A-current expression is regulated by activity but not by target tissues in developing lumbar motoneurons of the chick embryo

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ABSTRACT

The functional expression of A-type K\(^+\) channels (\(I_A\)) was examined in chick lumbar motoneurons (LMNs) at embryonic days 6 and 11 (E6 and E11). We observed a three-fold increase in \(I_A\) density between E6 and E11 in spinal cord slices and acutely dissociated LMNs. There was no change in current density, kinetics, or voltage-dependence of \(I_A\) in E11 homozygous limbless mutants or in E11 embryos in which hindlimbs were surgically removed at E6. Moreover, chronic in ovo administration of D-tubocurarine, which causes an increase in motoneuron branching on the surface of target muscles, had no effect on \(I_A\). Electrical activity played an important role in \(I_A\) regulation in LMNs in vitro and in ovo. Blocking spontaneous electrical activity of LMNs by chronic in ovo application of mecamylamine or muscimol reduced \(I_A\) by 80%. LMNs cultured in the presence of tetrodotoxin also failed to express normal densities of \(I_A\), even when the cultures also contained target tissues. The portion of \(I_A\) that remained after in ovo or in vitro blockade of activity inactivated more quickly than the \(I_A\) of LMNs that were allowed to discharge spikes. The developmental expression of LMN \(I_A\) increases significantly during development and this increase is activity-dependent but does not require interactions with target tissues. Ongoing activity also appears to regulate the kinetics of \(I_A\) inactivation.
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, as 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 (Spitzer and Lamborghini 1976; Dourado and Dryer 1992), whereas expression of others appears to require neurotrophic interactions with other cell types, ongoing electrical activity, or both (reviewed in Dryer et al. 2003). Although the precise spatiotemporal pattern of channel expression depends on the cell population being studied, the developmental expression of some channels, including Ca$^{2+}$-activated K$^+$ channels ($I_{K(Ca)}$) and A-type K$^+$ channels ($I_A$), requires cell-cell interactions in several neuronal populations (Dourado and Dryer 1992, 1994; McFarlane and Cooper 1992, 1993; Dourado et al. 1994; Raucher and Dryer 1994, 1995; Wu and Barish 1994; Cameron et al. 1998, 1999, 2001; Martin-Caraballo and Dryer 2002a, b).

In the present study, we have examined the developmental regulation of $I_A$ in lumbar motoneurons (LMNs) of the embryonic chick spinal cord. An initial description of this process was carried out by McCobb et al. (1990), who described the stage at which $I_A$ first appears, and the trajectory of the increase in the functional expression of these
channels during LMN development. Since then, a substantial body of literature has shown that trophic factors and cell-cell interactions can regulate developmental expression of $I_A$ (McFarlane and Cooper 1993; Dourado and Dryer 1994; Raucher and Dryer 1994; Dourado et al. 1994). Therefore, we have readdressed the issue of $I_A$ development in LMNs in order to determine cellular processes that regulate these channels. We have confirmed the greater than three-fold 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.
MATERIALS AND METHODS

Motoneuron labeling and dissociation

Labeling, dissociation, and culture of chick lateral motor neurons (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 37ºC (at E6, 0.1% trypsin for 15 min; at E11, 0.4% trypsin for 40 min) and then transferred to a cell culture medium consisting of Eagle’s minimal essential medium (BioWhittaker), 10% heat-inactivated horse serum, 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin. The ventral horns where then dissociated by trituration and plated onto poly-D-lysine-coated glass coverslips. Expression of $I_A$ in acutely dissociated LMNs was examined less than 4 hr after spinal cord dissociation by whole-cell recording. For experiments involving spinal cord-muscle co-cultures, Ell hindlimb muscles were removed and placed in a 0.5 mg/ml solution of type II collagenase for 15 minutes. After dissociation and plating, myotubes were incubated for 2 days before the addition of dissociated E6 LMNs. LMN-muscle co-cultures were maintained for 5 days in the presence or absence of 1µM tetrodotoxin (TTX).

Spinal cord slice preparation

Spinal cords were removed while immersed in a cold oxygenated sucrose saline consisting of (mM): sucrose (110), NaCl (60), KCl (3.0), NaH$_2$PO$_4$ (1.25), NaHCO$_3$ (28),
MgCl$_2$ (7), CaCl$_2$ (0.5), dextrose (5.0), ascorbate (0.6). They were then placed on a pre-made one-inch block of 1% agarose, and then covered with a 7% solution of ultra-low gelling temperature agarose (Sigma). The tissue was cooled for 5 minutes to allow the agarose to harden, and then 300-500 µm slices of the lumbar region of the spinal cord were made using a vibratome. $I_A$ expression was measured from LMNs 2-3 hr after slice preparation.

**Whole-cell recordings**

Dissociated LMNs where identified during patch clamp recordings using an Olympus IX70 inverted stage microscope equipped with epifluorescent optics and rhodamine filters. Recordings were made from DiI containing cells of diameter $\geq$ 14 µm (at E6) or $\geq$ 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. $I_A$ 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 and Dryer, unpublished observations). Data were analyzed using PClamp software (Axon Instruments). For whole-cell recordings from dissociated LMNs, the external saline solution was (mM): NaCl (145), KCl (5.4), MgCl$_2$ (0.8), CaCl$_2$ (5.4), tetraethylammonium chloride (10), glucose (5), and HEPES (13), pH 7.4 (with NaOH).
The pipette saline solution was (mM): KCl (120), MgCl₂ (2), HEPES-KOH (10), and EGTA (10), pH 7.4. For whole-cell recordings from slices, the external saline solution was (mM): NaCl (125), KCl (2.5), MgCl₂ (1.0), CaCl₂ (2.0), NaH₂PO₄ (1.25), NaHCO₃ (25), tetraethylammonium chloride (10), dextrose (25), pH 7.45. The pipette saline solution was (in mM): KCl (120), MgCl₂ (2), HEPES-KOH (10), and EGTA (10), pH 7.4. All external recording solutions contained 600 nM TTX in order 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 then 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, $V_{1/2}$ 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 Corp. Northampton, MA). Statistical analyses were carried out with Statistica® software (Statsoft, Tulsa, OK). In those experimental designs in which multiple experimental groups were compared to 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 to 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 hr later. The following drugs were applied daily from E6 until E10: d-tubocurarine (1 mg per day), mecamylamine (0.28 mg per day), and muscimol (0.1 mg twice per day). Drugs were prepared in a physiological saline containing (in mM): NaCl (139), KCl (3), MgCl$_2$ (1), CaCl$_2$ (3), and NaHCO$_3$ (17). Hindlimb removal was performed on E6 embryos, 16-18 hours 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 hours before electrophysiological recording.

Mecamylamine, muscimol, TTX, 5,8,11,14-eicosatetraynoic 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 ll 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 $I_A$ in cultured chick LMNs were originally described by McCobb et al. (1990). In that study, dissociated LMNs were cultured for 24 hr 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 $I_A$ 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. $I_A$ 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 tetraethylammonium (TEA) to block other outward currents (Fig. 1B). Mean current density and the time constant of $I_A$ inactivation ($\tau_{\text{inactivation}}$) 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 nineteenth century (Cajal 1897). Moreover, in most cases, it is possible to visualize the labeled motoneuron axons leaving the spinal cord through the ventral horn. \( I_A \) 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 (i.e. recordings were made within 2-4 hr of cell dissociation). LMNs tend to lose many of their distal dendritic processes during the cell isolation procedure and are therefore more electrically compact, although proximal dendrites are still present. Nevertheless, \( I_A \) current density and \( \tau_{\text{inactivation}} \) at both E6 and E11 were indistinguishable from that observed in slice preparations (Fig. 1C). In both cases, we observed a greater than three-fold 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 non-metabolizable 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 (Villarroel and Schwartz 1996; Keros and McBain 1997; Dryer et al. 1998; Colbert and Pan 1999; Kehl 2001; Holmqvist et al. 2001; Ramakers and Storm 2002).
**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 (\(I_{\text{K(Ca)}}\)) 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 one 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 then 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 \(I_{\text{K(Ca)}}\) 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 (\(\tau_{\text{inactivation}}\)) 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 (Prahlad et al. 1979; Carrington and Fallon 1988). By contrast, embryos heterozygous at this locus (L/l) 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 l/l and L/l embryos were excised at E11, dissociated, and $I_A$ was recorded acutely from fluorescently labeled LMNs. Typical examples of recordings from L/l and l/l LMNs are shown in Fig. 3B, and mean results from many cells are compiled in Fig. 3C, 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. 3C, 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 d-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 (Tang and Landmesser 1993; Oppenheim et al. 2000). We have previously shown that this treatment also increases the expression of whole-cell $I_{K[Ca]}$ in developing chick LMNs, whose expression depends on target-derived factors (Martin-Caraballo and Dryer 2002a, b). However, daily in ovo treatments with d-tubocurarine had no effect on $I_A$ density, kinetics, or voltage-dependence compared to LMNs from vehicle-treated embryos (Fig. 4A; 4B; Table 1). This result provides an additional line of evidence that $I_A$ and $I_{K[Ca]}$ 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 $I_{K_{Ca}}$ 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 Landmasser 1999; Usiak and Landmasser 1999), and a decrease in the developmental expression of $I_{K_{Ca}}$ (Martin-Caraballo and Dryer 2002a). As with the experiments already described using the neuromuscular nicotinic antagonist D-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 greater than 80% reduction in $I_A$ density compared to 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 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 five 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 five 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. 5A, 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.
DISCUSSION

We have examined the functional expression of transient A-type K^+ channels (I_A) in embryonic chick lateral motor neurons (LMNs) developing in vivo and in vitro. The density of I_A increased by more than three-fold between E6 and E11 in LMNs developing in ovo. This increase in I_A expression requires electrical activity within the LMNs that is driven by afferent 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 (Nerbonne et al. 1986; McFarlane and Cooper 1992; Dourado and Dryer 1992). A variety of mechanisms could trigger these changes, including a completely intrinsic program of channel expression (Spitzer and Lamborghini 1976; Dourado and Dryer 1992), regulation by cell-cell interactions via soluble (Dourado and Dryer, 1994; McFarlane and Cooper, 1993; Subramony and Dryer, 1997; Cameron et al. 1998) or insoluble (Raucher and Dryer 1994; Wu and Barish 1994) regulatory factors, or
regulation by activity (Muller et al. 1998; Liu and Kaczmarek 1998; Baines et al. 2001; Martin-Caraballo and Dryer 2002a).

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, as 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 \textit{in vivo} or \textit{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 (Van Hoorick et al. 2003; Boland 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 (Bahring et al. 2001; Takimoto et al. 2002; Shibata et al. 2003). 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 (Thompson 1985; Gu and Spitzer 1995; Sanes and Lichtman 1999; Buonanno and Fields 1999). Within this context, changes in the expression of $I_A$ and $I_{K_{Ca}}$ are likely to be significant, as they will change the waveform and temporal pattern of spike discharge in bursting LMNs (McCobb et al. 1990; Gao and Ziskind-Conhaim 1998). 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, and thereby regulating those aspects of neuromuscular maturation that depend upon specific patterns of motoneuron discharge.
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REFERENCES


Cameron JS, Dryer L, and Dryer SE. Regulation of neuronal K\(^+\) currents by target-derived factors: opposing actions of two different isoforms of TGF\(\beta\). Development 126: 4157-4164, 1999.


Phillips WD, and Bennett MR. Elimination of distributed synaptic acetylcholine receptor clusters on developing avian fast-twitch muscle fibres accompanies loss of polyneuronal innervation. *J Neurocytol* 16:785-797, 1987b.


Tabak J, Rinzel J, and O'Donovan MJ. The role of activity-dependent network
depression in the expression and self-regulation of spontaneous activity in the

Takimoto K, Yang EK, and Conforti L. Palmitoylation of KChIP splicing variants is
required for efficient cell surface expression of Kv4.3 channels. *J Biol Chem* 277:26904-
26911, 2002.

Tang J, and Landmesser L. Reduction of intramuscular nerve branching and
synaptogenesis is correlated with decreased motoneuron survival. *J Neurosci* 13:3095-
3103, 1993.

Thompson WJ. Activity and synapse elimination at the neuromuscular junction. *Cell Mol

Usiak MF, and Landmesser LT. Neuromuscular activity blockade induced by muscimol
and d-tubocurarine differently affects the survival of embryonic chick motoneurons. *J

modulation of Kv4 kinetics by KCHIP1 splice variants. *Mol Cell Neurosci* 24:357-366,
2003.

Villarroel A, and Schwarz TL. Inhibition of the Kv4 (Shal) family of transient K\(^+\)

Wisgirda ME, and Dryer SE. Characteristics of multiple voltage-activated K\(^+\) currents in
acutely dissociated chick ciliary ganglion neurones. *J Physiol (Lond)* 470:171-189,
1993.
FIGURE LEGENDS

FIG. 1. Macroscopic $I_A$ expression in E6 and E11 LMNs. *A:* LMNs filled with biocytin during whole-cell recording from a spinal cord slice preparation. E11 LMNs have a slightly larger cell body and more extensive dendritic branching compared to E6 LMNs. *B:* representative recordings from LMNs in spinal cord slices (*traces on the left*) and after acute dissociation (*traces on the right*) at E6 and E11 as indicated. The voltage protocol is shown above the current traces. Currents are indistinguishable in slices or dissociated cells. *C:* mean current density and the time constant of $I_A$ inactivation ($\tau_{\text{inactivation}}$) were compiled from many cells. Data were from currents evoked by a step pulse to –10 mV. There was a robust increase in $I_A$ current density from E6 to E11 LMNs ($p < 0.0001$) in both preparations. However, there was no change in $\tau_{\text{inactivation}}$ as a function of developmental stage ($p = 0.22$). *D:* representative recordings (*traces on the left*) and a compilation of mean current densities (*graph on the right*) of cells before and after superfusion of 5,8,11,14-eicosatetraynoic acid (ETYA), a non-metabolizable analog of arachidonic acid. ETYA (35 µM) blocked more than 80% of $I_A$ ($p < 0.0001$). In this and subsequent figures, the number of cells tested are listed above each test group, error bars represent s.e.m., asterisks indicate $p < 0.05$ and n.s. denotes no significant difference.
FIG. 2. Removal of hindlimb target tissues has no effect on $I_A$ expression. One hindlimb was removed at E6, at the onset of LMN-hindlimb synapse formation, and embryos were allowed to develop to E11, at which point LMNs were dissociated and examined by electrophysiology 2-3 hr later. $A$: families of A-currents evoked by depolarizing steps in LMNs contralateral (con, top left traces) and ipsilateral (-leg, top right traces) to the lesion. The bottom traces are currents evoked by a step pulse to -10 mV, shown with superimposed fitted single exponential curves with time constants as indicated. $B$: mean current density and $\tau_{\text{inactivation}}$ in LMNs from controls and operated embryos are indistinguishable ($p = 0.78$ for current density; $p = 0.46$ for $\tau_{\text{inactivation}}$). $C$: typical activation (left) and inactivation (right) curves fitted with a Boltzmann equation to obtain the $V_{1/2}$ and the slope factor, as described in Wisgirda and Dryer (1993). These data were derived from a representative cell. $D$: mean $V_{1/2}$ and the slope from activation and inactivation curves compiled from a number of cells. The voltage-dependence of $I_A$ is not affected by removal of target tissues ($p = 0.91$ for $V_{1/2}$ activation; $p = 0.49$ for $V_{1/2}$ inactivation; $p = 0.27$ for activation slope; $p = 0.24$ for inactivation slope).
FIG. 3. Absence of hindlimb target tissues does not affect $I_A$ expression in LMNs. 

$A$: E11 embryos heterozygous ($Ll$) or homozygous ($ll$) at the $limbless$ locus. Heterozygous embryos develop normal hindlimbs whereas homozygous mutants lack limbs. 

$B$: Families of current traces ($top$) are shown from representative LMNs dissociated from $Ll$ or $ll$ embryos. Sample traces evoked by a step pulse to $-10$ mV ($bottom$) are shown with superimposed fitted exponential curves. $A$-currents in these cells are indistinguishable.

$C$: Mean current density and kinetics are indistinguishable in LMNs dissociated from $Ll$ and $ll$ embryos ($p = 0.62$ for current density; $p = 0.23$ for $\tau_{\text{inactivation}}$). 

$D$: Mean voltage dependence parameters are unchanged by the lack of limb ($p = 0.93$ for $V_{1/2}$ activation; $p = 0.16$ for activation slope; $p = 0.53$ for $V_{1/2}$ inactivation; $p = 0.27$ for inactivation slope).
FIG. 4. Effects of electrical activity on the functional expression of $I_A$ in embryonic LMNs developing in vivo. A: Inhibition of electrical activity in LMNs by daily in ovo application (E6-E10) of the neuronal nicotinic antagonist mecamylamine (mecam) or the GABA<sub>A</sub> agonist muscimol reduced $I_A$ density compared to vehicle treated embryos (con) ($p< 0.0001$). However, chronic neuromuscular blockade by in ovo application of D-tubocurarine (D-TC), which increases LMN branching on the surface of the target tissue, had no effect on $I_A$ density ($p = 0.16$). B: sample traces of control (top left) and muscimol-treated (top right) embryos are shown with superimposed exponential curves with $\tau_{\text{inactivation}}$ as indicated. Note that the traces are shown on a different amplitude scale. $I_A$ inactivation is faster in the LMN from the muscimol-treated embryo. Mean $\tau_{\text{inactivation}}$ determined in many cells (bottom) is significantly faster in mecamylamine- and muscimol-treated embryos ($p< 0.002$), whereas $I_A$ kinetics in D-tubocurarine-treated embryos are not significantly different from vehicle-treated embryos ($p = 0.44$).
FIG. 5. Effects of electrical activity on the functional expression of $I_A$ in embryonic LMNs developing in vitro. E11 hindlimb muscles were dissociated and incubated for two days, after which E6 dissociated LMNs were added and the LMN-myotube co-culture was incubated for five days in the presence and absence of TTX. $A$: Families of current traces (top) and sample traces with superimposed exponential curves with $\tau_{inactivation}$ indicated (bottom) from representative LMNs co-cultured with muscle in the presence and absence of TTX. Note more rapid current inactivation in TTX-treated cultures. $B$: Addition of TTX to LMN-muscle co-cultures reduced mean $I_A$ current density ($p<0.0001$) and decreased mean $\tau_{inactivation}$ ($p<0.0001$).
Table 1. *Effects of in vivo drug application on voltage dependence of A-current*

<table>
<thead>
<tr>
<th>Drug Treatments</th>
<th>Activation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{1/2}$ (mV)</td>
<td>Slope ($k$)</td>
</tr>
<tr>
<td>control</td>
<td>-20.40 ± 2.57</td>
<td>15.64 ± 1.08</td>
</tr>
<tr>
<td>D-tubocurarine</td>
<td>-20.30 ± 2.80$^a$</td>
<td>18.23 ± 1.22$^a$</td>
</tr>
<tr>
<td>mecamylamine</td>
<td>-17.20 ± 2.90$^a$</td>
<td>18.28 ± 2.13$^a$</td>
</tr>
<tr>
<td>muscimol</td>
<td>-17.60 ± 4.88$^a$</td>
<td>17.28 ± 4.45$^a$</td>
</tr>
</tbody>
</table>

$^a$Not statistically significant at 0.05 level compared to controls by one-way ANOVA analysis. Drugs were administered daily from E6 until E11, at which time recordings were made from dissociated LMNs. Data were analyzed as described in Methods and in Wisgirda and Dryer (1993).
Figure 1

A

E6

E11

B

-120 -40 +30

E6 Slice E6 Culture

E11 Slice E11 Culture

C

Current Density (mA/cm²)

E6 Slice E6 Culture E11 Slice E11 Culture E6 Slice E6 Culture E11 Slice E11 Culture

D

Con ETYA

Current Density (mA/cm²)

Con ETYA

* ns (13) (12) (15) (11) (13) (12) (15)
Figure 3

A

B

C

D

\( \tau = 30.1 \text{ ms} \)

\( \tau = 32.1 \text{ ms} \)

\( E_{11} \text{ Acute} \)

\( n.s. \)

\( \sigma \)

\( v \)

\( I \)

\( F \)

\( \text{Slope} \)

\( \text{Activation} \)

\( \text{Inactivation} \)

\( 2 \text{ nA} \)

\( 100 \text{ ms} \)

\( \text{Current Density (mA/cm}^2) \)

\( \text{E}_{11} \text{ Acute} \)

\( \text{n.s.} \)

\( (9) \)

\( (10) \)

\( (11) \)

\( (12) \)

\( (13) \)

\( \text{Activation} \)

\( \text{Inactivation} \)
Figure 4

A

Current Density (mA/cm²)

Con  D-TC  Mecam  Muscimol

B

τ = 32.8 ms

τ = 12.8 ms

Con  D-TC  Mecam  Muscimol
Figure 5

A

Muscle

Muscle + TTX

τ = 28.1 ms

τ = 16.5 ms

B

Current Density (mA/cm²)

E11  E6+5  Muscle  Muscle+TTX

E11  E6+5  Muscle  Muscle+TTX

τ Inactivation (ms)

n.s.

E6+5  Muscle  Muscle+TTX

E6+5  Muscle  Muscle+TTX

(17)  (16)  (16)  (14)