Development of Larval Muscle Properties in the Embryonic Myotubes of *Drosophila melanogaster*

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The entire developmental history of muscle membrane electrogenesis can be observed in the embryonic myotubes of *Drosophila*. We have examined the development of ionic currents and muscle properties using whole-cell patch-clamp techniques throughout embryonic myogenesis. In the early stages of myogenesis, from myoblast fusion through to establishing epidermal insertions, the myotubes are electrically inert and are electrically and dye coupled to adjacent myotubes. Membrane electrogenesis begins in the mid-embryonic stages (early stage 16), when the myotubes abruptly uncouple, revealing the first of five prominent extrajunctional currents: a small, inward, voltage-gated calcium current (I_{Ca}). The uncoupling of the embryonic myotubes heralds the onset of extremely rapid electrogenesis; within several minutes both the fast, inactivating (I_A; *Shaker*) and delayed, noninactivating (I_K) outward potassium currents, the stretch-activated outward potassium current, and the junctional glutamate-gated inward current all appear and begin to develop in a current-specific manner. Very late in embryogenesis (late stage 17), the calcium-dependent, outward potassium currents [rapid, inactivating (I_{CF}; *slowpoke*) then delayed, noninactivating (I_{CS})] develop, completing the complement of macroscopic currents in the mature larval muscle. Hence, the voltage-gated currents (I_{Ca}, I_A, and I_K, respectively) appear relatively early, and the calcium-dependent currents (I_{CF}, I_{CS}) appear only very late during myogenesis. This developmental progression of current maturation is reflected in dynamic changes in the voltage responses of the embryonic membrane, from wholly passive response to current injection in the early, coupled myotubes to regenerating, overshooting action potentials in the mature embryonic muscle.

The earliest embryonic I_A current has a midpoint of inactivation 40 mV more negative than the I_A current in the mature embryo. As myogenesis proceeds, the inactivation curve develops a biphasic character, suggesting that a low-inactivation I_A channel is present in early development and progressively replaced by the mature form as development proceeds. The current at all stages can be completely eliminated in *Shaker* mutants (Sh^{KS133}). These findings suggest that an embryonic form of the *Shaker* I_A channel is present during early myogenesis. The prominent I_A current present in early development is almost entirely inactivated at the physiological resting potential; the significance and mechanism of this developmental shift are unclear.

[*Key words: Drosophila, myogenesis, muscle development, excitable membranes, potassium currents, Shaker, slowpoke, calcium currents, invertebrate embryogenesis*]

Within 12 hr, the musculature of the *Drosophila* embryo develops from unfused myoblasts to contractile, syncytial muscle fibers with all the characteristics of the mature larval muscles. During this period of rapid differentiation, the myoblasts fuse to form an intricate pattern of syncytial myotubes, the myotubes extend filopodial processes on the epidermis and establish specialized epidermal insertion sites, and the myotubes differentiate a wide array of specialized muscle properties to become functional, contractile larval muscles. In previous work (Bate, 1990), we have examined the earlier stages of this myogenic pathway: the patterning of the fusing myoblasts and the establishment of muscle-specific epidermal insertion sites. In the present study, we focus attention on the later stages of myogenesis: the development of muscle-specific properties in the fully patterned myotubes during late embryogenesis, with particular emphasis on the electrogenesis of the ionic currents responsible for the generation and regulation of the muscle contractile properties.

The ionic currents underlying *Drosophila* larval muscle properties have been the subject of intense study (Salkoff and Wyman, 1981; Wu and Haugland, 1985; Ganetzky and Wu, 1986; Singh and Wu, 1990). Five voltage-gated currents control muscle excitation in the mature larval muscles. These currents include an inward calcium current (I_{Ca}) and four outward potassium currents; two voltage-gated K^+ currents, the fast I_A and delayed I_K, and two calcium-gated K^+ currents, the fast I_{CF} and delayed I_{CS}. In addition, there is a glutamate-gated current at the neuromuscular junction (NMJ) (Jan and Jan, 1976a,b; Broadie and Bate, 1992). These elements combine in an excitation–contraction cascade that follows familiar lines: a nerve impulse triggers glutamate release at the NMJ, eliciting a nonspecific cation current at the postsynaptic membrane; the muscle is depolarized, thus opening voltage-gated calcium channels, mediating muscle contraction. Outward potassium currents, both voltage and calcium gated, open, counteracting the depolarizing calcium current and returning the muscle to its resting state.

Several of the ionic channels underlying these currents have been characterized at a physiological and genetic level. In particular, the I_A channel is known to be encoded by the *Shaker*

Table 1. Whole-cell currents in different genotypes at 16 hr AEL

	I_A	I_K	I_{Ca}
Wild-type	441 ± 38	164 ± 11	78 ± 14
Sh	(0)	168 ± 13	85 ± 12
slo	462 ± 47	176 ± 16	90 ± 17
Sh; slo	(0)	172 ± 24	74 ± 21

This table provides a comparison of whole-cell embryonic muscle currents (I_A, I_K, and I_{Ca}) at 16 hr AEL in the different genotypes used in this study. The *Sh*^{KS133} mutant specifically eliminates I_A but does not significantly alter I_K or I_{Ca}. Likewise, the *slo* mutant specifically eliminates I_{CF} (develops later; see Fig. 7) but does not significantly alter I_A, I_K, or I_{Ca}. Each value is the mean $\pm$ SD of at least five independent observations.

locus (Jan et al., 1977; Salkoff and Wyman, 1981) and the I_{CF} channel encoded by the *slowpoke* (*slo*) locus (Elkins et al., 1986; Elkins and Ganetzky, 1988). Mutations in both genes allow the specific removal of their gene products and subsequent physiological analysis of the remaining muscle currents. Conclusions based on these mutant analyses can be extended and confirmed with conventional ion-substitution and pharmacological blocking experiments (Singh and Wu, 1990). Studies using cultured embryonic myotubes (Zagotta et al., 1988) have shown that these cells are amenable to whole-cell patch-clamp analysis. We have developed techniques to dissect intact embryos throughout embryogenesis and expose the developing somatic muscles to a detailed analysis in whole-cell patch-clamp configuration using a combination of all these tools. This detailed characterization is unique to *Drosophila* and provides invaluable tools for the dissection of the complex whole-cell current.

In the present study, we employ these methods to dissect the physiological development of the maturing myotubes during *Drosophila* embryogenesis. The early embryonic myotubes have no detectable voltage-gated channels, but by the end of embryogenesis, the mature embryonic muscle has all five prominent extrajunctional current-carrying systems and a large synaptic current at the NMJ. We find that these currents appear and develop according to a strict developmental program. The earliest myotubes are electrically coupled and appear electrically inert, with no channel activity detectable in cell-attached patches. A small, inward calcium current is the first macroscopic current revealed after the myotubes uncouple. Uncoupling heralds the rapid onset of electrogenesis, but it is unclear if this sequence represents cause and effect, or is simply incidental. The voltage-gated potassium currents (I_A, I_K), a stretch-activated potassium current, and the synaptic glutamate-gated current all appear within minutes of myotube uncoupling. The calcium-gated potassium currents (I_{CF}, I_{CS}) do not appear during this initial stage of electrogenesis, arising only very late in embryogenesis and contributing negligibly to the whole-cell current.

Most of the embryonic currents appear indistinguishable from the mature currents in the larval muscles. A notable exception is the early embryonic I_A current, which has a midpoint of inactivation 40 mV more negative than the mature current. It appears that the I_A current is progressively replaced by a current with the mature inactivation profile during development. However, the I_A current at all stages can be eliminated with a null mutant allele of the *Shaker* locus (*Sh*^{KS133}), indicating that both embryonic and mature I_A channels are, at least in part, encoded by the *Shaker* gene. Since the initial I_A current has such a negative inactivation curve, it is apparently inactive at the physi-

ological resting potential. Thus, though the I_A current amplitude matures rapidly in early myogenesis, functionally the myotube may experience a much later and gradual onset of the mature I_A form. The mechanism and significance of these dynamic modifications are unclear.

The different components of the whole-cell current appear at very different times, develop with very different time courses, and differ in function according to a dynamic alteration in an inactivation profile. The development of these currents is reflected in the rapidly changing voltage response of the embryonic myotube membrane, which changes from a wholly passive response in early myogenesis, to a strongly suppressed response during mid-embryogenesis, and eventually to a regenerating, overshooting active response in the mature embryo. The functional significance of these changes, if any, remains to be investigated.

Materials and Methods

Current nomenclature

A variety of names have been used previously for the Ca^{2+}-gated K^+ currents in *Drosophila*. The fast, inactivating current has been designated I_{Acd} (Salkoff, 1983), I_C (Elkins et al., 1986), I_{Ac} (Wei and Salkoff, 1986), and I_{CF} (Singh and Wu, 1990). The slow, noninactivating current has been designated I_{Kc} (Wei and Salkoff, 1986), I_c (Gho and Mallart, 1986), and I_{CS} (Singh and Wu, 1990). We adopt the nomenclature of Singh and Wu (1990), chosen to maintain consistency with conventional names in other species, that is, I_{CF} for the fast, and I_{CS} for the slow Ca^{2+}-activated K^+ current.

Fly stocks

The wild-type *Drosophila melanogaster* strain Oregon R was used. A mutant strain [*foreclosed* (*fcl*^{244b})], which fails to close dorsally and has a weak cuticle, was used to facilitate study of late embryonic stages when a tough wild-type cuticle makes dissection difficult. However, in all cases, experiments with the mutant were confirmed using dissected and/or cultured wild-type Oregon R embryos.

A mutant allele of the *Shaker* locus, *Sh*^{KS133}, was used to eliminate the I_A current (Jan et al., 1977; Salkoff and Wyman, 1981; Wu and Haugland, 1985). The only available allele of the *slowpoke* (*slo*) locus was used to eliminate the I_{CF} current (Elkins et al., 1986; Elkins and Ganetzky, 1988). An *Sh;slo* double mutant, courtesy of Dr. Chun-Fang Wu (Singh and Wu, 1990), was used to eliminate both I_A and I_{CF}. Neither mutation causes a detectable alteration in any other current in the mature larval muscles (Singh and Wu, 1990). We found that this specificity was also true in the developing embryonic muscle (Table 1).

Preparation

Breeding flies were maintained on apple juice agar plates at 25°C and allowed to lay eggs overnight. The eggs were collected, dechorionated in commercial bleach, and placed in a plastic culture dish for staging. Embryos staged by morphological criteria (Campos-Ortega and Hartenstein, 1985; K. S. Broadie, unpublished observations) to a narrow well-defined developmental time window—usually between the first and second constrictions of the developing gut, a period of < 10 min at 25°C—were selected and allowed to continue development to the desired stage. All development times are reported in hours after egg laying (AEL) at 25°C and are recorded as decimals. Under these conditions, embryogenesis lasts 21 $\pm$ 1 hr.

All experiments were performed on dissected whole-embryo preparations. Younger embryos (<17 hr AEL) were transferred to polylysine-coated coverslips under normal saline, removed from the vitelline membrane, cut along the dorsal midline with a glass electrode, and attached to the coverslip surface with a gentle stream of saline. Older embryos (>17 hr) were transferred to coverslips coated with a thin layer of Sylgard under normal saline, cut along the dorsal midline with a fine metal blade, and pinned to the Sylgard with glass pins. The gut was then removed exposing the ventral nervous system, peripheral nerves, and somatic musculature. All experiments were performed on the large ventral internal-longitudinal muscle (muscle 6; Crossley, 1978) in anterior abdominal segments A2–A4.

Whole embryo culture and recording solutions

The late embryo (>17 hr AEL) has a tough cuticle that makes dissection and attachment to conventional substrates difficult. As a result, we have cultured dissected whole embryos from earlier, more amenable stages. Embryos were dissected at 16 hr AEL (as above) and cultured in a small drop (20 μl) of modified M3 (MM3) medium (Shields and Sang, 1977; Currie et al., 1988) in a humid chamber at 25°C. The MM3 culturing medium was similar to previous published reports with the following exceptions/additions: (1) the glutamate salts were replaced with equimolar aspartate salts, (2) 0.125 IU/ml insulin (Sigma) was added, and (3) 2% fetal calf serum (Sigma), filtered but not heat inactivated, was added. Under these conditions, the embryo preparations appeared to develop normally as judged by all available morphological, immunohistological, and physiological criteria, with the exception that the trachea fails to fill with air. Experiments with cultured preparations were independently confirmed with freshly dissected embryos.

Physiological recordings were performed with normal fly salines. The bath consisted of (in mmol/liter) 135 NaCl, 5 KCl, 4 $MgCl_2$, 2 $CaCl_2$, 5 TES (N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid), and 36 sucrose. The calcium concentration was varied as indicated in the figure captions: a calcium-free bath was prepared by removal of $CaCl_2$ and addition of 14 mM $MgCl_2$ and 0.5 mM EGTA; a high-calcium solution was prepared with 20 mM $CaCl_2$. The intracellular solution consisted of (in mmol/liter) 120 KCl, 20 KOH, 4 $MgCl_2$, 5 TES, 5 EGTA, 0.25 $CaCl_2$, 4 ATP, 4 GTP, and 36 sucrose. To study calcium currents, KCl was replaced with CsCl as indicated in the figure captions. The pH of all solutions was buffered at 7.15.

Isolation of ionic currents

The whole-cell voltage-gated current is composed of an inward calcium current (I_{Ca}) and four outward potassium currents (Fig. 1); two fast, inactivating currents, voltage gated (I_A) and calcium dependent (I_{CF}); and two delayed, noninactivating currents, voltage gated (I_K) and calcium dependent (I_{CS}). In these experiments, we have used mutations and ion substitutions to dissect these currents from the whole-cell response during voltage clamp.

Calcium current (I_{Ca}). CsCl was substituted for KCl in the patch pipette. Intracellular K^+ was replaced with Cs^+ via perfusion in whole-cell configuration for 5 min. In this configuration, no outward K^+ currents could be recorded in a Ca^{2+}-free bath. Thus, the inward I_{Ca} could be recorded in the absence of all four outward potassium currents.

Rapid, voltage-gated potassium current (I_A). External calcium was removed (see above), eliminating both the inward calcium current and the calcium-dependent potassium currents, I_{CF} and I_{CS}. I_A and I_K can be temporally separated. Alternatively, I_A can be specifically eliminated in Sh^{KS133} mutants and I_A amplitude obtained by subtraction (wild-type − Sh). There was no significant difference in I_A amplitude assayed with the two methods; the first method was routinely used.

Rapid, calcium-dependent potassium current (I_{CF}). Sh^{KS133} was used to eliminate I_A. I_{CF} can be temporally separated from the delayed currents (I_K, I_{CS}); I_{Ca} must be subtracted. Alternatively, I_{CF} can be specifically eliminated using the *slo* mutation and I_{CF} amplitude obtained by subtraction (wild-type − *slo*). There was no significant difference in I_{CF} amplitude assayed with the two methods; the first method was routinely used.

Delayed, voltage-gated potassium current (I_K). Sh^{KS133} was used to eliminate I_A. External calcium was removed, eliminating both the inward calcium current and both calcium-dependent potassium currents (I_{CF}, I_{CS}). In this configuration, I_K can be studied in isolation.

Delayed, calcium-dependent potassium current (I_{CS}). An *Sh;slo* double mutant was used to eliminate I_A and I_{CF}, respectively. I_{CS} was obtained by subtracting I_K and I_{Ca}, both of which can be studied in isolation.

In these experiments, only I_K and I_{Ca} could be studied directly in the absence of all other ionic currents. I_A could be studied in the presence of only the delayed I_K, which can be temporally resolved from the I_A peak. Values for I_{CF} and I_{CS} were obtained only from multiple current traces; I_{CF} can be specifically eliminated (*slo*), but I_{CS} can be measured only in recordings in which the other current components were identified and subtracted as detailed above.

L-Glutamate iontophoresis

The glutamate-gated current at the developing neuromuscular junction (NMJ) was studied by L-glutamate iontophoresis. A stock solution of 0.1 M L-glutamate (monosodium salt; Sigma) of pH 8 was made fresh
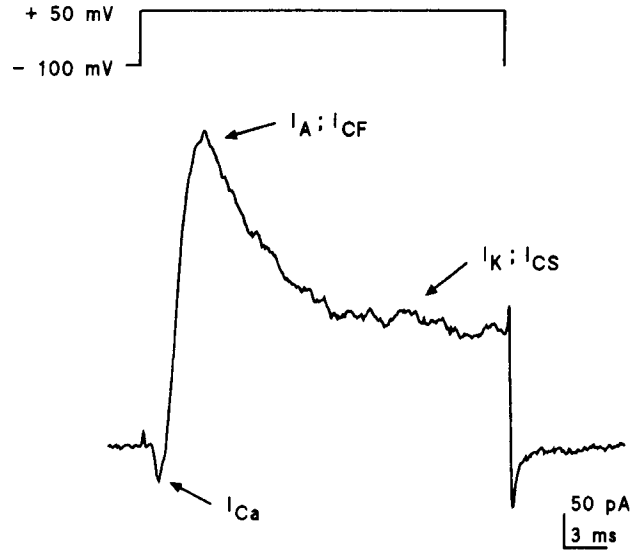

Figure 1. The whole-cell voltage-gated currents in the late embryonic myotube 6 (20 hr AEL). Five currents underlie the whole-cell response to a voltage step (−100 to +50 mV), an inward calcium current (I_{Ca}) and four outward potassium currents: two fast currents, voltage gated (I_A) and calcium dependent (I_{CF}); and two delayed currents, voltage gated (I_K) and calcium dependent (I_{CS}). The fast and slow K^+ currents have different activation rates and can be temporally separated. The fast K^+ currents (I_A, I_{CF}) rapidly inactivate and so do not contribute to the noninactivating current response, composed of the outward I_K and I_{CS} and the inward I_{Ca}. The inward Ca^{2+} current is largely masked by the outward K^+ currents.

daily. Iontophoretic pipettes were pulled with resistances of 50–75 MΩ. Glutamate was iontophoresed with short pulses (<1 msec) of negative current (10 nA; Farnell pulse-generating system) at the developing NMJ zone (preceding companion article, Broadie and Bate, 1992). Glutamate leakage between pulses was prevented with a small, positive backing current.

Electrophysiology: whole-cell patch-clamp techniques

For physiological experiments, the preparation was placed in a small perspex recording chamber and viewed in transmitted light with a compound microscope (Micro Instruments Ltd.) fitted with differential interference contrast (Nomarski) optics and a 40× water-immersion lens. Whole-cell recordings were made at room temperature (18–22°C) with patch pipettes pulled (Narishige patch-electrode puller) from borosilicate glass (fiber filled) with tips fire polished to final resistances of 5–8 MΩ. Whole-cell recordings were achieved using standard patch-clamp techniques (Hamill et al., 1981; Marty and Neher, 1983). Signals were amplified using an Axopatch-1D (Axon Instruments) patch-clamp amplifier, filtered with an 8-pole Bessel filter at 2 KHz, and either sampled on line or digitally stored on a modified digital audio tape recorder (Sony) for later analysis. Data were analyzed using pCLAMP version 5.51 software (Axon Instruments) on a Viglen III/33 MHz computer.

Seal resistances were typically >10 GΩ. Whole-cell configuration was achieved easily with slight suction, and input resistance of the myotube was in the range of 200–500 MΩ. With maximum currents less than 1 nA, series resistance errors (total current × series resistance) are kept to reasonable levels (usually <10 mV) and were not corrected. Myotubes with these characteristics, average diameter of 12 μm, and average length of 36 μm, should show reasonable space clamp. Series resistances were taken from the series resistance control on the amplifier and monitored throughout the experiment; typical values ranged from 10 to 25 MΩ. Cell capacitances were between 7 and 14 pF, increasing with developmental age, generating clamp time constants ($R_{series} \times C_{cell}$) averaging less than 0.25 msec. Though cell capacitance increased with developmental age, the size of the cell was not significantly increased. We hypothesize that this capacitance increase arises rather through the elaboration of the muscle tracheole system during late embryogenesis or some other changes in membrane properties. Because of this uncer-
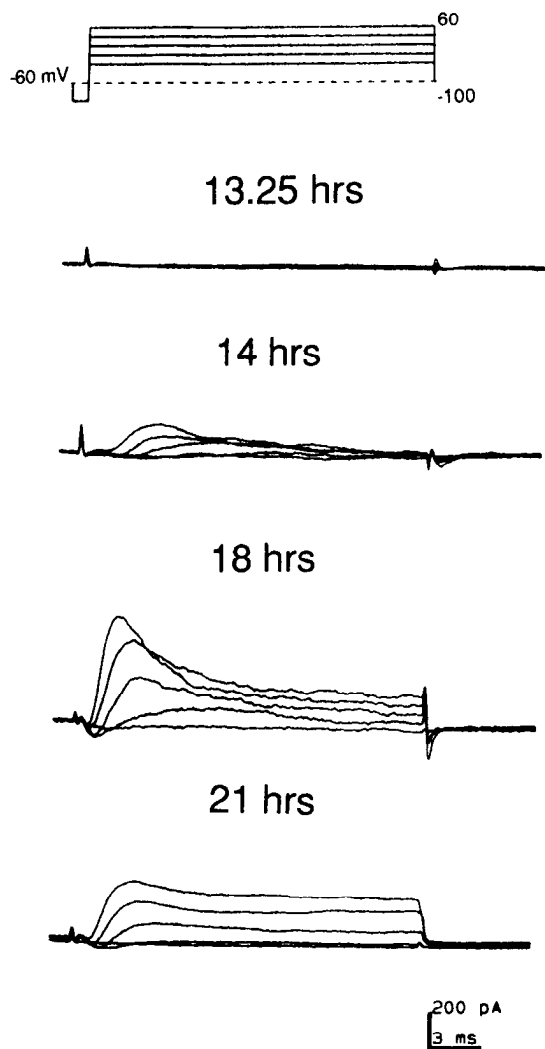
Figure 2 Development of the whole-cell voltage-gated currents in the embryonic myotube 6. At the earliest time point (13.25 hr AEL), immediately after the myotubes uncouple, no net current response is observed. Whole-cell currents appear soon thereafter (14 hr AEL), reach a peak in the late embryo (18 hr AEL), and decline prior to hatching (21 hr AEL). The outward K^+ currents largely mask the inward Ca^{2+} current, which is therefore difficult to discern, especially during early development. The myotubes are voltage clamped at -60 mV; a 2 sec, -100 mV prepulse precedes the voltage step to eliminate inactivation. Superimposed traces show voltage steps to $-20, 0, 20, 40$, and 60 mV. Each trace is the average of five trials.

tainty, current amplitude rather than current density is displayed in voltage-clamp recordings.

Two voltage-clamp protocols were used routinely to monitor voltage-gated currents: (1) an activation protocol that involved a 2 sec prepulse at -100 mV, to remove inactivation, and a voltage step to increasingly positive values in 20 mV increments; on-line leak subtraction was routinely used; and (2) a steady-state inactivation protocol that involved a 2 sec prepulse starting at 0 mV and decreasing in 20 mV increments, and a voltage step to a constant $+60$ mV. No leak subtraction was employed in this configuration.

Results

The embryonic musculature and development of muscular activity

Each hemisegment of the *Drosophila* embryo (A2–A7) has 30 syncytial muscle fibers innervated by two peripheral nerves: the anterior intersegmental nerve, which innervates the dorsal mus-

culature, and the posterior segmental nerve, which innervates the ventral muscles. In this developmental analysis, we have focused on a single identifiable muscle fiber: the large, ventral, internal-longitudinal muscle (muscle 6; Crossley, 1978) in anterior abdominal segments A2–A4. This muscle has been characterized physiologically and morphologically in the larva (Jan and Jan, 1976a,b; Singh and Wu, 1990) and morphologically examined in the embryo (Bate, 1990), and its size and position make it the most amenable muscle for embryonic manipulation.

Previous studies (Bate, 1990) have examined the origin and time course of early myogenic events in the *Drosophila* embryo. The embryonic myotubes arise through the fusion of mesodermal myoblasts during the second quarter of embryogenesis, followed by the establishment of epidermal insertion sites prior to dorsal closure (Bate, 1990). By 13 hr AEL, at the completion of dorsal closure (early stage 16), the mature muscle pattern is complete, but the muscles remain quiescent and are noncontractile when electrically stimulated or challenged with L-glutamate, the neurotransmitter at the NMJ. By 13.5 hr AEL, the myotube will contract when electrically stimulated, and endogenous contractions begin soon after (13.75–14 hr AEL). In the following period, endogenous muscle contractions increase gradually in frequency; initially only isolated myotubes contract, but by 14.5 hr AEL, coordinated, peristaltic contractions of entire hemisegments begin. At 16 hr AEL, an abrupt acceleration of peristaltic muscle contractions begins, and frequent muscle contractions, characteristic of larval locomotory movements, persist through the end of embryogenesis.

As in vertebrates (Dennis et al., 1981), the myotubes of *Drosophila* are electrically and dye coupled through much of their embryonic development (Johansen et al., 1989; Broadie and Bate, 1992), allowing the spread of depolarization laterally between fibers. Between 13–13.25 hr AEL, the myotubes abruptly uncouple (Broadie and Bate, 1992), immediately prior to the rapid maturation of muscular activity. We note that this abrupt uncoupling closely correlates with the initial stages of electrogenic development.

Development of whole-cell currents

As in the third instar larva, the muscles of the mature *Drosophila* embryo have five prominent voltage-gated extrajunctional ionic currents (Fig. 1): an inward calcium current and four outward potassium currents. When viewed in whole-cell configuration, these five components interact to generate a complex current response to a controlled voltage change. It is possible to follow the evolution of this response throughout myogenesis in the *Drosophila* embryo (Fig. 2). In the immature embryonic myotubes, prior to dorsal closure (stage 16, <13 hr AEL), the myotube membrane contains no voltage-gated channels as assayed in cell-attached patches (no activity in 25 patches) and the myotube appears electrically inert. When whole-cell voltage-clamp becomes feasible, after the myotubes abruptly uncouple (13–13.25 hr AEL), no whole-cell current is generated in response to voltage changes (Fig. 2). However, electrogenesis of voltage-gated currents begins soon thereafter (by 13.5 hr AEL). Initially (13.25–13.5 hr AEL), this activity is restricted to single-channel openings in response to a voltage step in cell-attached patches. During this period, we also observe for the first time the prominent stretch-activated K^+ currents identified by earlier workers (Aldrich et al., 1989; data not shown), but no quantitative assay was undertaken.

Whole-cell voltage-gated currents can first be recorded at 13.5 hr AEL. The initial whole-cell current has an activation thresh-
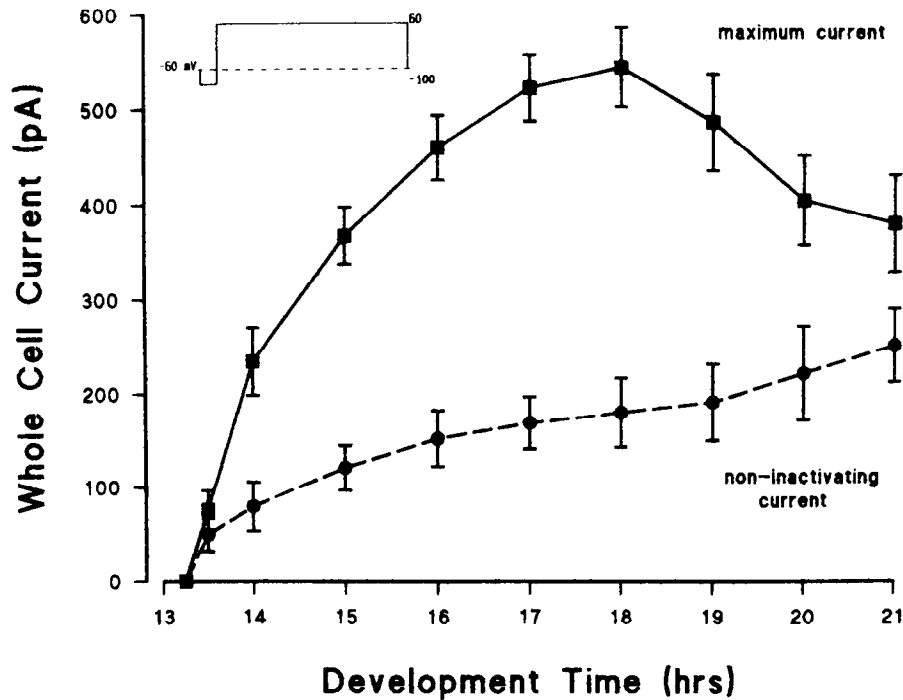
Figure 3. Development of the whole-cell voltage-gated current in the embryonic myotube 6. Maximum current represents peak current amplitudes; noninactivating current represents current amplitude 50 msec after voltage step. Both components appear concurrently at 13.25–13.5 hr AEL, immediately after myotube uncoupling. The noninactivating component increases gradually throughout late embryogenesis; the inactivating component increases more rapidly, peaks in the late embryo (18 hr AEL), and declines prior to hatching (21 hr AEL). The current is measured in response to a voltage step from a -100 mV prepulse (2 sec) to $+60$ mV. Each point shows the mean $\pm$ SD for at least 10 embryos ($N = 10$).

old of 0 mV and a maximum amplitude less than 100 pA ($+60$ mV; Fig. 2). From the earliest stages, three components of the current can be discerned: (1) an initial small, inward current; (2) a rapid, fast-inactivating outward current; and (3) a noninactivating outward current that persists for the length of the voltage step. All three components of the whole-cell current appear concurrently at 13.5–13.75 hr AEL (Figs. 2, 3). The inward component is often difficult to discern, as it is concealed by the opposing outward currents.

The whole-cell current rapidly matures over the next several hours (14–18 hr AEL; Figs. 2, 3). A continuous increase is observed in both the inward current and the inactivating and noninactivating components of the outward currents. Similarly, the inward current and noninactivating outward current continue to increase gradually for the remainder of embryogenesis (Figs. 2, 3). However, the developmental profile of the inactivating outward current is strikingly different (Figs. 2, 3): it initially (14–18 hr AEL) develops more rapidly, peaks (18 hr AEL), and declines markedly prior to hatching (21 hr AEL). The net effect is a whole-cell current profile that changes dynamically throughout late myogenesis.

Development of the rapid, inactivating potassium currents

From analysis of larval stages (Singh and Wu, 1990), it is known that the rapid, inactivating outward current is composed of two potassium currents: voltage gated (I_A) and calcium dependent (I_{CF}). These currents are carried through two channels encoded by the *Shaker* locus (I_A) and *slo* locus (I_{CF}), respectively. Mutations exist in both these structural genes that allow the selective elimination of the contribution of their gene products from the whole-cell current. We have exploited these tools to dissect the developmental profile of both I_A and I_{CF}.

It is easiest to study I_A development in a calcium-free extracellular bath, which eliminates the calcium current and the Ca^{2+}-dependent K^+ currents—specifically, for our purposes, the rapid I_{CF} (Fig. 4). This protocol leaves only the voltage-gated I_A and I_K intact, which can be temporally distinguished. Alternatively, the value of I_A can be obtained by subtracting the I_K component

that can be studied in isolation (see below). As previously reported in the mature larval muscle (Singh and Wu, 1990), I_A in the embryo is rapidly activated and inactivated by membrane depolarization; inactivation is essentially complete 50 msec after a voltage step to 60 mV, with remaining current negligible ($<2\%$ peak amplitude). As in cultured myotubes (Zagotta et al., 1989), we find that I_A activity diminishes rapidly in whole-cell recordings, possibly as the result of a long-lived inactivation state. I_A amplitude has usually decreased by 50% within 5 min of attaining whole-cell configuration (data not shown), though this rundown phenomenon is highly variable and seems strongly use dependent. To maximize consistency, recordings were made within 2 min of establishing whole-cell configuration.

I_A current can first be detected at 13.5–13.75 hr AEL (see Figs. 4, 7), soon after myotube uncoupling. Initially, I_A amplitude is small (<25 pA peak; $-100/+60$ mV voltage step), but it rapidly increases over the next several hours (14–18 hr AEL; see Figs. 4, 7), and by 18 hr AEL, a prominent current (480 $\pm$ 34 pA) is present. Throughout this period, I_A accounts for all the inactivating outward current; a null *Shaker* mutant (Sh^{KS133}) eliminates all the inactivating outward current in both normal and calcium-free salines (Fig. 4). At 18 hr AEL (see Figs. 4, 7), the I_A current peaks and a sharp decline in I_A amplitude (31 $\pm$ 12%) occurs in the last hours of embryogenesis prior to hatching (21 hr AEL). The mechanism of this rapid decline is unknown.

I_{CF} development was assayed in the Sh^{KS133} mutant, which completely eliminates the dominant I_A current in the embryonic myotubes (Fig. 4). I_{CF} can be temporally resolved from the delayed outward currents (I_K and I_{CS}). However, I_{CF} is a calcium-dependent current and must be preceded by a large calcium influx for its activation; therefore, I_{Ca} is a necessary contamination and must be subtracted to obtain I_{CF} amplitude (see below). The EGTA Ca^{2+} buffer in the intracellular pipette had no significant influence on the appearance or amplitude of I_{CF}. As reported in the larva (Gho and Mallart, 1986), I_{CF} in the embryonic myotubes, like I_A, is rapidly inactivated by membrane depolarization, with a time course very similar to I_A.

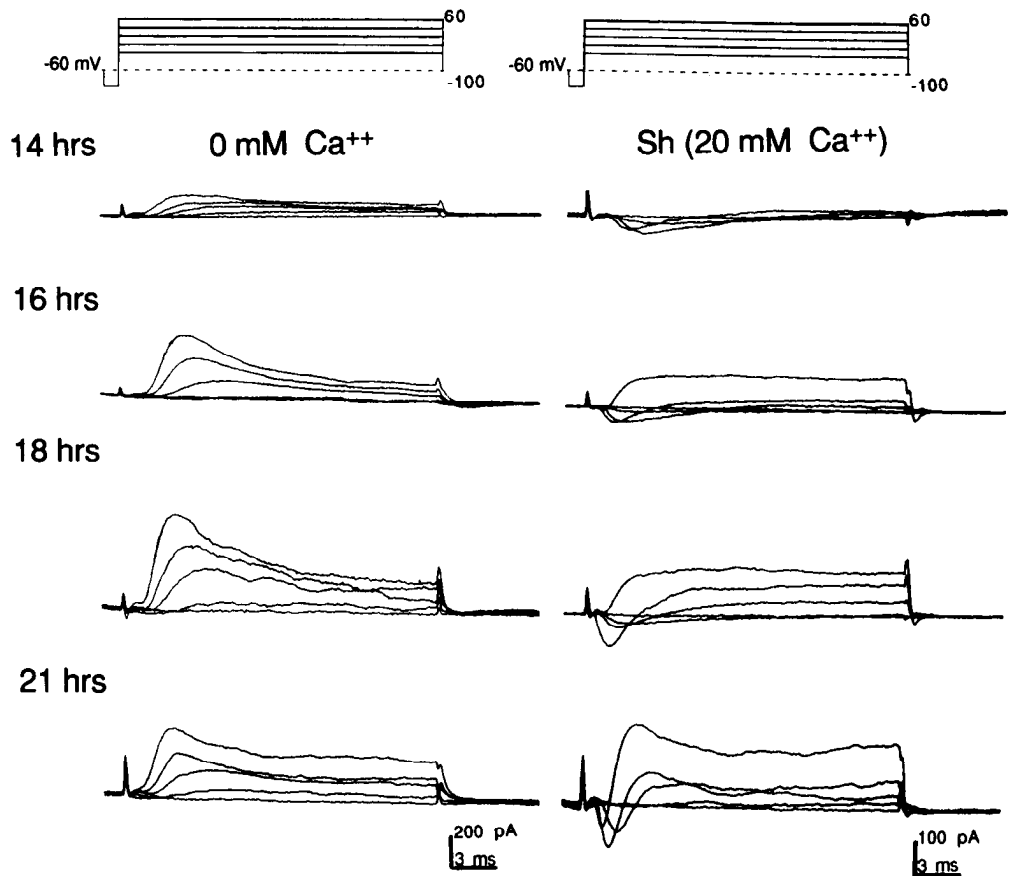Unlike I_A, I_{CF} is not present during the initial stages of elec-

Figure 4. Development of the fast potassium currents, I_A and I_{CF}. In the *first column*, calcium is removed from the extracellular medium to abolish the inward calcium current (I_{Ca}) and the calcium-dependent potassium currents (I_{CF} and I_{CS}). Only the voltage-gated potassium currents (I_A and I_K) remain, which can be temporally resolved. In the *second column*, a *Shaker* mutant (*Sh*[KS133]) abolishes I_A, and high external calcium (20 mM) is used to emphasize the fast, calcium-dependent potassium current (I_{CF}). The delayed currents, I_K and I_{CS}, remain but can be temporally resolved from the rapid I_{CF}. I_A appears early in development (by 14 hr AEL), peaks (18 hr AEL), and declines before hatching (21 hr AEL). I_{CF} is absent for most of embryogenesis, appearing late in development (18 hr AEL) and increasing slowly until hatching. The myotubes are voltage clamped at -60 mV; a 2 sec, -100 mV prepulse precedes the voltage step to eliminate inactivation. Superimposed traces show voltage steps to -20, 0, 20, 40 and 60 mV. Each trace is the average of five trials.

trogenesis (13.5–17 hr AEL) and plays no role in myotube physiology for most of embryogenesis (see Figs. 4, 7). I_{CF} current is first observed at 17–17.5 hr AEL. The initial current has a very low amplitude (<25 pA peak; $-100/+60$ mV voltage step) and appears negligible compared to the I_A current (>400 pA peak) at this time (see Figs. 4, 7). Again, unlike I_A, the amplitude only increases gradually after this initial appearance, and at hatching (21 hr AEL) I_{CF} has a peak amplitude <100 pA (see Figs. 4, 7). Though I_A has declined in the interim (18–21 hr AEL), I_A amplitude at hatching is still many times greater than I_{CF} amplitude.

Development of the delayed potassium currents

From the larval stages, it is known that the delayed, inactivating component of the outward current is composed of two potassium currents: voltage gated (I_K) and calcium dependent (I_{CS}). Unlike the rapid, inactivating currents (I_A, I_{CF}), these currents have not been characterized at a genetic level and no mutations are known that eliminate them specifically. Nevertheless, owing to their delayed onset relative to the fast currents, and owing to the rapid inactivation of the fast currents, it is possible to isolate these two components temporally. We found that both I_A and I_{CF} inactivated rapidly in the embryonic myotubes. Inactivation was essentially complete 50 msec after the voltage step and the remaining current was negligible or absent. On the other hand, I_K and I_{CS} reached a peak amplitude by 50 msec after the voltage step and did not inactivate even after a prolonged period (500 msec). The delayed currents themselves are differentiated on the basis of calcium dependency.

It is easiest to examine I_K in a calcium-free bath, which eliminates the calcium current and both calcium-dependent K$^+$ currents. If a *Shaker* mutant (*Sh*[KS133]) is used to eliminate I_A, I_K can be examined in isolation (Fig. 5). I_K appears soon after myotube uncoupling (13.15–13.25 hr AEL), early in electrogenesis and concurrent with the appearance of I_A (see Fig. 7). Initially (13.5–14 hr AEL), I_K amplitude increases rapidly, though more slowly than I_A, such that I_A amplitude soon becomes several times greater than I_K (16 hr AEL; see Fig. 7). During late development (>15 hr AEL; see Figs. 5, 7), I_K increases much more gradually, increasing continuously through the end of embryogenesis. There is no indication of a late decline in I_K amplitude, which appears to be an exclusive characteristic of I_A. At hatching (21 hr AEL; see Fig. 7), I_K has a whole-cell amplitude >200 pA ($-100/+60$ mV voltage step), nearly as large as I_A and second only to I_A among the myotube voltage-gated currents.

As in the larva (Singh and Wu, 1990), quinidine blocks I_K in the embryonic myotubes. In Ca^{2+}-free saline, quinidine at 10 μmol/liter reduced I_K amplitude by up to 50% but did not significantly alter I_A (data not shown). On the other hand, quinidine at 1 mmol/liter still left considerable I_K current (up to 30%) and we were never able to block I_K completely. This may indicate subpopulations of I_K with differing sensitivity to quinidine-blockade or a complex blocking mechanism. As a consequence of this uncertainty, we never used quinidine block to estimate I_K amplitude or to eliminate the I_K component from multicurrent recordings.

I_{CS} is difficult to examine. The noninactivating component of the whole cell can be measured with and without external Ca^{2+} to determine the Ca^{2+}-dependent I_{CS} contribution. An *Sh;slo* double mutant is used to eliminate all traces of the rapid, in-
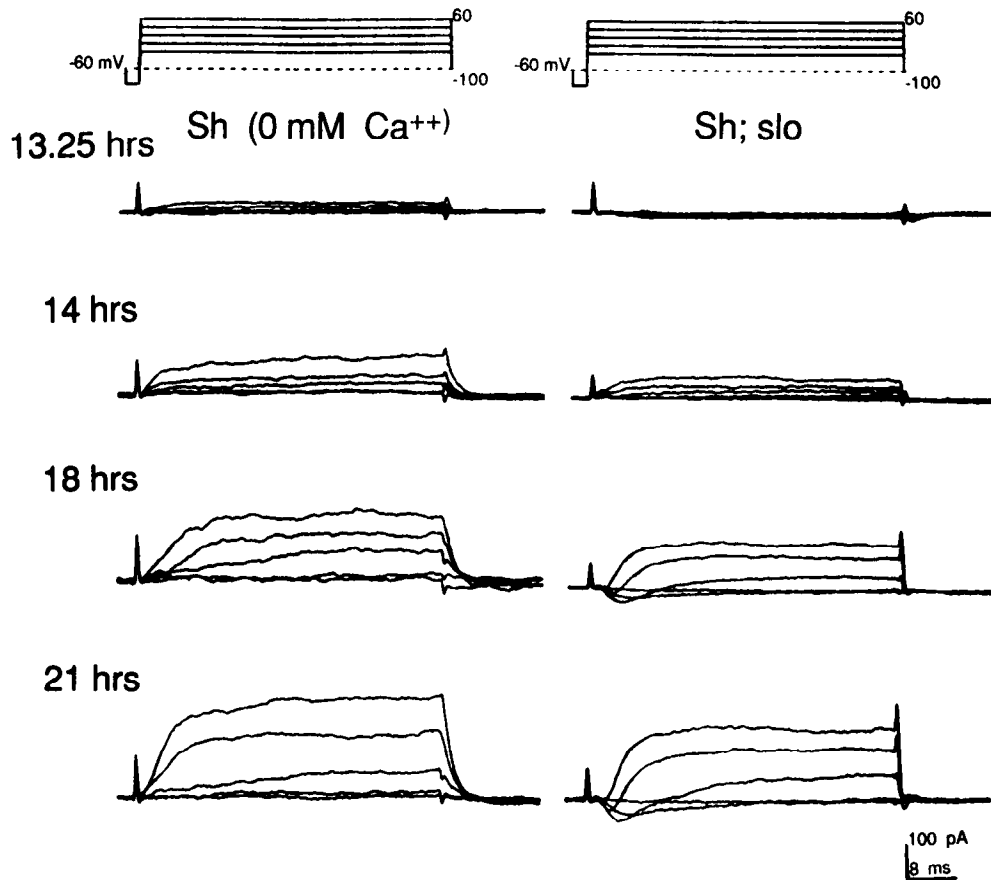
Figure 5. Development of the delayed potassium currents, I_K and I_{CS}. In the *first column*, a *Shaker* mutant (Sh^{KS133}) abolishes I_A; removing external calcium (see Materials and Methods) abolishes both the inward calcium current (I_{Ca}) and the calcium-dependent potassium currents (I_{CF} and I_{CS}). Hence, I_K can be followed in isolation. In the *second column*, an *Sh;slo* double mutant eliminates both of the rapid, inactivating currents, I_A and I_{CF}. The remaining noninactivating current represents the sum of the outward I_K and I_{CS}, and the inward I_{Ca} (2 mM Ca^{2+} in bath). I_{CS} amplitude can be obtained by subtracting I_K (*first column*) and I_{Ca} (see below), both of which can be studied in isolation. I_K appears early in development (by 14 hr AEL) and increases steadily throughout embryogenesis. I_{CS} is absent for most of embryogenesis, appearing late in development (18 hr AEL) and increasing slowly until hatching. The myotubes are voltage clamped at -60 mV; a 2 sec, -100 mV prepulse precedes the voltage step to eliminate inactivation. Superimposed traces show voltage steps to -20, 0, 20, 40, and 60 mV. Each trace is the average of five trials.

activating current (Fig. 5), but I_K, I_{Ca}, and I_{CS} remain, all non-inactivating and with similar time courses. Therefore, I_{CS} development was measured by subtracting the contributions of I_K (Fig. 5) and I_{Ca} (Fig. 6), both of which can be obtained in isolation. I_{CS} has a very similar developmental time course to I_{CF} in that it is absent for most of embryogenesis, appears only late in muscle development, and contributes negligibly to the whole-cell current even at hatching (Figs. 5, 7). I_{CS} appears slightly later than I_{CF}, at 18–18.5 hr AEL (Fig. 7). It has a very low initial amplitude and matures only gradually, such that at hatching it has an amplitude of <50 pA ($-100/+60$ mV voltage step) and is the smallest of the four outward potassium currents (Figs. 5, 7).

Development of the inward currents: the calcium current and glutamate-gated synaptic current

Muscle contraction in the mature larva is mediated by an inward calcium current (Jan and Jan, 1976a,b; Singh and Wu, 1990). Similarly, muscle contraction in the mature embryo can be blocked by calcium channel blockers or in a calcium-free saline. It is possible to block all outward potassium currents by replacing intracellular K^+ with Cs^+ in whole-cell configuration (Fig. 6A). In this configuration, an inward, noninactivating cal-

cium current can be recorded. In Ca^{2+}-free saline, this current is abolished and no voltage-gated currents are recorded (data not shown), indicating that no other inward currents are present and that blockage of the potassium conductances is essentially complete. In particular, we did not find evidence for an inward sodium current in the embryonic muscle as has been reported in the mature larval muscle (Yamaoka and Ikeda, 1988).

The calcium current (I_{Ca}) is the first macroscopic voltage-gated current revealed when the embryonic myotubes uncouple at 13–13.25 hr AEL (Fig. 7). Indeed, a large calcium current (>50 pA peak; Fig. 6A) is measurable within 1 min of myotube uncoupling, indicating that I_{Ca} is probably present prior to uncoupling. Since we have been unable to voltage clamp the coupled fibers, it is unclear exactly when I_{Ca} first appears. I_{Ca} magnitude develops continuously for the remainder of embryogenesis (Figs. 6A, 7), approximately doubling in size between myotube uncoupling (13.25 hr AEL) and hatching (21 hr AEL). I_{Ca} has the most graded maturation of all the muscle currents (Fig. 7).

For completeness, we consider the glutamate-gated current at the developing neuromuscular synapse, which has been the subject of a considerably more detailed analysis (preceding companion article, Broadie and Bate, 1992). The glutamate-gated current was assayed by iontophoresis of L-glutamate at the de-
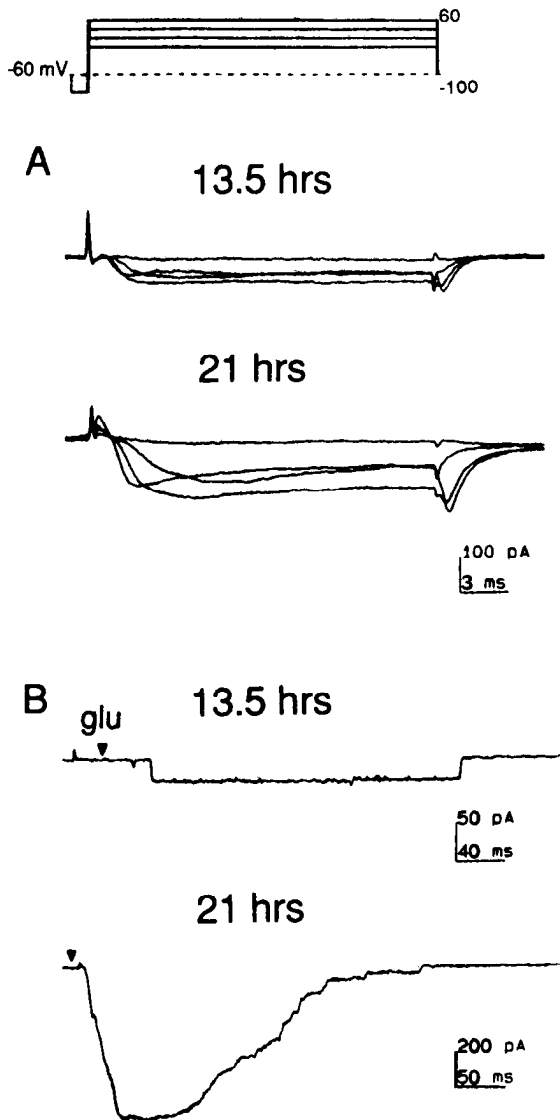
Figure 6. Development of the inward currents: the voltage-gated calcium current (I_{Ca}) and the L-glutamate–gated synaptic current at the developing NMJ. *A*, The calcium current was examined in the absence of the outward potassium currents by replacing intracellular K^+ with Cs^+ in whole-cell configuration (see Materials and Methods). High external calcium (20 mM) was used to emphasize the inward I_{Ca} in these traces. I_{Ca} is the earliest detectable current in the embryonic myotubes, shown here at 13.5 hr AEL, soon after myotube uncoupling. I_{Ca} increased only gradually through the later stages of embryogenesis (21 hr AEL, hatching). The myotubes are voltage clamped at -60 mV; a 2 sec, -100 mV prepulse precedes the voltage step to eliminate inactivation. Superimposed traces show voltage steps to 0, 20, 40, and 60 mV. Each trace is the average of five trials. *B*, The glutamate-gated current was assayed by the iontophoresis of L-glutamate at the developing NMJ (Broadie and Bate, 1992). A small glutamate current was first observed at 13.25–13.5 hr AEL, soon after myotube uncoupling. The amplitude of this current increases rapidly throughout the later stages of embryogenesis and, at hatching (21 hr AEL), is the largest conductance in the mature muscle. Myotube is voltage clamped at -80 mV. Each trace is a single representative trial; at least 10 embryos were examined at each time point.

veloping synaptic zone. In common with I_A and I_K, glutamate-gated current first develops within several minutes following myotube uncoupling at 13–13.25 hr AEL. The initial current is small (<20 pA at -80 mV; Fig. 6*B*) and appears to represent the opening of a single, large-conductance, glutamate-gated

channel (Broadie and Bate, 1992). However, the current rapidly increases in amplitude and has reached an amplitude of >250 pA (at -80 mV) within 2 hr (by 15.5 hr AEL), and so parallels the rapid maturation of the I_A current (data not shown, but see Broadie and Bate, 1992). A more gradual but still rapid increase continues through to the end of embryogenesis, and at hatching (21 hr AEL; Fig. 6*B*) the synaptic glutamate-gated current is the largest conductance in the mature embryonic muscle.

Development of myotube voltage responses

In parallel with the maturation of the whole-cell currents, the voltage response of the embryonic myotube varies dramatically with developmental stage (Fig. 8). During the initial stages of myogenesis, while the myotubes are still coupled, they have a completely passive response to current injection (Fig. 8); most of the current flows through the nonrectifying gap junctions, and very little voltage change is elicited in the injected myotube. Immediately after uncoupling (13–13.25 hr AEL), current injection elicits a much greater change in myotube voltage, but the response remains largely passive. In the early myotube (13.5–14 hr AEL), the first voltage response to depolarizing current is observed, with a small inward rectification with a threshold of -10 to -20 mV (Fig. 8). With increased current injection, the delay initially observed in this response is removed and the myotube is rapidly depolarized for the length of the stimulus.

Surprisingly, given the current development, no evidence for a hyperpolarizing response is observed at any time during these early stages (Fig. 8). Although I_A is prominent in voltage-clamp recordings, it is almost completely inactivated at the physiological resting potential (-46 mV; see below) and so does not contribute significantly to the response shown (see below). The influence of I_A can only be seen if the myotube is hyperpolarized to below -80 mV by injection of constant current. At this artificial potential, the myotube displays a large, though transient, rapid outward rectification (data not shown). At early stages, the inward I_{Ca} is dominant to the outward I_K, and this, presumably, explains why a small inward rectification is the observed voltage response in these early stages.

A second development in voltage response commences by 15 hr AEL and reaches maximal effect by 18 hr AEL (Fig. 8); that is, there is an increasing restriction of voltage depolarization to below 0 mV. During earlier periods (<15 hr AEL), increasing injection of positive current depolarizes the myotube to increasingly positive values. After this period (starting 15 hr AEL), increasing injection of positive current will initially depolarize the myotube, but depolarization is restricted to 0 mV. By 18 hr AEL, even the injection of relatively large currents (>200 pA) will not depolarize the myotube above 0 mV (Fig. 8).

During late embryogenesis (>18 hr AEL), a third stage of voltage responsiveness develops (Fig. 8). During this period (18–21 hr AEL), the myotube gradually develops an active, regenerating voltage response to depolarizing current injection. Initially (18.5–19.5 hr AEL), this development is signaled by sporadic, overshooting potentials with a threshold of approximately $-10/-20$ mV and peak of $+10$ to $+15$ mV; however, in general, voltage responses are still restricted to below 0 mV as with earlier stages. Slightly later (20–21 hr AEL; Fig. 8), this restriction has been completely eliminated; the myotube shows a regenerative, active potential with a threshold of $-10/-20$ mV, peak of $+20$ to $+30$ mV, and duration of 50–60 msec. In the hatching embryo (21 hr AEL), the membrane voltage response to injected depolarizing current consists of rapid, action potential-like spikes
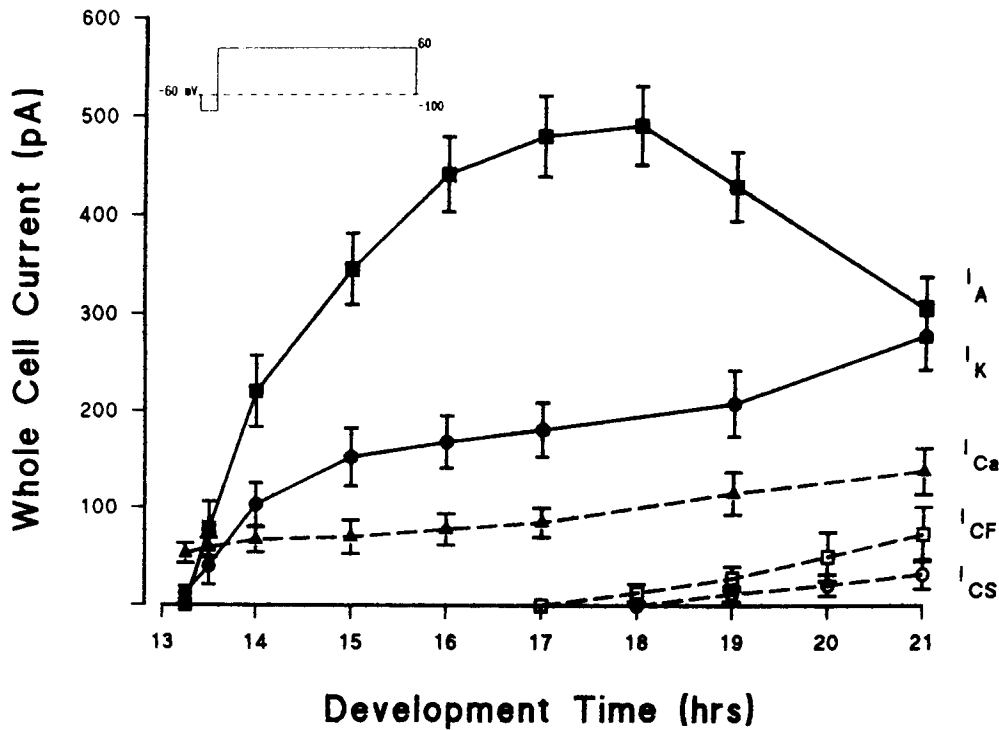
Figure 7. Development of the five components of the whole-cell voltage-gated current: I_{Ca}, I_A, I_{CF}, I_K, and I_{CS}. The calcium current is the first to appear in the embryonic myotubes. The voltage-gated currents, I_A and I_K, both appear early in development; I_K increases steadily through development, but I_A increases rapidly, peaks, and declines sharply prior to hatching. The calcium-dependent currents, I_{CF} and I_{CS}, are the last to appear, arising late in embryogenesis and achieving only a relatively small amplitude by hatching (21 hr AEL). The peak current is measured in response to a voltage step from a -100 mV prepulse (2 sec) to $+60$ mV. Each point shows the mean $\pm$ SD for at least 10 embryos ($N = 10$). Current separation techniques are detailed in the Materials and Methods.

that overshoot 0 mV and continue without adaptation for the length of the current injection.

An embryonic I_A current encoded by Shaker *with a negatively shifted inactivation range*

Four of the five embryonic myotube currents (I_{Ca}, I_K, I_{CF}, and I_{CS}) have characteristics similar to the mature currents described in the third instar larva (Singh and Wu, 1990); that is, they have grossly similar activation/inactivation ranges assayed at the whole-cell level. In contrast, the I_A current in the early embryonic myotube has a midpoint of inactivation 40 mV more negative than the mature I_A current, though the activation curve remains similar (Figs. 9, 10). Nevertheless, I_A current at all stages of development can be completely eliminated by a mutation at the *Shaker* locus (Sh^{KS133}; Fig. 4), indicating that the channels underlying the I_A current throughout myogenesis are encoded, at least in part, by the *Shaker* gene. We conclude that an embryonic and mature form of the *Shaker* channel exist in the developing *Drosophila* muscles.

During the initial stages of I_A electrogenesis (13.5–14 hr AEL), the I_A current has a very negative inactivation curve (Figs. 9, 10); the first significant removal of inactivation occurs at -50 to -60 mV, and complete removal of inactivation is not achieved even after a 2 sec prepulse at -80 mV. During the mid-stages of I_A development (16–18 hr AEL; Figs. 9, 10), the inactivation curve develops a biphasic character. The initial stage (0 to -60 mV) is similar to the mature relationship, with significant removal of inactivation by -20 mV. The second stage (-60 to -100 mV) is similar to earlier development, in that a substantial removal of inactivation commences at -60 mV and continues through -100 mV (Figs. 9, 10). Even by 18 hr AEL, significant I_A current remains inactive after a 2 sec prepulse at -80 mV. Thus, it appears that at least two populations of channels are present that differ in their inactivation range. By hatching (21 hr AEL), an inactivation curve similar to that in the larva is observed (Figs. 9, 10), with a midpoint of inactivation at -35

mV and complete removal of inactivation by -60 mV. It appears that two forms of the I_A current are expressed progressively: an early form with a very negative inactivation curve is progressively replaced with the mature form of the current as embryogenesis proceeds. The mechanism and significance of this developmental shift remain to be investigated.

The earliest I_A current (13.5–15 hr AEL), is strongly inactivated at the physiological resting potential (-46 ± 6 mV). This suggests that the early rapid maturation of the I_A current may be misleading in that most or all of this current may be functionally inactive. Therefore, the myotube may experience a much later and more gradual onset of the I_A current as the mature I_A current, with a much more positive inactivation curve, develops (Fig. 10). The physiological role of the abundant, early I_A current remains obscure.

Discussion

Development of muscle currents

The development of the voltage-gated muscle currents in the *Drosophila* embryo follows a clear progression: first the inward Ca^{2+} current (I_{Ca}) appears, then the voltage-gated outward K^+ currents (I_A, I_K), and finally the calcium-dependent K^+ currents (I_{CF}, I_{CS}). Before the myotubes uncouple (13–13.25 hr AEL), I_{Ca} appears to be the only macroscopic current present in the myotubes, though we were unable to perform voltage-clamp experiments on the early, coupled muscles. Uncoupling correlates with the rapid onset of electrogenesis, with I_A, I_K, and the synaptic L-glutamate–gated currents all appearing within several minutes. The calcium-dependent K^+ currents do not appear until much later, near the end of embryogenesis.

Each current matures with a characteristic time course. For most of these currents, this maturation is unremarkable, involving a gradual increase in current amplitude from first appearance through to the end of embryogenesis. A striking exception is the I_A current encoded by the *Shaker* locus, which matures rapidly upon appearance, peaks in the late embryo, and

13 hrs

14 hrs

18 hrs

21 hrs

20 mV

100 ms

14 hrs

-60 mV

60

-100

100 pA

3 ms

18 hrs
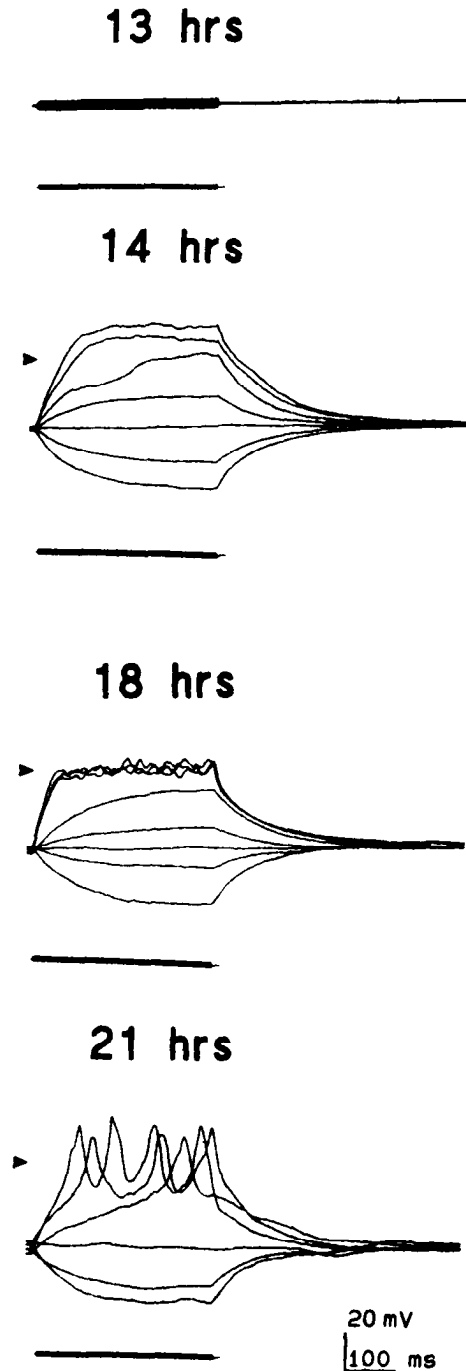
100 pA

3 ms

21 hrs

100 pA

3 ms

Figure 8. Development of the whole-cell voltage response in current-clamped embryonic myotubes. Constant current was injected from −50 pA in 25 pA increments; *bar length* indicates injection duration; resting potential, −46 ± 6 mV; *arrowheads* indicate 0 mV. During early myogenesis (13 hr AEL), the myotubes are electrically coupled and show a completely passive response to current injection. By 14 hr AEL, the myotubes are uncoupled but show only a small positive rectification in response to depolarizing current. By 18 hr AEL, a larger outward rectification is apparent, but voltage responses are restricted to 0 mV, even in response to large depolarizing currents (+150 pA). At hatching (21 hr AEL), the myotube has developed a dynamic, regenerating potential response to depolarizing current injection. The threshold of this potential response is −10 to −20 mV; the peak, +20 to + 30 mV; and the regenerating potential continues for the duration of current injection. Each trace is a single representative trial; at least 10 embryos were tested at every time point.
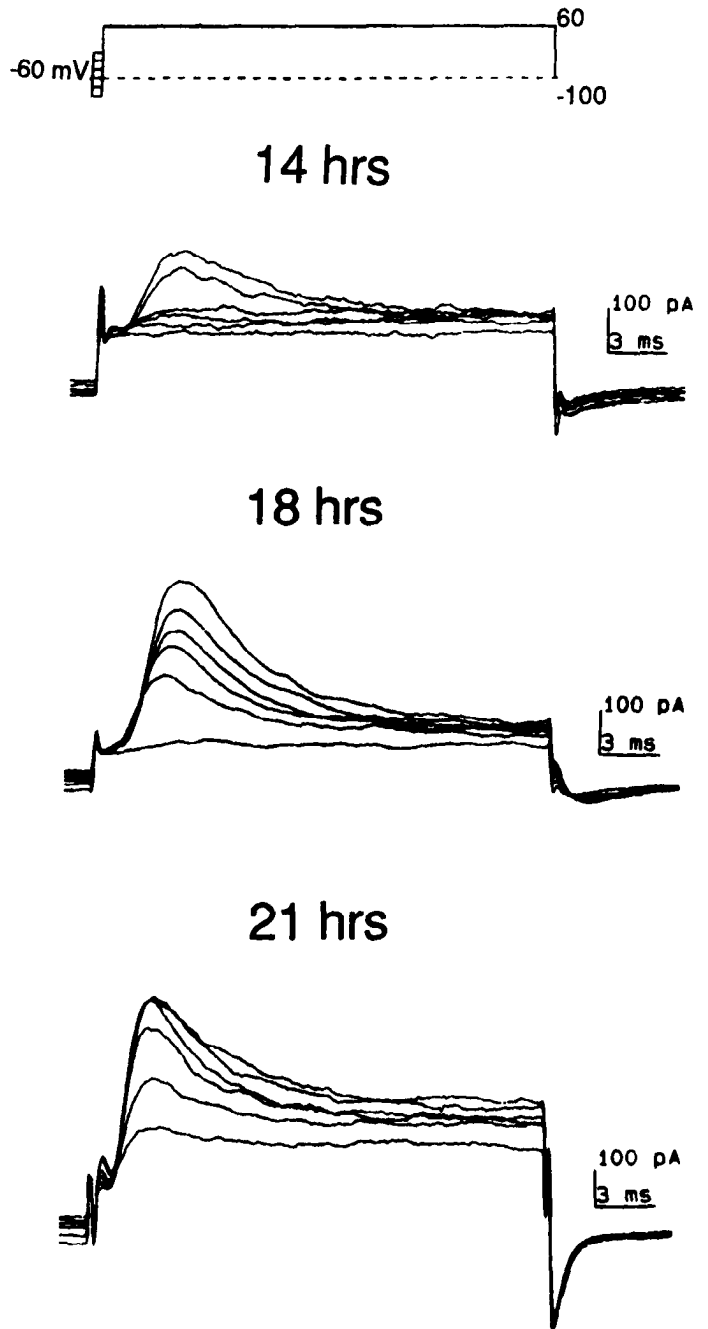
Figure 9. Development of the steady-state inactivation profile of whole-cell currents in the embryonic myotubes (myotube 6). A prepulse (2 sec), starting from 0 mV and decreasing in −20 mV increments (0, −20, −40, −60, −80, and −100 mV), was followed by a voltage step to +60 mV. In young myotubes (14 hr AEL), the whole-cell current was strongly inactivated even at −60 mV. By 18 hr AEL, the midpoint of inactivation has shifted more positive and a prepulse of −20 mV is sufficient to activate significant current. However, complete removal of inactivation is not achieved even with a −80 mV prepulse. At hatching (21 hr AEL), the midpoint of inactivation has become much more positive; complete removal of inactivation is achieved by −60 mV. Notice the difference in time-to-peak between early (14 hr) and late (21 hr) stages; the intermediate stage (18 hr AEL) appears to be composed of two populations, with high inactivation (−60 to −100 mV) showing a time-to-peak similar to early stages and low inactivation (−20, −40 mV) showing a time-to-peak similar to mature stages. Superimposed traces are the average of five trials each.

current (pA)

18 hrs

21 hrs

14 hrs

600

500

400

300

200

18 hrs

21 hrs

14 hrs

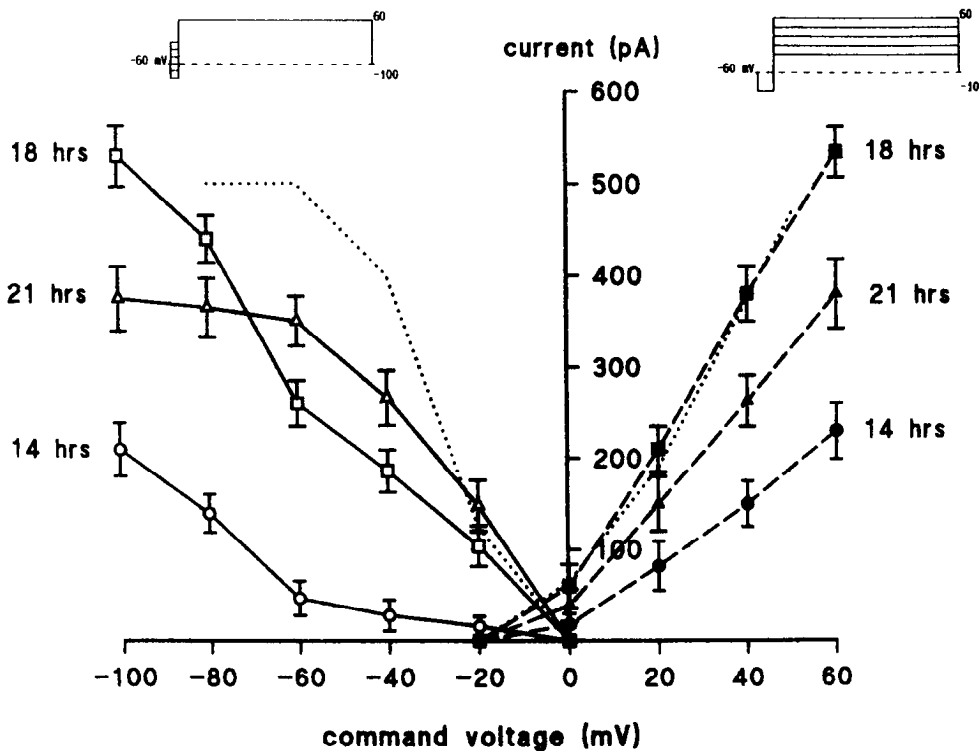-100 -80 -60 -40 -20 0 20 40 60

command voltage (mV)

Figure 10. A comparison of the development of the activation and inactivation curves of the embryonic myotubes. The activation range (*solid symbols*) is on the *right*; the inactivation range (*open symbols*) is on the *left*. The protocols are presented as displayed earlier (see Figs. 2, 9). The midpoint of the inactivation curve changes dramatically with development: in early development (14 hr AEL), I_A is strongly inactivated even at −60 mV; at 18 hr AEL, the inactivation curve is biphasic with an initial inactivation curve (0 to −60 mV) similar to the mature form, but with a late inactivation component (−60 to −100 mV) similar to earlier development; by hatching (21 hr AEL), the inactivation curve is monophasic with a midpoint of −35 mV and saturation reached by −60 mV. Hence, a low-inactivation I_A current is present during earlier myogenesis and progressively replaced by the mature form as development proceeds. The *dotted relationships* reflect the activation and inactivation curves from mature cultured embryonic myotubes (Solc et al., 1987) and are included for comparison. Each point is the mean ± SD of at least 10 embryos (*N* = 10).

declines markedly prior to hatching. This late decline in I_A amplitude may be attributed to removal of an "embryonic" I_A channel (see below). The function of this unusual developmental time course remains unclear.

In the present study, we have limited our developmental analysis to well-characterized currents present in the mature *Drosophila* larval muscles (Singh and Wu, 1990). However, previous studies on cultured embryonic myotubes (Aldrich et al., 1989) have identified as many as six distinct classes of potassium channels, including two A-type channels, one delayed rectifier, two nonactivating channels, and a stretch-activated channel, none of which are calcium dependent. It is possible that the two A-type channels represent the early and late I_A types we have described in whole-cell recordings. Likewise, it is possible that the whole-cell I_K described here is composed of subpopulations of different I_K channel types. Indeed, our inability to eliminate I_K completely with quinidine suggests populations of I_K channels with different quinidine sensitivities. On the other hand, previous studies on cultured embryonic myotubes (Leung and Byerly, 1991) have identified only a single type of Ca^{2+} channel, whose properties suggest that it is the same as that shown in whole-cell recordings in the present study. A similar study has not been conducted on the Ca^{2+}-gated K^+ channels in the embryo, so it remains to be demonstrated whether the whole-cell currents described here are composed of single- or multiple-channel populations.

The development of muscle currents in the pupa [flight muscles (DLMs)] and the embryo (abdominal muscle 6) of *Drosophila* is remarkably similar. In both cases, the voltage-gated K^+ currents (I_A, I_K) arise very early in development and the calcium-dependent K^+ currents (I_{CF}, I_{CS}) arise only very late, immediately prior to hatching (embryo) or following eclosion (pupa) (Salkoff, 1985; Elkins and Ganetzky, 1988). Hence, the voltage-gated K^+ currents are the only repolarizing currents

present during development. The primary difference between the DLMs and the embryonic muscles is in the development of the inward calcium current (I_{Ca}). In the embryo, this is the first macroscopic current to appear; in the pupa, I_{Ca} appears only very late in development, immediately prior to eclosion (Salkoff, 1985). Presumably, therefore, the DLMs are noncontractile during development whereas the embryonic muscles not only are contractile, but are vigorously active throughout late development. This vigorous movement is a characteristic of embryonic development in all characterized species, vertebrate (Blackshaw and Warner, 1976; Dennis et al., 1981) and invertebrate (Bentley et al., 1979; Broadie et al., 1991), and has been suggested to play a role in muscle development, NMJ development, or both. Hence, it is likely that the late maturation of I_{Ca} is a specialization in the adult flight muscles and that the early maturation of I_{Ca} in the embryo represents the more general case.

There has been considerable speculation about the apparent functional redundancy of the voltage-gated (I_A, I_K) and calcium-dependent (I_{CF}, I_{CS}) K^+ currents. Both appear to function to repolarize the muscle to a resting state after excitation, and both have similar physiological properties (Elkins and Ganetzky, 1988; Singh and Wu, 1990). It is interesting in this light to note that the calcium-dependent K^+ currents are absent for most of embryogenesis, appear only soon before hatching, and even in the hatchling embryo contribute negligibly to the repolarizing K^+ current. It appears, therefore, that the voltage- and calcium-dependent K^+ currents play developmentally distinct roles in the muscles, with the voltage-gated I_A/I_K acting in isolation in the developing embryo and I_{CF}/I_{CS} contributing significantly only to the mature state. In mature muscles, I_{CF} appears to play a dominant role in repolarization while I_A is secondary (Elkins and Ganetzky, 1988; Singh and Wu, 1990). Indeed, the complete elimination of I_A has only a small effect on the time course of repolarization (Elkins and Ganetzky, 1988), though I_A plays an

important role in the delay of action potential initiation. Therefore, the early developmental role of I_A appears to be replaced, at least in part, by I_{CF} in the mature muscle. The reason for this developmental progression is unclear.

It has been speculated that there may be an evolutionary relationship between the voltage- and calcium-gated K^+ channels. Specifically, it has been suggested that I_{CF} may have evolved as a more precise form of repolarizing control in muscle, as it is directly regulated by calcium entry, sharing a common signal with muscle contraction (Salkoff, 1985). Control of intracellular calcium may be regarded as the crucial link between electrical signals and cellular activity. In development, the feedback mechanism in this circuit is indirect, proceeding through I_A/I_K activated solely by membrane depolarization. In the mature muscles, a direct link exists between cytoplasmic calcium levels and muscle repolarization/relaxation via the calcium-dependent K^+ currents. In principle, this direct link forms a more precise and efficient feedback system for controlling Ca^{2+} entry and thus muscular activity.

Development of the muscle voltage response

The developmental progression of voltage responsiveness in the embryonic myotubes is similar to that observed during the pupal development of the adult DLMs (Salkoff, 1985). An early passive voltage response is replaced by a small rectifying response in mid-stages, and is followed by the development of an active, regenerating response very late in development, just prior to hatching (embryo) or following eclosion (pupa). The primary differences are as follows: (1) The early rectifying responses have opposite polarities, outward in pupal muscles and inward in embryonic muscles. Presumably, this reflects the very late development of I_{Ca} in the pupa relative to the embryo. It is noteworthy that in both cases a contribution of I_A is not observed, as it is wholly inactivated in early muscle development (Salkoff, 1985), though the mechanisms may differ (see below). (2) The "depolarization restriction" transiently observed in mid-stages (16–18 hr AEL) of the embryo is not reported in the pupal muscles. This possibly reflects the rather later development of I_K in the pupa relative to the embryo (Salkoff, 1985), though it is unclear how the "depolarization restriction" at 0 mV is produced.

In newly eclosed adult flies, I_A plays the primary role in muscle (DLM) repolarization during action potential generation, but this role is quickly taken by the developing I_{CF} (Elkins and Ganetzky, 1988). Thus, elimination of I_A in early development affects action potential duration and frequency, as I_{CF} elimination does in the mature fly, but elimination of I_{CF} in the immature fly has only very slight effects (Elkins and Ganetzky, 1988). These results suggest a developmental change in the relative amplitudes of I_A and I_{Ca} currents controls early action potential generation in the DLMs. A wholly analogous case is observed in the late embryo, where an apparent decline in I_A amplitude and increase in I_{Ca} amplitude precede the ability to generate action potentials. It seems, therefore, that the generation and regulation of muscle action potentials are very similar in embryonic and pupal development. We note, however, that at the physiological resting potential (-46 mV), I_A amplitude actually increases continuously throughout embryogenesis (Fig. 10), so the apparent decline in I_A amplitude observed in our voltage-clamp protocol does not appear to be involved in this process. Therefore, it is more probable that I_{Ca} attains some necessary threshold amplitude for the generation of action po-

tentials only toward the end of embryogenesis. Though I_{CF} and I_{CS} appear soon before action potential generation in the embryonic muscles, they are unlikely to be involved in the generation of this response, as they are of small amplitude and, in the larva at least (Singh and Wu, 1990), appear antagonistic to action potential generation.

Most reports suggest that the mature larval muscles are incapable of generating action potentials, responding to depolarizing current passively (Jan et al., 1977; Wu and Ganetzky, 1988). However, we report that mature embryonic muscles respond to constant depolarizing current with a train of regenerating action potentials. There are two possible explanations for this apparent discrepancy. First, it is possible that larval muscles do generate action potentials; experiments with aerated larval preparations (Yamaoka and Ikeda, 1988), or with artificially hyperpolarized muscle resting potentials (Singh and Wu, 1990), have shown that larval muscles are capable of firing action potentials. However, we must stress that nonaerated embryo preparations at the physiological resting potential readily produce trains of action potentials whereas the larval preparations manifestly do not under similar conditions. Second, the difference may be explained by the influence of the calcium-dependent K^+ currents (I_{CF}, I_{CS}). Experiments in the mature larva have shown that I_{CF} is the major contributor to muscle repolarization and acts to suppress action potentials (Singh and Wu, 1990). In mutations eliminating I_{CF} (*slo*), all-or-none action potentials are much more readily produced in the mature larval muscles (Singh and Wu, 1990). Both I_{CF} and I_{CS} have a small amplitude in the hatchling embryo and contribute negligibly to the repolarizing K^+ current. Hence, the conditions are similar in the mature embryonic muscle to larval muscles with a null *slo* mutation, and the muscles respond similarly by generating action potentials. At present, we cannot distinguish the above two possibilities. It is of interest to establish whether the ability to generate action potentials is a transient stage of muscle development or is the mature state of the larval muscles.

Although we can, perhaps, explain the dynamic changes in muscle voltage response during embryogenesis, it is much more difficult to speculate on their functional significance. In early development, for example, it has been suggested that the lateral spread of depolarization between coupled myotubes plays a role in regulating or synchronizing the early stages of muscle development in vertebrate embryos (Dennis et al., 1981), and may be similarly important in *Drosophila*. Likewise, it is possible that later voltage response regulation may play a role in muscle development. For example, the depolarization restriction in mid-myogenic stages (16–18 hr AEL) correlates with the onset of vigorous muscle activity and may play a role in moderating or regulating muscle activity during early, delicate stages. A similar argument might suggest that the development of action potentials in late embryogenesis may signal or regulate late-developing muscle characteristics. So far, there has been no experimental verification of these intriguing correlations.

An embryonic form of Shaker?

Variants of the I_A current are present in many different tissues of the fly in both larval (Aldrich et al., 1989; Schwarz et al., 1990) and adult stages (Elkins and Ganetzky, 1988; Hardie et al., 1991). Many of these variants show differences in their voltage operating range. This rich diversity of I_A currents raises the possibility that varying forms of I_A channels may be expressed, not only in different tissues, but in the same tissue at

developmentally distinct periods and, perhaps, with distinct functional consequences. Some of these I_A channels may be encoded by different structural genes (Solc and Aldrich, 1988; Wei et al., 1990), but at least two, one in larval and pupal muscle (Salkoff, 1985; Zagotta et al., 1988; Singh and Wu, 1990) and one in pupal and adult photoreceptors (Hardie et al., 1991), are encoded by the *Shaker* locus and can be eliminated by a null mutation of the *Shaker* structural gene.

In the present study, we have revealed two variant forms of the I_A current that are expressed in a progressive fashion in the developing embryonic myotubes. The earlier, "embryonic" form has a midpoint of inactivation 40 mV more negative than the mature form found in the hatchling embryo and larval muscles. Initially, only the "embryonic" I_A current is present. During late embryogenesis, the inactivation curve is biphasic, indicating two populations of I_A channels, embryonic and larval, and in the hatchling embryo only a single I_A current remains, sharing similar inactivation characteristics with the mature larval I_A current. The I_A current at all stages can be eliminated by a mutation in the *Shaker* locus (Sh^{KS133}). We conclude that an embryonic form of the *Shaker* channel is present in the young embryonic myotubes and is progressively replaced by the mature form during late embryogenesis.

It has recently been demonstrated that the voltage dependence of different *Shaker* channels can vary dramatically. *Shaker* channels in both the adult *Drosophila* photoreceptors (Hardie et al., 1991) and pupal neurons (Baker and Salkoff, 1990) have a voltage operating range shifted 30–40 mV more negative than that of *Shaker* channels in larval muscle. Likewise, we have seen a negative shift (of similar magnitude; 40 mV) in the inactivation range of a *Shaker* whole-cell current in the embryonic myotubes relative to the inactivation range in the larval muscles.

Previous work has shown that I_A diversity may arise through alternate mRNA splicing (Kamb et al., 1988; Schwarz et al., 1988), heteromultimeric K$^+$ channel structure (Haugland and Wu, 1990; Isacoff et al., 1990; Covarrubias et al., 1991), and multiple structural genes (Tempel et al., 1988; Covarrubias et al., 1991). At least 10 transcripts are encoded by alternative splicing of mRNA from the *Shaker* locus, at least in *Xenopus* oocytes (Kamb et al., 1988), but despite this potential diversity, only two kinetically distinct potassium channels have actually been shown to be encoded by *Shaker* in *Drosophila* (i.e., larval muscle and adult photoreceptors; Hardie et al., 1991). These different *Shaker* channels, as predicted, are encoded by different mRNA transcripts (Hardie et al., 1991). In the present study, we have the possibility of a third example: a distinct I_A current, encoded by the *Shaker* locus, that may arise though alternative splicing during early embryonic muscle development. This possibility has not yet been rigorously tested and requires further investigation.

The I_A current is one of the first voltage-gated currents to appear in the embryonic myotubes, matures much more rapidly than other currents, and so is the most prominent current in the voltage-clamped myotubes for most of embryogenesis. However, the inactivation characteristics of the early I_A current render it almost entirely inactive at the physiological resting potential. Moreover, during the early, dramatic increase in I_A amplitude, almost all of this current remains inactive under physiological conditions. It is only relatively late in development, with the emergence of the mature form of I_A, that the myotube experiences a substantial I_A current active under physiological conditions. These findings pose the interesting question

of a function, if any, for this early, dramatic increase in I_A amplitude. It is possible that the I_A current is more active normally than under experimental conditions; perhaps it has a long time course of inactivation that can be removed by remaining at the resting potential for an extended period of time (>5 sec). Alternatively, unknown mechanisms may hyperpolarize the myotube under normal conditions and so activate the abundant I_A current. For example, it has been reported that tracheal aeration of the mature larval muscles lowers the resting potential to -80 mV, considerably lower than the -30 to -60 mV reported in unaerated preparations (Yamaoka and Ikeda, 1988). However, in vigorously aerated (O_2 supplied in bath), freshly dissected embryos (<3 min), we never saw resting potentials more negative than -60 mV, though tracheal aeration of the embryo was not attempted. Even if we accept a resting potential of -60 mV, we find that most of the I_A current remains inactivated at this potential (Fig. 10). Thus, the question of the functional significance of the early embryonic I_A current, if any, remains unresolved and awaits further investigation.

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