co-cultures contained 1 μM TTX to block spontaneous action potential discharge in the LMNs, while a second group was maintained in normal culture medium. After 5 days in vitro, $I_A$ was assayed in Dil-labeled LMNs in both groups. As an additional comparison, $I_A$ was also measured in acutely isolated E11 LMNs. We observed that LMNs co-cultured for 5 days with target tissues expressed an $I_A$ indistinguishable from that of E11 LMNs in terms of both kinetics and current density; in other words, channel development appeared to proceed normally. However, blockade of activity by TTX caused an almost 60% reduction in LMN $I_A$ density, even in the presence of target tissues (Fig. 5B). Moreover, the $I_A$ that was present in the TTX-treated LMNs had faster inactivation kinetics (Fig. 5, A and B); indeed, the currents were indistinguishable from those observed after blockade of activity in vivo (Fig. 4B). Therefore ongoing activity within LMNs appears to regulate both the amplitude and the kinetics of A-currents in developing lumbar motoneurons.
We have examined the functional expression of transient A-type $K^+$ channels ($I_A$) in embryonic chick LMNs developing in vivo and in vitro. The density of $I_A$ increased by more than threefold between E6 and E11 in LMNs developing in ovo. This increase in $I_A$ expression requires electrical activity within the LMNs that is driven byafferent inputs during normal in vivo development. Activity also appears to play a role in regulating the kinetics of $I_A$ inactivation. Somewhat surprisingly, we found no evidence for regulation of the expression, voltage dependence, or kinetics of $I_A$ by interactions with target tissues in the hindlimb using procedures that we have previously shown to cause profound changes in the expression of $I_{K(Ca)}$ (Martin-Caraballo and Dryer 2002a).

A previous study indicated that LMNs are capable of expressing $I_A$ by E6, although these channels are present at a relatively low density at that stage, and that current density increased substantially by E11 (McCobb et al. 1990). We confirmed this developmental pattern in spinal cord slice preparations as well as in LMNs examined shortly after dissociation. The kinetics and voltage dependence of $I_A$ do not change over this period of time in LMNs. Similar developmental changes in $I_A$ density have been observed in other cell types (Dourado and Dryer 1992; McFarlane and Cooper 1992; Nerbonne et al. 1986). A variety of mechanisms could trigger these changes, including a completely intrinsic program of channel expression (Dourado and Dryer 1992; Spitzer and Lamborghini 1976), regulation by cell–cell interactions via soluble (Cameron et al. 1998; Dourado and Dryer 1994; McFarlane and Cooper 1993; Subramony and Dryer 1997) or insoluble (Raucher and Dryer 1994; Wu and Barish 1994) regulatory factors, or regulation by activity (Baines et al. 2001; Liu and Kaczmarek 1998; Martin-Caraballo and Dryer 2002a; Muller et al. 1998).

We have previously shown that expression of macroscopic $I_{K(Ca)}$ in chick LMNs is regulated in part by interactions with target tissues and also by ongoing synaptic activation of the LMNs (Martin-Caraballo and Dryer 2002a). Therefore we were somewhat surprised to find that $I_A$ expression and gating were completely normal in LMNs from embryos that lack hindlimb target tissues as a result of surgical removal of a limb or due to the "limbless" mutation. Consistent with this, we observed that chronic neuromuscular blockade, which increases LMN sprouting along the surface of the muscle fibers, also failed to alter expression or kinetics of $I_A$. Thus three separate sets of in vivo experiments indicate that functional expression of $I_A$ in developing chick LMNs occurs independent of exposure to target-derived trophic molecules, in marked contrast to the regulation of LMN $I_{K(Ca)}$ channels (Martin-Caraballo and Dryer 2002a,b).

Nevertheless, LMN $I_A$ expression does not proceed according to a purely internal program and does require external influences. Specifically, ongoing synaptic activation of LMNs plays an important role in regulating LMN $I_A$ channels, because in ovo pharmacological blockade of excitatory synaptic inputs, or hyperpolarization of the LMNs, eliminates the increase in $I_A$ density that normally occurs between E6 and E11. A similar pattern was observed in vitro, where blockade of motoneuron activity with tetrodotoxin reduced $I_A$ expression in LMNs cultured with hindlimb muscles. We have previously observed activity-dependent regulation of $I_{K(Ca)}$ in LMNs using several of these experimental designs (Martin-Caraballo and Dryer 2002a).

The molecular basis for the effect of electrical activity on $I_A$ expression is not known. However, the portion of $I_A$ that is expressed after activity blockade in vivo or in vitro inactivates...
more quickly than $I_A$ channels expressed during normal development. There is now a growing literature indicating that various Kv4 channels are expressed as complexes containing Ca$^{2+}$-binding ancillary subunits known as KChIPs (An et al. 2000) and members of the DPPX family of proteins (Nadal et al. 2003). There are a number of known KChIP genes, some of which occur in multiple splice variants (Boland et al. 2003; Van Hoorick et al. 2003). All known KChIPs regulate various aspects of Kv4 gating, especially the kinetics of inactivation (Shibata et al. 2003). There is also evidence that KChIPs regulate the trafficking of Kv4 channels, leading to changes in steady-state expression levels and stability, at least in heterologous expression systems (Bahringer et al. 2001; Shibata et al. 2003; Takimoto et al. 2002). DPPX also affects both the trafficking and gating of Kv4 channels (Nadal et al. 2003). Thus it is possible that electrical activity in LMNs acts through KChIPs, DPPX, or ancillary subunits with a similar function, to regulate surface expression of functional A-channels, and indirectly, to regulate their kinetics.

Regulation of ion channel expression by ongoing electrical activity provides a mechanism for feedback loops to control the formation and maintenance of developing neural networks (Katz and Shatz 1996). For example, embryonic chick spinal motoneurons are normally driven to fire spontaneous bursts of activity throughout the developmental stages studied here (reviewed by O’Donovan et al. 1998). Spontaneous activity in spinal LMNs occurs at developmental stages that coincide with significant maturation of the neuromuscular system (O’Donovan and Landmesser 1987), including the elimination of polyneuronal innervation of fast-twitch muscle fibers in chick hindlimb (Phillips and Bennett 1987a,b). Moreover, synapse elimination, gene expression, and several other aspects of neuromuscular junction differentiation have been shown to depend on specific patterns of spike discharge in motoneurons (Buonanno and Fields 1999; Gu and Spitzer 1995; Sanes and Lichtman 1999; Thompson 1985). Within this context, changes in the expression of $I_A$ and $I_K(Ca)$ are likely to be significant, because they will change the waveform and temporal pattern of spike discharge in bursting LMNs (Gao and Ziskind-Conhaim 1998; McCobb et al. 1990). Activity-dependent changes in the expression of $K^+$ channels can therefore provide a mechanism for self-regulation of the pattern of spontaneous spike discharge (Tabak et al. 2001). In summary, these results are consistent with a model in which activity in LMNs drives changes in the functional expression of $I_A$ and $I_K(Ca)$ channels, possibly by acting on auxiliary subunits of the functional channel complexes. This in turn could cause changes in the waveform and temporal pattern of spike discharge, thereby regulating those aspects of neuromuscular maturation that depend on specific patterns of motoneuron discharge.

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