The Differentiation of Excitability in Embryonic Chick Limb Motoneurons

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The well-documented role of neuromuscular activity as a regulator of motoneuron and muscle development raises important questions about the differentiation of excitability in motoneurons. We have recently described changes in expression of voltage-dependent calcium currents that take place during neuromuscular development in the chick embryo (McCobb et al., 1989). We now report similar analyses, using whole-cell patch-recording methods, of the major currents underlying action potential generation in the same motoneurons. Studies were conducted on identified hindlimb motoneurons isolated from the spinal cord at 3 very different stages of chick hindlimb development. Motoneurons could generate overshooting action potentials at the earliest stage studied [embryonic day 4 (E4)]. However, large changes in densities of several voltage-dependent ionic currents occurred thereafter. E6 and E11 motoneurons had progressively larger I_{Na} densities and, consequently, greater action potential amplitudes. Densities of 2 potassium currents, I_K and I_A, increased on separate schedules. The relatively late and much larger increase in I_A resulted in a substantial developmental decline in action potential duration. These changes, which will greatly affect motoneuron output to muscle by affecting Ca^{2+} entry through voltage-gated channels, occur at the same time that activity-dependent developmental changes occur in the neuromuscular system.

The development of excitability appears, for at least some cells, to be a complex progression of events, as various voltage-dependent ionic currents that underlie specific features of excitability develop on independent schedules (Salkoff and Wyman, 1981; Bader et al., 1983, 1985; Barish, 1986; Nerbonne et al., 1986; Krieger and Sears, 1988; O'Dowd et al., 1988; McCobb et al., 1989; Nerbonne and Gurney, 1989). Such complexity raises many questions. For example, is the expression of various ion channel genes under differential control, and if so, what is the nature of the respective regulatory variables? Furthermore, because functional activity itself regulates many "downstream" features of nervous system development, can specific ionic currents be identified that play deciding roles in regulating the

expression of other genes that shape the nervous system? Obviously, specific hypotheses are needed to address these ideas. In the interest of framing such hypotheses, we have used patch-clamp techniques to characterize excitability in an important developmental model.

Both synaptic and developmental interactions between vertebrate somatic motoneurons and their target muscle have been studied extensively. These studies document the importance of (1) trophic interactions, and (2) muscle activity as regulators of many aspects of both nerve and muscle phenotype during development and regeneration (Dennis, 1981; Brown, 1984; Betz, 1987; Purves, 1988). How motoneuron excitability might fit into the regulatory schemes is unknown. However, aspects of motoneuron phenotype, such as cell death (Laing and Prestige, 1978; Pittman and Oppenheim, 1978, 1979), axon terminal growth and branching (Dahm and Landmesser, 1988), and elimination of extraneous synapses (Srihari and Vrbová, 1978; Ding et al., 1983), are potentially subject to influence by motoneuron activity both directly, as activity alters intracellular calcium levels, and indirectly, as motoneuron output limits muscle contractile activity.

Mature motoneurons and late immature motoneurons have been subjected to a wide variety of electrophysiological recording procedures (Barrett et al., 1980; Mallart, 1984; Schwindt and Crill, 1984; Fulton and Walton, 1986; Ziskind-Conhaim, 1988). However, early motoneurons have only become accessible as retrograde labeling has been combined with patch-clamp recording from dissociated cells (O'Brien and Fischbach, 1986a, b, c, d; Fruns et al., 1987; McCobb et al., 1989). Whole-cell patch-clamp recording, because of the tight seal of electrode to the membrane and low-access resistance to the cell interior (Hamill et al., 1981; Sakmann and Neher, 1984), enable resolution and quantification of voltage-gated currents that are superior to those possible with techniques applied previously even to mature motoneurons. The purpose of the present study was to apply this technique to motoneurons isolated from as wide a span of neuromuscular system development as possible, allowing the identification of changes that take place during development *in vivo*.

The earliest age studied was embryonic day 4 (E4, stages 22–24; Hamburger and Hamilton, 1951), which is soon after motoneuronal axons begin to grow out into the largely undifferentiated mesenchyme of the early limb buds, making them amenable to retrograde labeling (Fig. 1). The most dramatic phase of limb differentiation occurs before E11 (stage 37). By this point, the leg is much larger, is virtually mature in form, has contractile muscle and functional synapses, and is spontaneous-
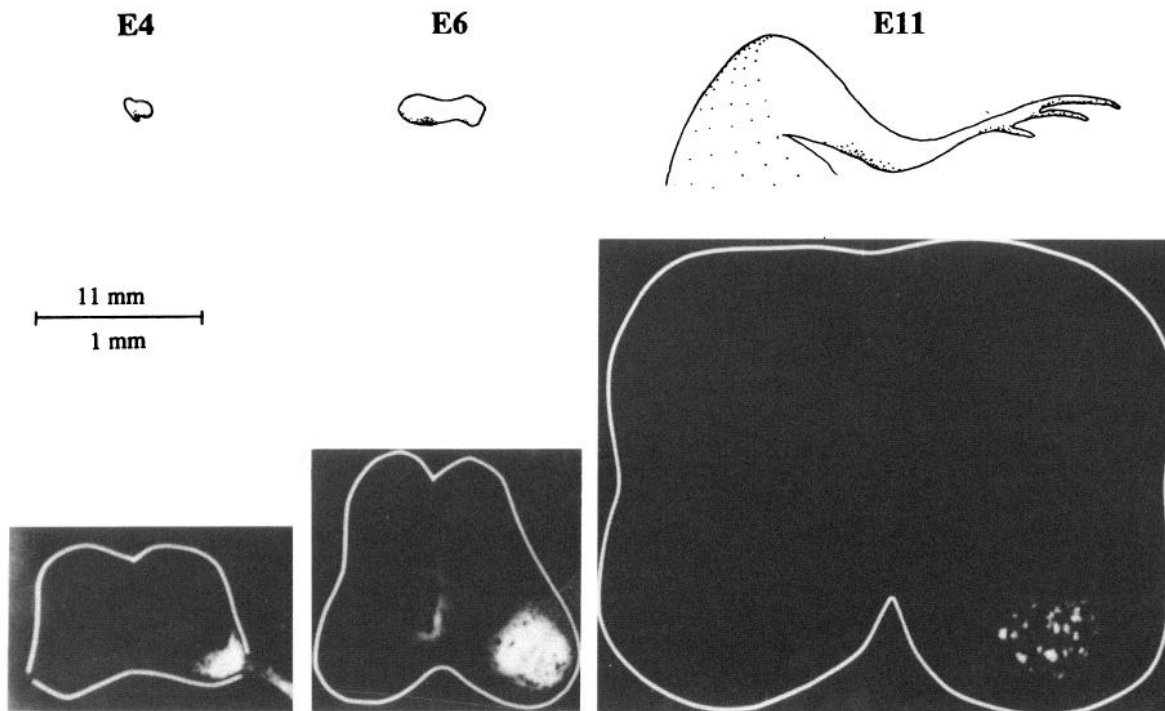
Figure 1. Retrograde labeling of chick hindlimb motoneurons with fluorescent DiI over the wide span of limb development studied. *Line drawings* illustrate limb size and shape at E4, E6, and E11 (stages 24, 29, and 37; Hamburger and Hamilton, 1951). *Photographs* are of fluorescence-illuminated cryostat sections of spinal cords of embryos at the same stages as seen in the line drawings (for clarity, the margins of the cords have been traced). DiI labeling was restricted to the lateral motor column region on the same side as the injected limb. E6 and E11 cords were dissected out before processing, but the E4 cord was fixed and sectioned within the embryo. The ventral root with labeled motor axons can be seen leading out of the right side of the E4 cord.

ly active (Hamburger, 1977; Jacobson, 1978). A relatively discrete phase of cell death, during which the motoneuron pool is reduced by ~50%, occurs between E6 and E9 (Hamburger, 1975; Laing, 1982) and is therefore bracketed by our measurements at E6 and E11. We previously reported that large changes in the expression of voltage-dependent Ca^{2+} currents occur in these motoneurons between E4 and E11 (McCobb et al., 1989), and we now report that the action potential mechanism and major underlying currents are undergoing equally important changes.

Materials and Methods

Retrograde labeling of limb motoneurons with the carbocyanine dye DiI. Embryonic chick limb motoneurons were retrogradely labeled *in ovo* with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Junction City, OR), with some modification of the procedure previously described (McCobb et al., 1989; see also Honig and Hume, 1986). Beginning on the morning of embryonic day 0 (E0), fertile eggs were incubated at 36–37°C. Dye injections were made on the evening of E3, E5, or E10, and spinal cord dissociations were done the following morning (i.e., E4, E6, or E11). For injection, a hole was made in the shell over the air space, and the shell membrane was torn carefully away from extraembryonic membranes to avoid rupturing blood vessels. Forceps were used to tear a hole in the amnion of E4 and E6 embryos. A glass micropipet (a patch electrode with a broken tip) was used for dye injections into E4 and E6 embryonic limbs. The micropipet was mounted on an empty 10-ml syringe, which supplied air pressure for ejecting the dye. In E11 embryos, the amnion was penetrated with the point of a pair of forceps; a hindlimb was then grasped at the foot and pulled through the hole, and muscles of the thigh and foreleg were injected through a 30-gauge needle with DiI suspended in saline and ethanol. Dye injection was done only into hindlimbs, thus minimizing the diversity of developmental stages in the motoneurons that would result from the rostrocaudal progression of spinal cord development. Eggs were sealed with plastic tape and returned to the in-

cubator overnight. DiI was dissolved in 100% ethanol at 5 mg/ml. This solution was then diluted with 4 times as much of a standard saline solution (composition of solutions given below) containing 0.1% bovine serum albumin, yielding a fine suspension of DiI (1 mg/ml) in 20% ethanol and 80% saline.

Embryos were staged according to Hamburger and Hamilton (1951). E4 embryos were at stages 22–24, E6 embryos at stages 28–29, and E11 embryos at stage 37. To verify the selective labeling of neuronal somata in the lateral motor column region, cords from each stage were fixed in 2% paraformaldehyde, cryoprotected in a graded sucrose series, and cryostat-sectioned. As seen in Figure 1, fluorescent labeling was restricted to the ventrolateral region of the ipsilateral cord.

Spinal cord dissociation and motoneuron culture. The procedures used for obtaining limb motoneurons from E4, E6, and E11 embryos were slightly modified from those described in detail by McCobb et al. (1989). Dissections were made in saline. The spinal cords of staged embryos with dye-injected limbs were ventrally exposed. Tungsten needles were used to sever spinal nerve roots; the meninges were then stripped off, and the cord was transferred to Ca^{2+}- and Mg^{2+}-free saline (CMFS) at room temperature. E6 and E11 cords were immediately cut into approximately 1-mm³ pieces and transferred to a dram vial. E4 spinal cords were transferred intact. CMFS was replaced with trypsin solution, and the tissue was incubated at 37°C with gentle agitation for the appropriate length of time. E4 embryos were incubated in 0.05% trypsin (Sigma type XI) for 10 min, E6 embryos were incubated in 0.1% for 20 min, and E11 embryos were incubated in 0.40% for 45–60 min. All trypsin solutions were made in CMFS. Tissue was rinsed and triturated in motoneuron plating medium (MnP), which lacks embryo extract, that was preincubated in the tissue culture incubator (37°C, 5% CO_2). A series of increasingly smaller-tipped fire-polished Pasteur pipets was used for trituration. Success was evidenced by translucent cell bodies and the retention of long stumps of broken processes on acutely dissociated neurons (Fig. 2). Neurons were plated in 35-mm dishes or rectangular perfusion chambers (1 × 4 cm). The substratum was coated before neuron plating with a thin layer of Matrigel (Collaborative Research Inc., Bedford, MA). MnP was replaced with motoneuron nutrient medium (MnN), which contains embryo extract, 1–2 hr after plating,
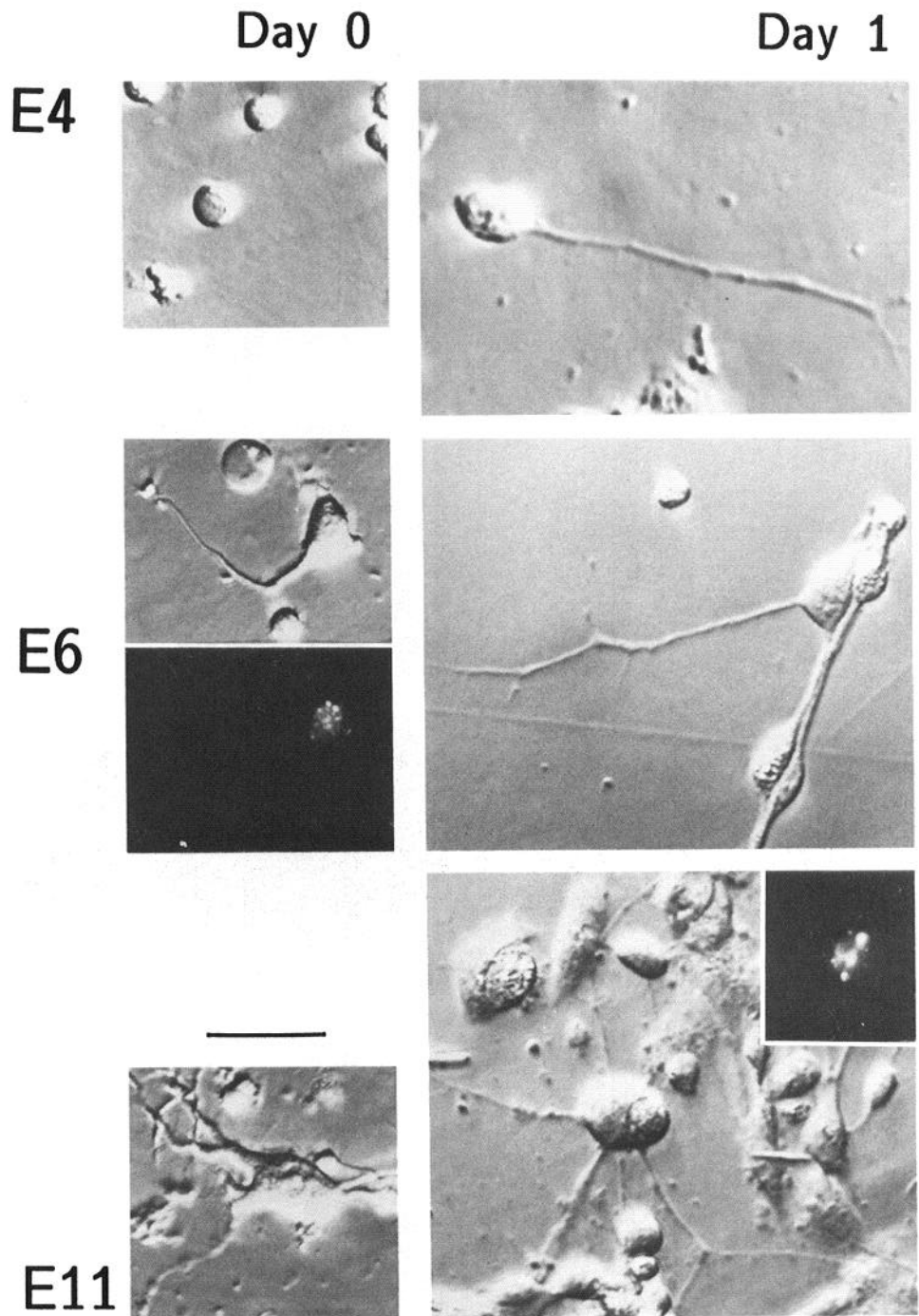
Day 0　　　　　　Day 1

E4

E6

E11

Figure 2. Identified chick limb motoneurons in cell culture viewed with Hoffman modulation contrast optics. *Left,* Acutely isolated motoneurons from E4 (stage 24), E6 (stage 29), and E11 (stage 37) embryos. Stumps of broken axons and dendrites are typically seen on E6 and E11 motoneurons but are not always obvious on E4 motoneurons. A fluorescence micrograph of the E6 motoneuron showing DiI labeling appears just *below* the Hoffman micrograph. *Right,* Motoneurons representing the same embryonic stages photographed on the day following isolation. *Inset* on the E11 motoneuron illustrates DiI fluorescence of the cell body. Filters for rhodamine dyes were used to visualize DiI. Scale bar, 50 μm

and cultures were left in the incubator overnight for electrophysiological analysis on the day following dissociation (Fig. 2). Both visual inspection and cell capacitance measurements indicated an increase in motoneuron size with development. Mean capacitance values for E4, E6, and E11 motoneurons were 18.6 ± 0.7, 26.4 ± 0.9, and 31.8 ± 2.0, respectively (mean $\pm$ SEM; $n = 50$, 50, and 51 neurons). For comparisons between stages (as described below), ionic currents were expressed as current densities, obtained by dividing current amplitudes by cell capacitance. Within age groups, no correlation was found between current density and cell capacitance, suggesting that cell size per se did not confound the measurement of current density.

Electrophysiological recording. Motoneurons identified by fluorescence were studied using conventional whole-cell patch-clamp recording techniques. Patch electrodes were pulled from capillary glass and had

resistances from 2.5 to 5MΩ when filled with internal solution. Pipets were coated with wax to minimize electrode capacitance. A List L/M EPC-7 patch-clamp amplifier was used. After rupture of the patch to produce whole-cell voltage-clamp mode, pipet series resistance and linear capacitance of the cell membrane were calculated from the time constant of, and area beneath, the capacitive transient obtained during control pulses of -20 mV from the holding potential. Series resistance values were typically between 4 and 12 MΩ and the membrane capacitance could be charged with a time constant between 100 and 400 μsec. Electronic compensation was used to reduce series resistance and membrane potential settling time to 40–70% of precompensation values.

When symptoms of poor space clamp could not be satisfactorily eliminated with series resistance compensation adjustments, the cell was excluded from quantitative analysis of currents. With sodium currents,

inadequate space clamp was indicated by regenerative events during intermediate test steps and a steepening of the current–voltage relationship in the activation range. The measurement of I_A was more critically dependent on good space-clamp parameters than that of I_K. Because activation kinetics are voltage dependent, the voltage gradient resulting from poor space clamp results in a delay of current activation, and this delay increases with distance from the pipet. Because I_A is only briefly activated before it inactivates, the effect of the delay is to reduce the magnitude of the peak current. In cases of poor space clamp, what appeared to be I_A on the basis of both rapid-activation kinetics and sensitivity to block by 4-aminopyridine (4-AP) was relatively insensitive to inactivation by depolarizing prepulses. To determine whether we were underestimating I_A in early motoneurons, we measured potassium currents in E4 and E6 motoneurons after only 4–6 hr in culture, when very little neurite outgrowth had occurred. Densities of I_A and I_K were similar to those measured after 1 d in culture.

Linear components of leak and capacitative current, though small, were eliminated from test-pulse traces by digital subtraction of the scaled control currents. Current density measurement data are expressed as mean ± SEM. For quantitative measurements of Na^+ currents, K^+ currents were reduced by including 5 mM 4-AP in the external saline and 70 mM Cs^+ in the internal pipet solution. K^+ currents were measured with KCl in the internal pipet solution and 1 μM TTX in the external solution to eliminate I_{Na}.

For current-clamp recordings, a seal was first established in voltage-clamp mode, and the membrane was ruptured in either voltage- or current-clamp mode. Very similar values of the resting membrane potential were obtained by the 2 procedures. Action potentials were evoked with 2-msec-duration pulses of 100–900 pA. Action potential duration was measured as half repolarization time (HRT), defined as the time between the peak of the action potential and the point at which the membrane potential returned to the value halfway between the peak and starting potential. The more conventional measurement of action potential duration was not used because the stimulus artifact extended beyond that point in many of the low-amplitude action potentials of E4 neurons, in particular. Action potentials were compared at a variety of holding potentials.

Effects of drugs were examined during continual perfusion of the small-volume recording chamber or by releasing small quantities from a hole in the tip of a U-shaped polyethylene tube, which was accomplished by closing off the vacuum at one end of the tube (McCobb et al., 1989). TTX, tetraethylammonium ion (TEA$^+$), and 4-AP were dissolved in saline, and pH was adjusted as necessary. All experiments were conducted at room temperature (~20°C).

Solutions. Saline: 146 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH, 7.4 with NaOH); CMFS: 155 mM NaCl, 5mM KCl, 10 mM HEPES (pH, 7.4 with NaOH); MnP: 90% Dulbecco's Modified Eagle's Medium (DMEM) (GIBCo, Grand Island, NY), 10% horse serum with 100 U/ml penicillin and 100 μg/ml streptomycin; MnN: 85% DMEM with glutamine, 10% heat-inactivated horse serum: 5% chick embryo extract with 100 U/ml penicillin and 100 μg/ml streptomycin; external recording solutions: standard saline (as above) for current clamp, saline with 1 μM TTX for K^+ currents, saline with 5 mM 4-AP for Na^+ currents; internal pipet solutions: 140 mM KCl, 5 mM $MgCl_2$, 10 mM K_2EGTA, 10 mM HEPES (pH, 7.4 with KOH; used for both current clamp and K^+ current recordings); and 70 mM Cs-aspartate, 70 mM KCl, 5 mM $MgCl_2$, 5 mM Cs_2EGTA, 5 mM K_2EGTA (pH, 7.4 with CsOH; used for Na^+ current recordings).

Results

Sodium currents in chick motoneurons

Characterization. Figure 3 illustrates typical sodium currents recorded with a series of test-voltage steps (V_{test}) from a holding potential (V_{hold}) of −80 mV. Na^+ currents were found to be highly sensitive to block by TTX at all stages, including E4 (Fig. 3*C*). TTX at 100 nM virtually eliminated sodium currents (n = 9) and at 10 nM, reduced the sodium current by approximately 50% (n = 3). The absence of a relatively TTX-resistant component in motoneurons is in contrast to dorsal root ganglion neurons (Bossu and Feltz, 1984) and myotubes (Weiss and Horn, 1986). No obvious developmental changes in voltage depen-
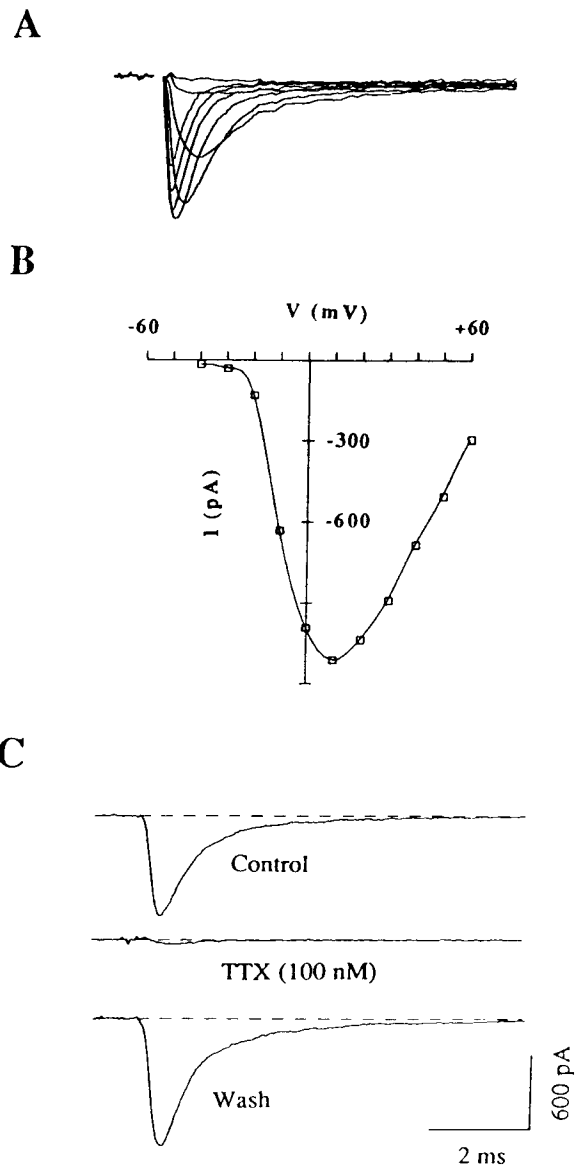

Figure 3. Na^+ currents measured with whole-cell voltage clamp in early chick limb motoneurons *in vitro* (E4, stages 22–24) identified by retrograde labeling before dissociation. *A,* Currents evoked with a series of voltage steps (to between −30 and +40, in 10-mV increments) from a holding potential (V_{hold}) of −80 mV. *B,* Peak current-voltage relationship for the currents displayed in A. *C,* Na^+ currents measured in E4 and later motoneurons show consistently high sensitivity to block by TTX. Virtually complete block, as seen here, was achieved with 100 nM TTX.

dence or kinetics of activation or inactivation were observed. We saw no evidence for multiple components of I_{Na}.

Developmental changes in expression of Na^+ currents. Quantitative comparisons of sodium current expression in motoneurons isolated from different staged embryos were made by measuring the maximum current amplitude elicited during a series of voltage steps from a holding potential of −80 mV. Current amplitudes were normalized for cell size by dividing by cell capacitance, and are therefore expressed as current densities. Sodium current density in chick limb motoneurons was found to increase substantially during development. Motoneurons isolated from E4 chicks already expressed appreciable current (mean peak current density, 66.6 ± 9.0 pA/pF; n = 21); however,
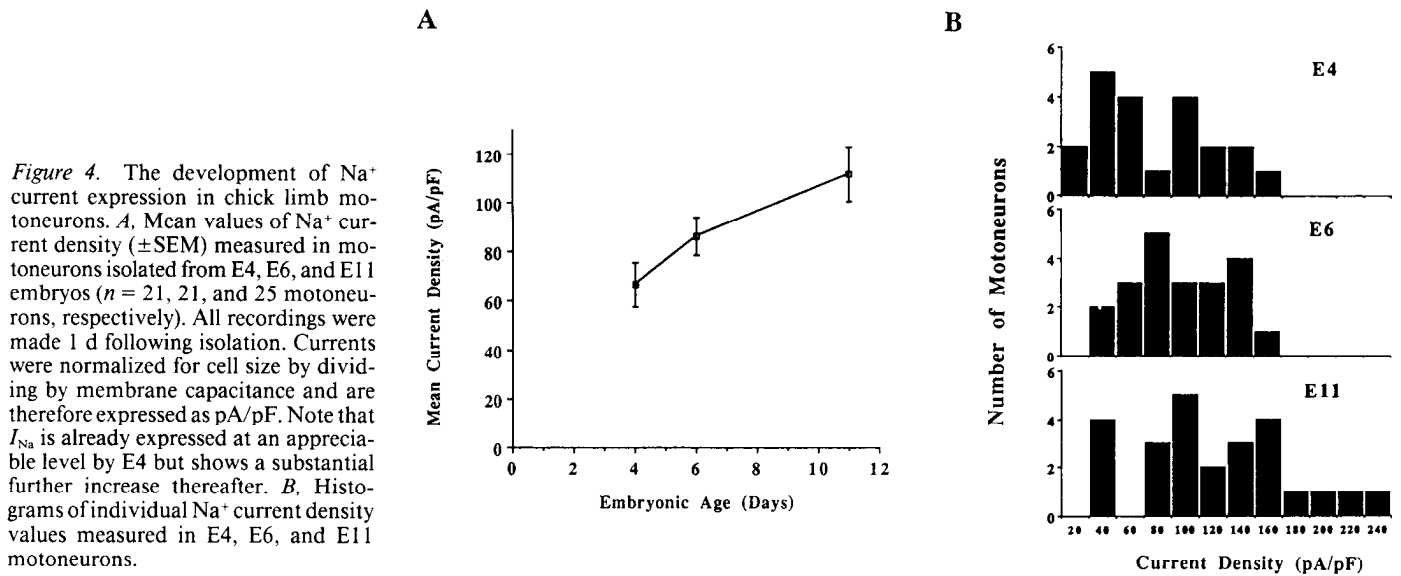
A

B

Figure 4. The development of Na^+ current expression in chick limb motoneurons. *A,* Mean values of Na^+ current density (±SEM) measured in motoneurons isolated from E4, E6, and E11 embryos (n = 21, 21, and 25 motoneurons, respectively). All recordings were made 1 d following isolation. Currents were normalized for cell size by dividing by membrane capacitance and are therefore expressed as pA/pF. Note that I_{Na} is already expressed at an appreciable level by E4 but shows a substantial further increase thereafter. *B,* Histograms of individual Na^+ current density values measured in E4, E6, and E11 motoneurons.

motoneurons isolated from E6 chicks expressed greater I_{Na} densities (86.2 ± 7.6 pA/pF; n = 21). A more moderate increase was seen between E6 and E11, with mean density reaching 112 ±11.1 pA/pF (n = 25; Fig. 4).

Potassium currents

Characterization. The voltage-dependent potassium currents observed in embryonic chick limb motoneurons could be resolved into 2 distinct components: a transient current similar to A-type potassium current in other cells (I_A; Rogawski, 1985), and a sustained current (referred to here as I_K). Figure 5 illustrates potassium currents recorded from a motoneuron in response to a series of voltage steps from a holding potential of −100 mV. Holding at more depolarized potentials briefly (prepulse duration, 100 msec) before stepping to the same test potentials was found to inactivate I_A selectively. The I_A component was found to activate much more rapidly than I_K (time to peak, ~2–3 msec for I_A, compared to ~10–20 msec for I_K) and to

A

Total K$^+$ Current
(V$_{hold}$ = -100 mV)

V_{test} =
+50
-30

B

Non-inactivating Current (I$_K$)
(V$_{hold}$ = -30 or -40 mV)

V_{test} =
+50
-30

5 nA
50 ms

C

V_{test} =

Inactivating Current (I$_A$)

+50
+40
+30
+20
+10
0
-10
-20
-30

D

I_K
I_A

Current (pA)
8000
6000
4000
2000
0
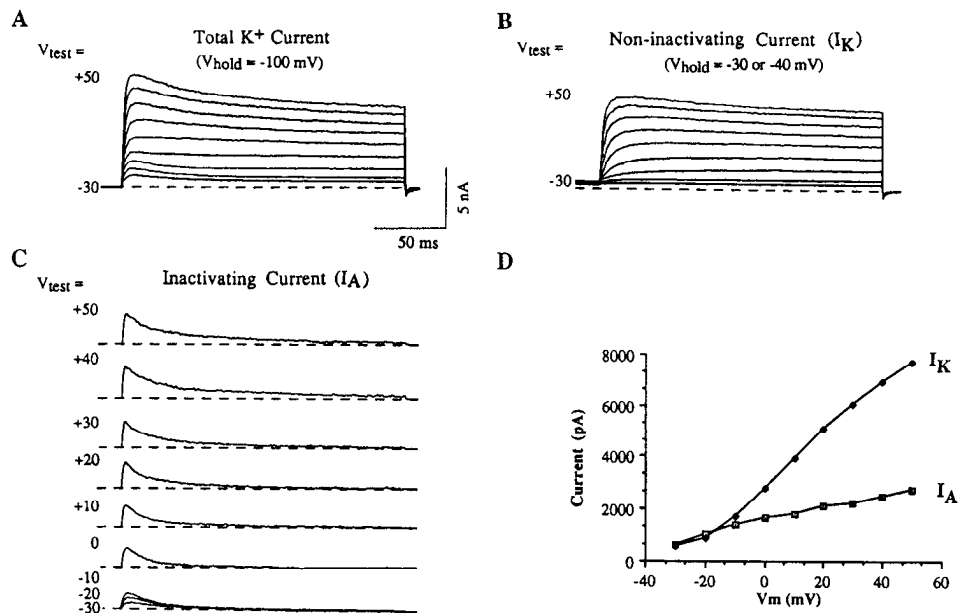-40 -20 0 20 40 60
Vm (mV)

Figure 5. K$^+$ currents measured in an E6 chick limb motoneuron. *A,* Total K$^+$ current elicited by a series of steps to test potentials of −30 to +50 (in 10-mV increments) applied from V$_{hold}$ = −100 mV. *B,* K$^+$ currents elicited when the same series of test potentials used in *A* were preceded by a 100-msec prepulse to −30 mV or −40 mV. Note that this current, which is not inactivated by depolarizing prepulse potentials, decays little during the 200-msec-duration test step. Based on the similarity of its kinetics and voltage dependence to delayed-rectifier-type K$^+$ currents in other cells, it will be referred to here simply as I_K. Prepulse potentials of −30 mV were used for test steps to −10 and above, and of −40 mV for steps to −30 and −20. Prepulses to −30 mV activated a small amount of K$^+$ current, as indicated by the fact that the illustrated traces begin slightly above the zero-current baseline. Similar non-zero current "pedestals" are also evident in Figures 6 and 10. *C,* Difference currents (records in *B* subtracted from those in *A*) reveal the component of total K$^+$ current that is inactivated by the 100-msec prepulse described for *B.* This transient and readily inactivated K$^+$ current strongly resembles A-type potassium current in other cells and will be referred to here as I_A. *D,* Peak current–voltage relationship of K$^+$ current components shown in *B* and *C.*

A   Total K$^+$ Current (from -80 to +20 mV)   **B**   Total K$^+$ Current (from -100 to +20 mV)

a

a

Control

Control, Wash

4-AP

TEA$^+$

Non-inactivating (I$_K$) Current (from -40 to +20 mV)   Non-inactivating (I$_K$) Current (from -30 to +20 mV)

b

b

Control

Control, Wash

4-AP

TEA$^+$

Inactivating (I$_A$) Current   Inactivating (I$_A$) Current

c

c

Control

TEA$^+$

4-AP

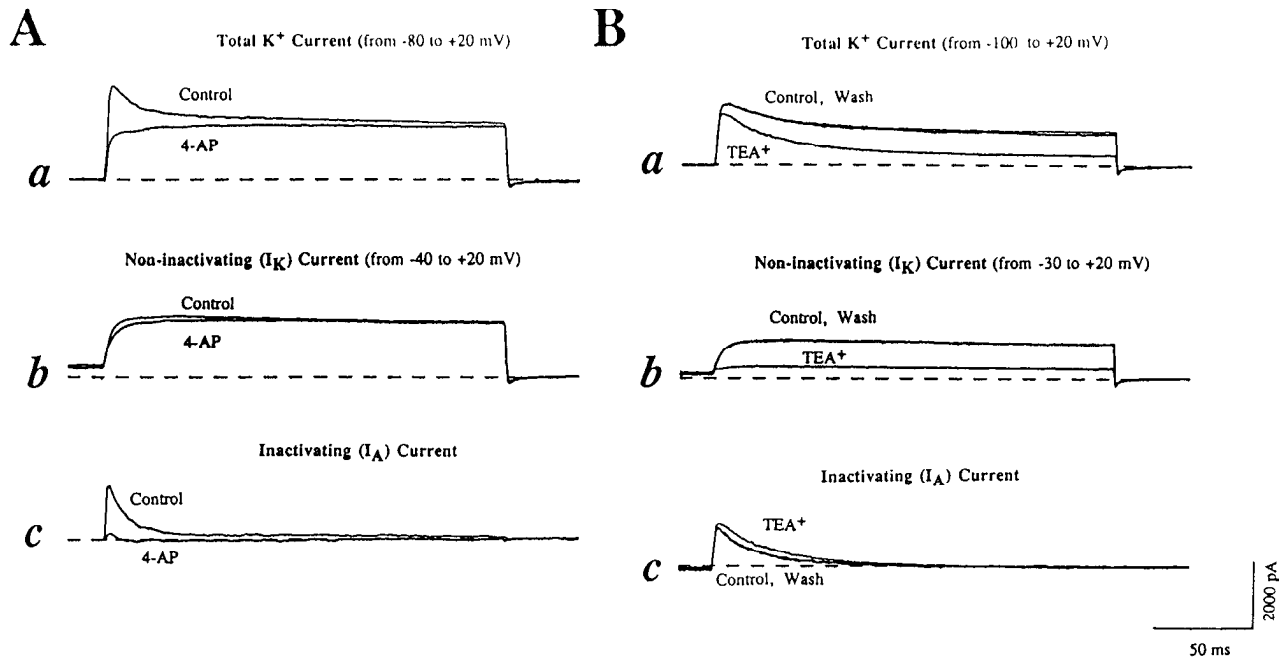Control, Wash

2000 pA

50 ms

Figure 6. Inactivating (I_A) and noninactivating (I_K) components of the total K$^+$ current can be distinguished pharmacologically as well as by inactivation. *A*, I_A is selectively blocked by 4-AP (5 mM). *a*, Total K$^+$ current elicited by a step from $V_{hold} = -100$ mV to $V_{test} = +20$ mV, before and in the presence of 4-AP. *b*, 4-AP has essentially no effect on I_K, the noninactivating current elicited by a step to +20 that is preceded by a 100-msec prepulse to −40 mV. *c*, Difference currents (*a*, *b*) showing the nearly complete block of I_A by 4-AP. *B*, I_K is selectively reduced by TEA$^+$ (25 mM). *a*, Total K$^+$ elicited by a step from $V_{hold} = -100$ to $V_{test} = +20$ mV before, during, and after exposure to TEA$^+$. *b*, TEA$^+$ causes a large reduction in I_K, elicited when the test step to +20 mV is preceded by a 100-msec prepulse to −30 mV. Note that this noninactivating current is reduced by approximately the same amount as the component of the total current, which is sustained until the end of the test pulse in *a*. *c*, The difference currents (*a*, *b*) demonstrate that the inactivating component (I_A) is not reduced by TEA$^+$. The small apparent enhancement of I_A by TEA$^+$ probably reflects the improved space-clamp conditions resulting from the block of I_K.

exhibit rapid decay during a sustained test potential. The time constant of I_A decay during a step to +20 mV was typically ~30 msec. Partial inactivation of the current could be observed in some cases at holding potentials as negative as −90 mV. The current was typically half-inactivated at −60 or −50 mV and could be completely inactivated by 100-msec prepulse potentials to −40 or −30 mV. The inactivating component could be eliminated by exposure to 4-AP (concentration, 5 mM) with no reduction of the noninactivating current component. This further substantiates the identification of the inactivating component as I_A (Fig. 6). I_A was not reduced by extracellular TEA$^+$ even at the high concentration of 25 mM.

The current referred to as I_K was not inactivated by brief depolarizing prepulses that eliminated I_A, decayed with a much slower time constant during sustained steps (typically 12–14 sec at +20 mV), and was not reduced in magnitude by 5 mM 4-AP (Fig. 6). I_K was substantially (~80–90%) but not fully blocked by 25 mM TEA$^+$.

Because EGTA (10 mM) was included in the internal solution, it seems unlikely that Ca^{2+}-activated K$^+$ currents ($I_{K(Ca)}$) would have contributed to the K$^+$ current we measured. Indeed, neither I_K nor I_A exhibited features of $I_{K(Ca)}$, such as N-shaped dependence of peak current on test potential. Very similar currents were also recorded with 10 mM BAPTA internally. Neither reducing nor elevating external Ca^{2+} concentrations (to 0 and 10 mM, respectively) had any effect on the K$^+$ currents recorded from single cells (with the possible exception of small shifts in voltage dependence).

Developmental changes in expression of K$^+$ currents. Both I_A

and I_K were present in at least some chick limb motoneurons at all stages of development studied (Fig. 7), and no changes in kinetic, voltage dependence, or pharmacological properties were observed during development. However, a large change in density of I_A did occur during development, while a relatively small change was seen in density of I_K. Quantitative comparisons of I_A and I_K expression in motoneurons isolated from different stage embryos were made according to the following criteria: potassium currents were elicited by stepping for 200 msec to a test potential of +20 mV (V_{test}), first from a holding potential of −100 mV, and then from a prepulse potential of −30 mV (duration 100 msec); I_K was measured as the current sustained until the end of the +20-mV test step that was preceded by the −30-mV prepulse; I_A was measured as the peak of the difference current obtained by digital subtraction of the 2 test currents and thus represents the current component inactivated by the prepulse at −30 mV.

At E4, potassium current was almost exclusively comprised of I_K, with I_A either small or absent. Therefore, 16 of 17 neurons expressed an I_A density of less than 10 pA/pF. By contrast, in E11 motoneurons, the smallest observed density of I_A was 23 pA/pF, and the largest was 143 pA/pF. I_A densities measured in E6 motoneurons were consistently intermediate between those measured in E4 and E11 motoneurons (Fig. 7B).

As illustrated in Figure 7A, an increase of approximately 16-fold was observed in mean density of motoneuron I_A between E4 and E11. Peak current densities at E4, E6, and E11 were 3.9 ± 1.4, 26.8 ± 3.5, and 65.5 ± 8.6 pA/pF ($n = 17$ at each age). During the same developmental span, I_K increased by only ap-
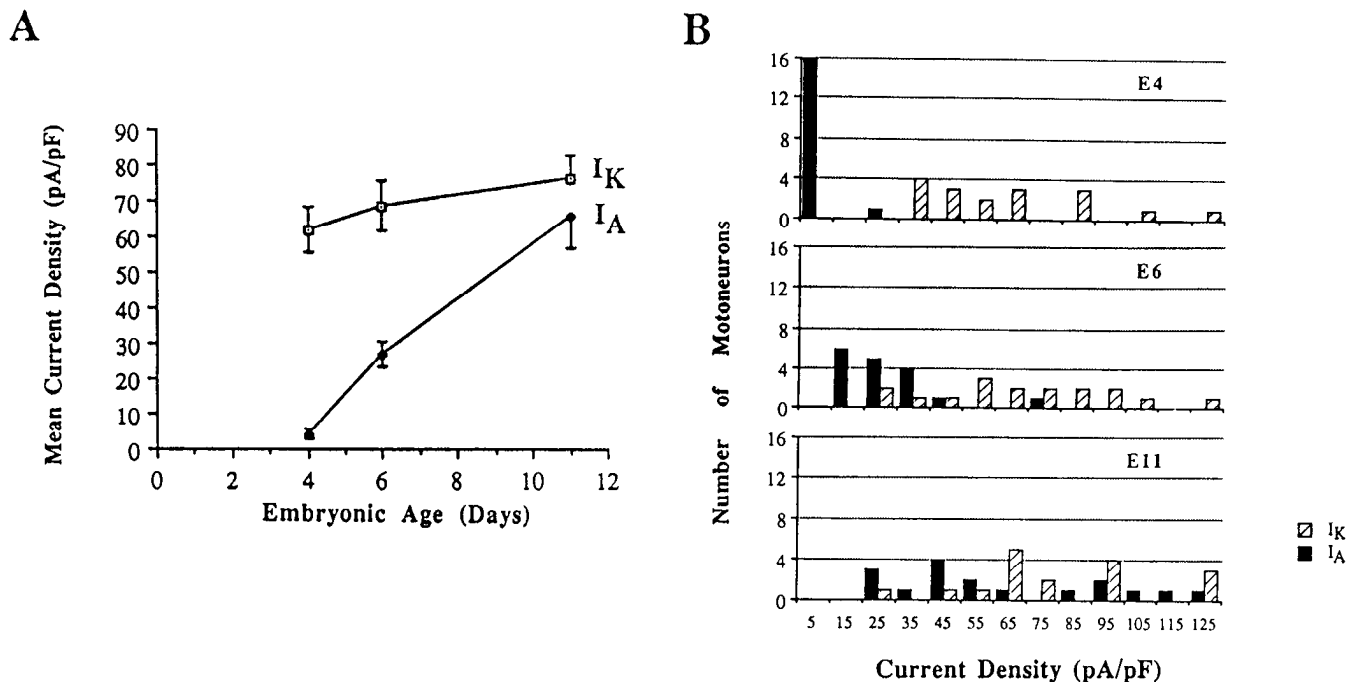
Figure 7. Development of K⁺ currents in chick limb motoneurons. *A,* Mean values (±SEM) of current densities (pA/pF) measured in motoneurons isolated at E4, E6, or E11 (*n* = 17 motoneurons for each stage). Currents were measured after 1 d in culture. Note that the expression of I_K is already appreciable at E4 and that the density of this current increases by only approximately 25% during the period from E4 to E11. By contrast, I_A is small or absent at E4 and increases in mean density by more than 16-fold by E11. Currents were recorded for 200-msec test pulses to +20 mV (V_{hold} = −100 mV) with and without a 100-msec duration prepulse to −30 mV. I_A was measured as the peak of the difference between currents with and without the prepulse, and I_K was measured as the current sustained until the end of the test pulse that was preceded by a prepulse. *B,* Histogram of I_A (*solid bars*) and I_K (*hatched bars*) densities measured in motoneurons from E4, E6, and E11 chick embryos. The distribution of I_K densities changed little during development, while I_A density values increased substantially between E4 and E6 and again between E6 and E11.

proximately 25%. Mean densities of I_K were 61.9 ± 6.3, 68.6 ± 6.9, and 76.2 ± 6.8 pA/pF, respectively (*n* = 17 at each stage).

Action potential maturation in chick limb motoneurons

Nearly all healthy motoneurons isolated from E4 and later-stage embryonic chicks were capable of generating overshooting action potentials in response to depolarizing pulses during current clamp recording. Action potential amplitudes increased with development between E4 and E11. Action potentials of E4 motoneurons peaked at between −2 mV and +30 mV (mean, 15.7 ± 3 mV; *n* = 12), whereas action potentials of E11 motoneurons peaked between +16 and +49 mV (mean, 27.3 ± 5 mV; *n* = 7). Action potential repolarization was typically much more rapid in E11 motoneurons than in earlier motoneurons. The time required for repolarization to a value halfway between the peak voltage and the voltage from which action potentials were evoked (between −81 and −85 mV) was measured (HRT; Fig. 8). HRT ranged from 1.6 to 8.4 msec for E4 motoneurons and from 1.0 to 1.8 ms for E11 motoneurons. Mean HRTs were 3.7 ± 0.6, 2.0 ± 0.2, and 1.5 ± 0.1 msec for E4, E6, and E11 motoneurons, respectively (*n* = 12, 13, and 12 motoneurons). The same developmental decrease in HRT was observed for action potentials evoked from −61 to −65 mV; however, the difference was smaller, presumably because these more depolarized potentials caused partial inactivation of I_A, a current that plays an important role in action potential repolarization (Fig. 9).

Pharmacological agents were used to determine which ionic currents control the form of the action potential. Motoneurons were inexcitable in TTX (1 µM), indicating that regenerative excitation is attributable to sodium current, with minimal contribution of calcium current. This is consistent with the relatively small size of calcium currents measured in these neurons (McCobb et al., 1989). Action potential duration was controlled by both potassium currents. Under conditions in which both I_A and I_K would be active, I_A played the dominant role in repolarization. This is illustrated in Figure 9: the addition of 4-AP alone caused significant prolongation of the action potential (Fig. 9*A,* upper panel), whereas the addition of TEA⁺ alone did not (Fig. 9*A,* lower panel). However, when I_A is not present (because it is blocked by 4-AP), the addition of TEA⁺ does cause broadening of the action potential (Fig. 9*B*).

The voltage dependence of I_A inactivation may play an important role in regulation of the duration of motoneuron action potentials. Action potentials elicited from a negative holding potential were much briefer than those elicited from a more positive potential (Fig. 9*C*). That the difference results from inactivation of I_A is supported by the similarity of the effect of 4-AP on action potential duration: as seen in Figure 9*C,* 4-AP (3 mM) prolonged the action potential elicited from −85 mV to about the same degree as did changing the holding potential to −54 mV. In voltage clamp experiments, I_A was found to be virtually eliminated by holding at −50 mV. This is consistent with the idea that voltage-dependent inactivation of I_A is largely responsible for the voltage dependence of action potential duration.

Figure 10 summarizes the above-described developmental changes in motoneuron action potentials, potassium currents,

and sodium currents by presenting representative recordings from motoneurons at the 3 embryonic ages studied. Action potential amplitude and duration show progressive increases and decreases, respectively, with development. The increase in the transient component (I_A) of the potassium current parallels the decrease in action potential duration, and the increase in I_{Na} parallels the increase in action potential amplitude. Note that only a small part of the increase in absolute current magnitudes, as shown here, can be explained by the developmental increase in motoneuron size (see Materials and Methods), while the larger part results from increases in current densities.

Discussion

Current- and voltage-clamp techniques were used to characterize the state of electrophysiological differentiation of chick limb motoneurons beginning at the earliest time that they can be positively identified and to chart changes occurring as the limb neuromuscular system proceeds through the early, and most important, phase of its differentiation. Three major currents appear to dominate the action potential mechanism of these neurons: a highly TTX-sensitive sodium current (I_{Na}), a relatively sustained, delayed-rectifier-type potassium current (I_K), and a more rapidly-activating, transient potassium current (I_A). Comparisons of current densities between motoneurons from embryos of different stages revealed large developmental changes. Consistent with the early appearance of TTX-sensitive action potentials, appreciable I_{Na} expression was observed in motoneurons isolated as early as E4. Substantial increase occurred thereafter as well, with a consequent increase in action potential amplitude. I_K expression was also already high at E4 but showed a more moderate increase thereafter, compared with I_{Na}. The development of I_A expression markedly differed from that of I_{Na} and I_K. I_A was small at E4 and increased 16-fold by E11. The developmental decline in the duration of the repolarization phase of the action potential can be explained by this increase in I_A. Interestingly, the expression of the 3 current types develops on different schedules, further supporting the idea that expression of individual currents can be under differential regulatory control (McCobb et al., 1989).

The sequence of development of ionic currents in chick limb motoneurons, in which Ca^{2+} current maturation (McCobb et al., 1989) lags behind I_{Na} and I_K, appears more similar to that reported for avian neural crest cells (Bader et al., 1983, 1985), rat sympathetic neurons (Nerbonne et al., 1986; Nerbonne and Gurney, 1989), and mouse spinal neurons (Krieger and Sears, 1988) than to that reported in amphibian spinal neurons (Barish, 1986; O'Dowd et al., 1988). In the latter neurons, the early development of $I_{Ca^{2+}}$ relative to I_{Na} and I_K enables the generation of very long duration ($\sim$100 msec) Ca^{2+}-dependent action potentials before much briefer Na^+-dependent action potentials (Baccaglini and Spitzer, 1977; Spitzer, 1985; Barish et al., 1986; O'Dowd et al., 1988). In the context of neuromuscular development, such Ca^{2+} action potentials could play an important regulatory role if they were to occur. In chick limb motoneurons, however, the very early development of I_K expression would tend to preclude long-duration action potentials. Furthermore, N- and L-type Ca^{2+} current density is very small at E4, the earliest stage studied (McCobb et al., 1989). At E4, T-type Ca^{2+} current density is larger than that of N- and L-types, but still small compared to I_{Na}. The subsequent developmental decrease in T-current density implies that any regenerative events de-
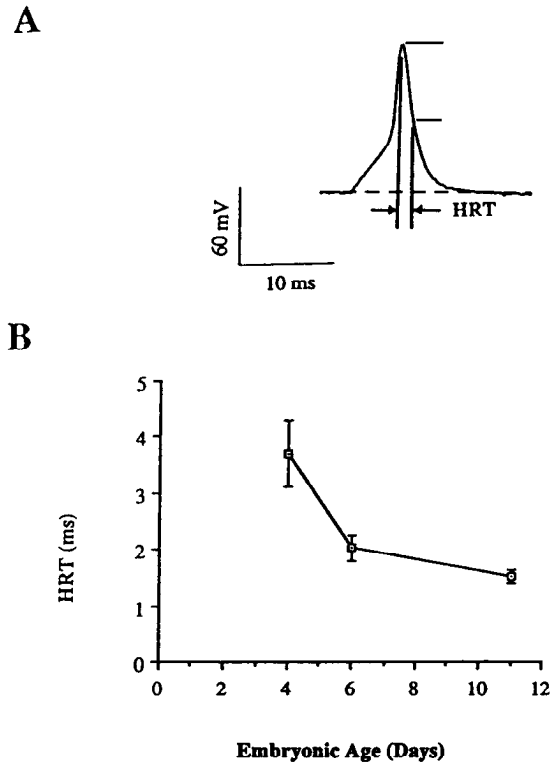

Figure 8. Developmental change in action potential duration. *A*, HRT is defined as the time between the peak of the action potential and the time at which the membrane potential has returned to the level halfway between the peak and the potential prior to the action potential stimulus. *B*, Mean (±SEM) HRT values for action potentials elicited from E4, E6, and E11 motoneurons (n = 12, 13, and 12, respectively). For each neuron, several action potentials were evoked from holding potentials ranging from −81 to −85 mV, and HRT values were averaged. See Figure 10 for representative action potential waveforms at the different stages.

pendent on this current would have to occur very early in motoneuron maturation.

I_A activates more rapidly than I_K and thus brings about more rapid action potential repolarization. Therefore, while early development of I_K prevents very long duration action potentials, action potential duration still declines substantially with the development of I_A. Furthermore, the voltage-dependent inactivation of I_A provides more mature motoneurons with a mechanism for adjusting action potential duration as a function of membrane potential. The relatively late appearance of I_A and its effects on the action potential have also been reported in rat sympathetic neurons (Nerbonne et al., 1986) and unidentified spinal neurons of the mouse and *Xenopus* (Krieger and Sears, 1988; Ribera and Spitzer, 1990). Interestingly, in *Drosophila,* flight muscle I_A develops before the delayed rectifier current rather than after it (Salkoff and Wyman, 1981).

The developmental changes in whole-cell current density reported here are most easily explained by changes in ion channel numbers rather than modification of single channel properties (e.g., conductance, open probability, ion selectivity). While measured single channel properties change with neuronal development (Blair and Dionne, 1985; Harris et al., 1988; Yool et al., 1988), it is not clear whether such changes result from expression of new channels or modification of properties of channels already expressed. In the present studies, the consistency, over
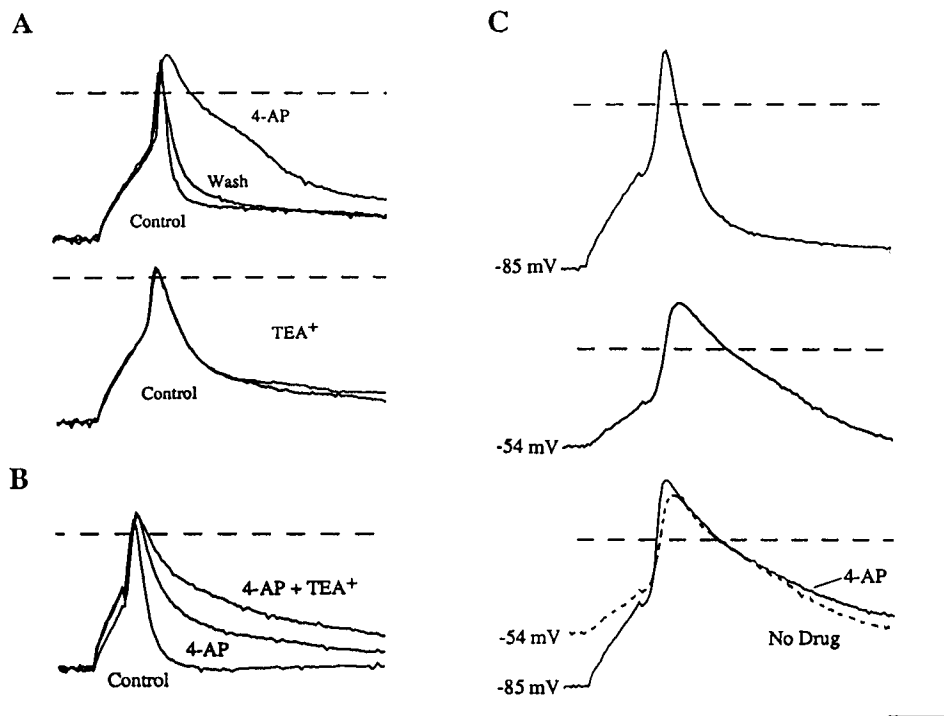
Figure 9. Contributions of 4-AP-sensitive (I_A) and TEA$^+$-sensitive (I_K) currents to action potential repolarization in motoneurons. *A*, Action potentials in an E6 motoneuron were evoked from a holding potential of -80 mV before, during, and after exposure to 5mM 4-AP (*top set of traces*) and 25 mM TEA$^+$ (*bottom set of traces*). TEA$^+$ alone had little effect on action potential repolarization, whereas 4-AP alone prolonged the action potential considerably. *B*, Although TEA$^+$ alone has little effect on action potential repolarization, TEA$^+$ (25 mM) does cause a substantial prolongation when it is added after I_A has been blocked by 4-AP. *C*, Depolarized holding potentials produced action potential broadening comparable to that produced by 4-AP. In the absence of 4-AP, an action potential elicited from a holding potential of -54 mV (*center panel*) is broadened relative to the action potential elicited from -85 mV (*top panel*). The *bottom panel* compares the action potential elicited from -54 mV in the absence of drug (replotted as the *dashed trace*) with an action potential elicited from -85 mV in the presence of 3 mM 4-AP (*solid trace*). The similarity of the repolarization phase of the 2 traces suggests that depolarization-induced broadening of the action potentials results from inactivation of I_A. Calibration bars: horizontal, 10 msec; vertical, 30 mV.

development, of voltage dependence, kinetic, and pharmacological features of the 3 current components suggests that changes in channel expression outweigh modification of channel properties and are sufficient to explain the observed changes in the action potential.

While longitudinal studies of individual developing neurons would be ideal, we believe that the population studies reported here present a valid picture of the general progression of motoneuron development. Between E4 and E6, the population is relatively stable, because only a small percentage of motoneurons are born after E4 (Hamburger, 1977). After E6 and before E11, approximately 50% of the population succumbs to cell death (Hamburger, 1975, 1977). Although we cannot exclude the possibility that cells fated to die *in vivo* may not have survived to be sampled *in vitro*, it seems likely that the data for E4, at least, and probably E6, as well, represent motoneurons of both fates. We have not yet been able to resolve a bimodal distribution of any electrophysiological feature that might predict the fate of individual cells.

Ideally, the quantitative measurements reported here would have been made immediately after spinal cord dissociation to avoid the complication of possible changes in ionic currents in culture. However, acutely isolated neurons were too delicate to record from reproducibly, and recordings were made more easily and consistently when the neurons were given a day to recover from the trauma of dissociation. Voltage- and current-clamp recordings made on acutely dissociated motoneurons did give

results qualitatively similar to those obtained a day later. Motoneurons acutely isolated from E4 embryos exhibited I_{Na} and action potentials (as well as I_K), supporting the very early differentiation of excitability per se. Cells allowed to remain in culture longer than 1 d after dissociation grew neurites extensive enough to interfere with the attainment of good voltage control (space clamp). Therefore, we do not yet know whether expression of currents changes quantitatively with time *in vitro*.

Necessarily, the procedures for spinal cord dissociation differed between embryonic stages (see Materials and Methods). Could the differences contribute to differences in measured current densities? We were consistent for each developmental stage in sampling the healthiest motoneurons in each of a minimum of 5 separate, and by visual inspection comparably healthy, experimental cultures. The trends of developmental increases in I_{Na} and I_A densities were pronounced and were seen in both the absolute range of values observed and the mean values. Furthermore, the different schedule of changes in I_{Na}, I_A, and I_K make it very unlikely that the developmental patterns result from treatment differences. Ca^{2+} currents measured in motoneurons from the same stages showed a pattern even less likely to arise from treatment differences, with developmental increases in N- and L-type Ca^{2+} currents coinciding with a large decrease in T-type current (McCobb et al., 1989)

How might changes in intrinsic excitability of motoneurons affect neuromuscular system development? Excitability and action potential waveform are very important determinants of
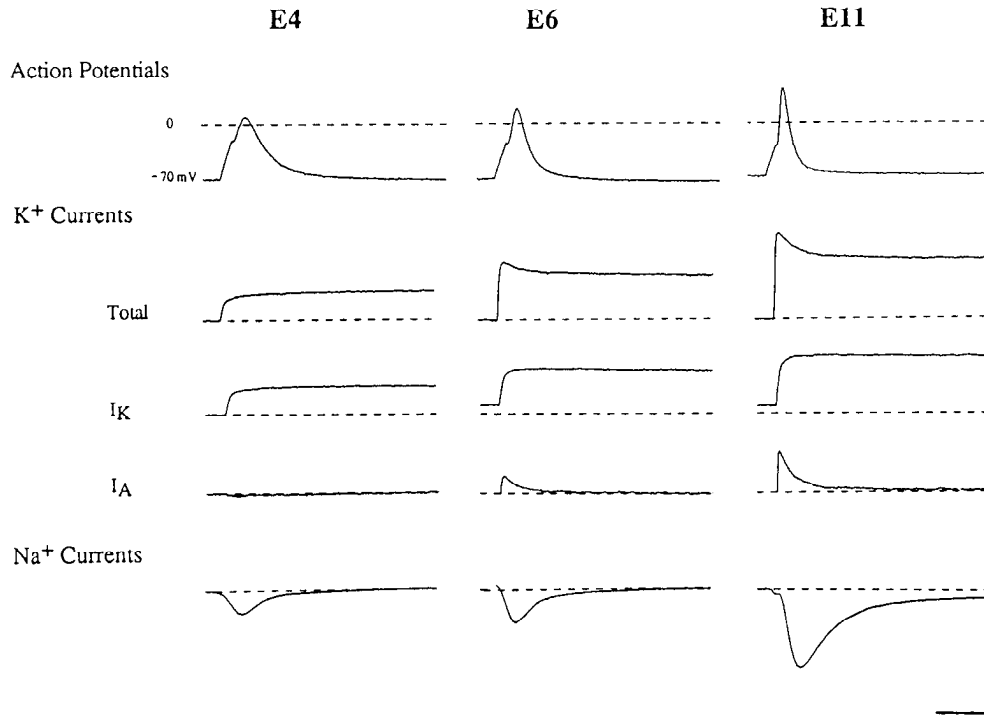
E4   E6   E11

Action Potentials

Figure 10. Summary of changes in electrophysiological properties of chick hindlimb motoneurons. Representative examples of action potentials and ionic currents recorded from motoneurons isolated at embryonic ages are indicated. Note the progressive increase in action potential amplitude and decrease in duration. Action potentials were elicited from a holding potential of −70 mV. Potassium currents show a large proportionate increase in the rapidly activating transient component, identifiable as I_A. Sodium current amplitude increases progressively with development. Horizontal calibration bar: 10 msec, 75 msec, and 2 msec for action potentials, potassium currents, and sodium currents, respectively. Vertical calibration bar: 52 mV for action potentials, 2 nA for all currents.

Ca^{2+} entry through voltage-gated channels. These features are changing substantially even as Ca^{2+} currents themselves are differentiating (McCobb et al., 1989). This adds support to the suggestion already put forth that changes in voltage-dependent Ca^{2+} entry in motoneurons might control neuromuscular differentiation (McCobb and Beam, 1990). Motoneuronal cell death, for example, could be affected by Ca^{2+} entry as a result of either indirect or direct mechanisms. An *indirect* mechanism is suggested because voltage-dependent Ca^{2+} entry controls trans-synaptic activation of muscle contraction, which in turn influences 'motoneuron survival during the period of normal cell death *in vivo*. Specifically, blocking activity increases survival (Laing and Prestige, 1978; Pittman and Oppenheim, 1978, 1979), while increasing activity decreases survival (Oppenheim and Núñez, 1982). This raises the possibility that the timing of differentiation of excitability in motoneurons triggers cell death by enabling the onset of neuromuscular transmission. *Directly,* increasing Ca^{2+} entry might either make cells more vulnerable to death (Choi, 1987) or protect against death (as does entry through L-type channels in several neuronal types *in vitro*; Collins and Lile, 1989; Koike et al., 1989). It is interesting to speculate that the development of I_A (and rapid repolarization) might play a role that counterbalances the effect of increasing excitability. Thus, by limiting Ca^{2+} entry during the action potential, this current might decide the fate of individual motoneurons with respect to cell death.

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